Robust cadmium removal and tolerance by a magnetotactic bacterium isolated from the sediment of an open-pit mine tailings lake

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The consequences of rampant and improper disposal of mine tailings in the Philippines are the rise of heavy-metal contaminated bodies of water, the concomitant destruction of the environment, and the emergence of health hazards to people in surrounding communities. In response, this study aimed to isolate and screen magnetotactic bacterium from the sediment of a man-made open-pit mine tailings lake in Luneta, Antamok, Itogon, Benguet, Philippines for cadmium (Cd) removal. The bacterial strain isolated and purified by magnetotaxis using capillary racetrack method and grown in the selective medium Magnetospirillum Growth Medium (MSGM), designated here as strain UPB-MAG05, was inoculated at an initial concentration of 1.67 x 10^7 CFU/ml in MSGM added with different initial Cd concentrations at 0.0, 0.43, 0.67, 0.98, 1.35, and 1.84 ppm. Cd removal was measured through the change in Cd concentration in the cell-free culture supernatants using Atomic Absorption Spectroscopy (AAS) at time intervals 24, 72, 120, and 168 hours post-inoculation. Cells in the presence of Cd grew more favorably than the negative control measured at $OD_{600\text{-}650nm}$. Cells grown at 0.67 ppm Cd showed the highest cell growth. Furthermore, cell growth was still observed at 1.84 ppm exhibiting the highest Cd uptake with 48.81% decrease in Cd concentration in the cell-free supernatant after 24 hours. This suggests that this is the extreme Cd concentration tolerated by strain UPB-MAG05 which is 360-fold higher than the toxic level in humans. In general, this study reported that the Philippine magnetotactic bacterial strain UPB-MAG05 has a promising potential as a bioremediating agent for Cd-polluted waters.

KEYWORDS

bioremediation, cadmium, magnetotactic bacteria, open-pit mine tailings

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INTRODUCTION

The process of mining mineral resources entails the excavation and chemical extraction of relatively pure heavy metal components. The latter operation yields ore wastes, also known as mine tailings, which are often combined with water and are subsequently disposed (DeWitt 2000). Improperly-disposed mine tailings flow downstream emptying into natural large bodies of water such as rivers, lakes, and seas. This kind of water pollution destroys natural flora and fauna and triggers the emergence of diseases that affect human populations in the immediate vicinity as well as in communities downstream where the contaminated water might run through (Cariño and Rood 1992). Cadmium, one of the heavy metals that pollute the water environment around mining sites (Dagdeviren 2009), enters the food chain through its accumulation in many organisms notably mollusks, crustaceans, and some plants. Significant increase in cadmium levels in aquatic environments is a compelling reason for an urgent need to manage the situation through bioremediation, a relatively cost-effective way of using microorganisms to clean up contaminated sites as opposed to physico-chemical techniques that can detoxify heavy metal pollutants (De Witt 2000, Sharma 2012). Bacteria, fungi, and algae can be bioremediating agents that modify toxic substances to non-toxic forms (Coelho et al. 2015).

Magnetotactic bacteria assimilate high concentrations of iron from the environment around 200 times more than the typical bacteria to form magnetic particles of magnetite (Fe_3O_4) or greigite (Fe_3S_4) (Uebe and Schuler 2016) making them potential efficient biosorbent agents of accumulated metals from mine tailings (Coelho et al 2015). Magnetotaxis, the magnetotactic behavior of the bacteria, is exhibited by the cells by swimming along the Earth's geomagnetic fields or responding to any externally applied magnets (Aruajo 2015, Bazylinski and Frankel 2004). Magnetotactic bacteria thrive at the oxic-anoxic transition zone (OATZ) and downward to the anaerobic sediment of aquatic environments where soluble iron exists driving their multiple iron transport system robustly activated (Calugay 2003, Calugay 2004, Suzuki 204). They are a morphologically and phylogenetically diverse group of bacteria existing with different cell morphotypes typically as spirilla, vibroid, coccoid, and rod (Bazylinski and Frankel 2004) but all share the common traits of requiring low or zero oxygen (De Long et al. 1993). Magnetosomes have high affinity to heavy metal ions present in the substrate making them suitable agents for heavy metal bioremediation in water samples (Tanaka 2016, Arakaki 2002).

The Cordillera Administrative Region (CAR) located in the mountainous northern part of the Philippines is known for its rich mineral resources. Consequently, mining is an important industry in the region, ranking third behind forestry and agriculture. Small-scale operations in Luneta, Antamok, Itogon, Benguet, CAR, pose severe health risks to the local community as people are exposed to the hazardous man-made open-pit mine tailings lake or dam approximately 2 kilometers in diameter formed from decades of waste accumulation with estimated tens of thousands of tons of tailings. Miscarriages of pregnant women, diseases of the skin, respiratory tract infections, and blood poisoning have been reported as results of their exposure to toxic fumes emanating from the surrounding mining mills (Cariño and Rood 1992). Microorganisms thriving in sediments of the open-pit mine tailings lake may have acclimatized and adapted resulting in the emergence of resistance or tolerance to high concentrations of toxic heavy metals. It can be assumed, subsequently, that metal-loving magnetotactic bacteria present in the site are more efficient for bioremediation of heavy metal-polluted waters than those isolated from pristine habitats. This study, therefore, was designed to determine the presence of magnetotactic bacteria in the mine tailings lake and their potential as robust bioremediating agents due to their ability to survive under high metal concentrations. The utilization of magnetotactic bacteria as bioremediating agent is practical and convenient as their magnetosensitivity or the ability to orient and migrate to a magnetic field allows easy recovery of cells through magnetic separation method. Although the biogeographic distribution of magnetotactic bacteria is wide in all continents of the Earth (Lefevre 2013, Bazylinski and Frankel 2004), their existence in numerous aquatic environments of the Philippine archipelago, including wastewaters such as mine tailings lakes, remains unexplored.

This study aimed to isolate and screen magnetotactic bacteria from the sediment of a man-made open-pit mine tailings lake in Luneta, Antamok, Itogon, Benguet, Philippines for cadmium removal.

MATERIALS AND METHODS

Isolation of magnetotactic bacteria

Sediment samples from 5 to 10 cm depth from the surface were collected from the open-pit mine tailings lake in Luneta, Antamok, Itogon, Benguet, Philippines. Sterile glass jars with 250 ml capacity were filled to the brim with water collected near the sediment, closed tightly and stored at room temperature in the dark to inhibit the growth of photosynthetic microorganisms. Magnetotactic bacteria were selectively isolated from non-magnetic bacteria from the sediment sample through magnetotaxis induced by attaching a cylindrical neodymium-boron magnet (diameter = 1.5 inch; height = 0.5 inch) outside of the sampling jar at the sediment-water interface and then stored at room temperature. The biofilm that formed around the magnet after a week was collected with a Pasteur pipette and resuspended in 15 ml filter-sterilized natural habitat water obtained just above the sediment from the collection site. The natural habitat water medium was prepared by centrifuging the collected water at 10,000 rpm for 5 minutes and the supernatant was filtered using a 0.2 µm pore size, 29 mm diameter Cellulose Ester (CE) membrane syringe filter (Sartorius, Goettingen, Germany) to eliminate other microorganisms and magnetic debris. A magnet was again attached above the conical bottom of the 15 ml Falcon tube containing the natural water habitat medium. The resulting biofilm formed around the magnet after 3 days was re-suspended in 1.5 ml natural water habitat medium in a microcentrifuge tube. Motile cells were confirmed by wet mount and hanging drop by light microscopy.

Purification of magnetotactic bacteria by capillary racetrack

To further ensure the isolation of magnetotactic bacteria, the magnetized samples were subjected to a magneto-aerotactic process called Capillary Racetrack method (Wolfe et al 1987). The set-up consisted of a sterile Pasteur pipette filled with filtered natural habitat water positioned horizontally. The flame-sealed thin end resting on top of the neodymium-boron magnet represents the anaerobic-magnetic zone while the opposite wide end plugged with cotton previously dipped into the 1.5 ml magnetotactic bacteria suspension described above represents the aerobic zone.

Magnetotatic bacteria are either microaerobic, strict anaerobes or facultative anaerobes and therefore are magneto-aerotactic and will swim towards the flame-sealed anaerobic-magnetic zone of the Pasteur pipette away from the cotton-plugged aerobic zone. Magneto-aerotactically-separated bacteria which formed as a speck at the end of the thin sealed end of the Pasteur pipette after 3 days were recovered by using a sterile hypodermic needle and transferred into a 1.5-ml tube containing filtered

natural habitat water medium. The magneto-aerotactically collected cells were again checked microscopically as described above.

Cells were purified by standard streak-plate method on solid chemically defined and selective medium for magnetotactic bacteria, Magnetospirillum Growth Medium (MSGM) (ATCC: medium 1653) (Matsunaga et al. 1991) and stored in anaerobic containers. Purified colonies were subsequently put in 30-ml MSGM vials filled to the brim stored at room temperature in anaerobic containers. After one week, cells were transferred into 15 ml Falcon tubes filled to the brim with different concentrations of Cd and then kept in anaerobic containers.

Measurement of Cd removal from cell-free culture supernatant by Atomic Absorption Spectroscopy (AAS)

An initial cell density of 1.67 x 10^7 CFU/ml of the magnetotactic bacterial isolate measured by 0.5 Standard McFarland Turbidity Test was inoculated in 15 ml MSGM with different Cd concentrations using cadmium salt, cadmium chloride ($CdCl_2$), at 0.0, 0.43, 0.67, 0.98, 1.35, and 1.84 ppm at room temperature under anaerobic conditions as described above.

Cd removal was evaluated by measuring the change in Cd concentration in the cell-free culture supernatant of the culture medium. Cell-free culture supernatants were obtained by centrifugation at 6500 rpm for 30 minutes at 4ºC. Cd concentrations in the supernatants were measured by AAS at time intervals of 24, 72, 120, and 168 hours post-inoculation. Cell growth was measured from the cultures at the aforementioned time intervals at $OD_{600\text{-}650nm}$ prior to centrifugation. All measurements were done in triplicates.

Statistical Analysis

A two-way ANOVA was used to determine the statistical significance of the differences of Cd^{2+} concentrations in the supernatants among different initial concentrations of Cd^{2+} in the medium as well as the effect of time intervals using GraphPad Prism 7.04 Software. Tukey's multiple comparisons test was also utilized to confirm the significance of the differences observed between groups.

RESULTS AND DISCUSSION

Isolation, purification, and cultivation of magnetotactic bacterium

After magnetizing the sediment samples, subsequent use of the Capillary Racetrack method of separating magnetic cells from non-magnetic cells, growth in the selective medium MSGM, a total of 10 magnetotactic bacterial isolates were obtained from the sediment samples of the man-made open-pit mine tailings lake in Antamok, Itogon, Benguet, Philippines. Cocci cells were most prevalently observed with spiral and rod-like cells also detected but in fewer numbers. One out of the 10 purified isolates, designated here as strain UPB-MAG05, showed the strongest magnetotaxis by forming distinct biofilms on the attached magnet on the side of the culture tubes at the shortest period of 3 days. Settling of cells occurred near the region where the magnet was attached with gravity showing no influence on settling at the bottom of the tube unlike in typical non-magnetic bacteria. This was observed repeatedly after cells were subcultured from one tube to another after 3 to 7 days indicating strong magnetotaxis. This strain, subsequently, was chosen for the Cd removal assay.

Cell growth of strain UPB-MAG05 under different concentrations of cadmium

An inverse relationship between the growth of strain UPB-MAG05 and Cd^{2+} concentration was found (Fig. 1). In cultures

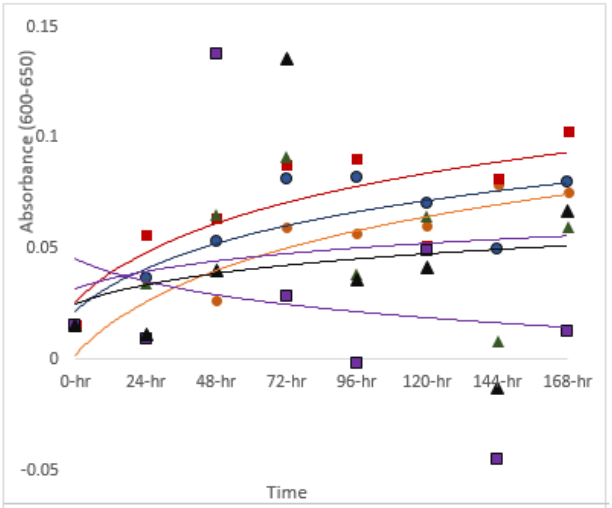

Figure 1: Growth of strain UPB-MAG05 inoculated in different cadmium concentrations (0.0 ppm, 0.43 ppm, 0.67 ppm, 0.98 ppm, 1.35 ppm, and 1.84 ppm), at room temperature under anaerobic conditions. Growth was measured daily for 7 days.

with higher Cd concentrations, a trend of decreasing cell growth was observed. Cells still thrived but decreased in number at the two highest concentrations of Cd, at 1.35 ppm and 1.84ppm, implying that the Minimum Inhibitory Concentration (MIC) is higher than 1.84 ppm. Remarkably, 1.84 ppm is 360 times higher than the borderline toxic level of cadmium in humans which is 0.005 ppm as set by the Agency for Toxic Substances and Disease Registry. Among all concentrations tested, cells grew most favorably at 0.43 ppm and 0.67 ppm, considerably above the toxic level, suggesting a robust tolerance or resistance of strain UPB-MAG05 to toxic levels of Cd.

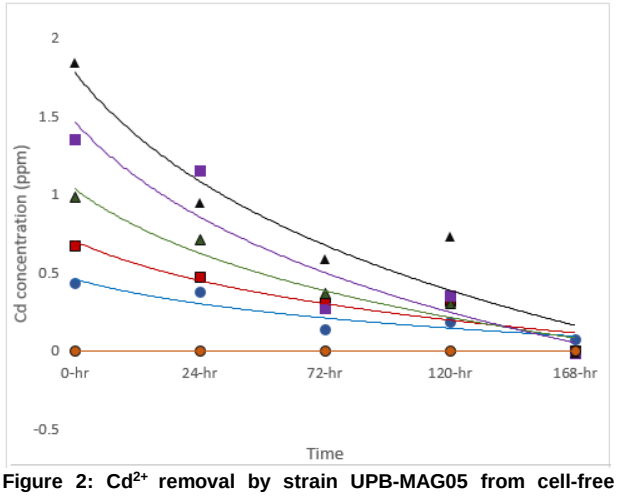

Figure 2: Cd^{2+} removal by strain UPB-MAG05 from cell-free culture supernatants with different initial cadmium concentrations (0.0 ppm, 0.43 ppm, 0.67 ppm, 0.98 ppm, 1.35 ppm, and 1.84 ppm).

Cd removal from cell-free culture supernatants by strain UPB-MAG05

An abrupt decrease in Cd with higher concentrations of 0.98, 1.35 and 1.84 ppm, was observed relative to lower concentrations of 0.43 and 0.67 ppm which was exhibited during a 24 to 72-hr period (Figure 2). This could be due to the fact that negatively charged bacterial cell walls bind to the positively charged Cd^{2+} ions, immobilizing the metal and inhibiting its intracellular toxic effects which is the fundamental strategy to avoid the toxic effects of Cd^{2+} ions in bacterial species.

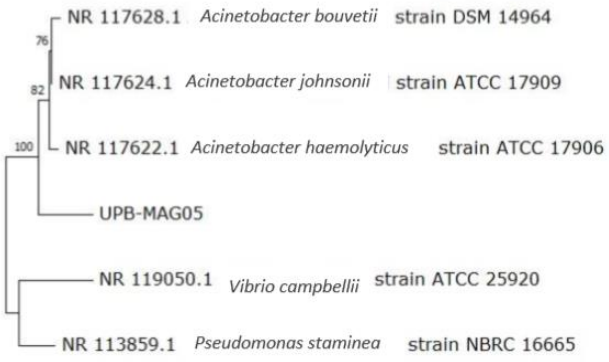

Figure 3: Phylogenetic tree showing genomic affiliation of UPB-MAG05 strain with taxonomically highly related species identified based on 16S rRNA sequences through MEGA 07 Software.

Therefore, in high concentration of Cd^{2+}, the high amount of Cd^{2+} ions will facilitate more interactions with bacterial cell wall resulting to high uptake of Cd^{2+} ions (Dlugonski 2016). More specifically, 1.84 ppm of Cd resulted to a 48.81% decrease at 24 hours post-inoculation, followed by 0.67 ppm with 29.45%, 0.98 ppm with 27.93%, then 1.35 ppm with 14.62%, and lastly by 0.43 ppm with 11.53%.

The two-way ANOVA showed that the exposure time of isolate to the medium with Cd^{2+} and the initial concentration of Cd^{2+} in the medium had significant effects on the Cd^{2+} levels in the cell-free supernatant with p values of <0.0001 and 0.0334, respectively (p<0.05). The statistical analysis likewise estimated a significant interaction between the exposure time of isolate and the initial concentration of Cd^{2+} in the medium with a p value of 0.0109 (p<0.05). Further, a post hoc Tukey's multiple comparisons test showed that 0.43 ppm vs. 1.35 ppm, 0.43 ppm vs. 1.84 ppm, 0.67 ppm vs. 1.35 ppm, and 0.67 ppm vs. 1.84 ppm differed significantly at p values 0.0003, 0.0112, 0.0014, and 0.0474, respectively, at 24h post-inoculation. Only 0.43 ppm and 1.84 ppm were significantly different at 48h after inoculation at p value 0.0139 (p<0.05). There were no significant differences between concentrations at p>0.05in various pairs at all time intervals.

The strain UPB-MAG05 may have developed resistance through bioaccumulation of cadmium ions inside the cell, particularly on the membrane fraction inside the cytoplasm, thus decreasing the Cd^{2+} concentration similar to the findings of Iriwati et al. (2016) on heavy metal biosorption by the copper-resistant bacterium *Acinetobacter* sp. IrC2.

In the study conducted by Arakaki et al. (2002), the bacterial strain *Desulfovibrio magneticus* precipitated less than 95% of cadmium at an initial concentration of 1.3 ppm in the growth medium. In particular, mechanism for cadmium recovery is not a simple adsorption on the cell wall. Results from studies on fractionated cells showed that majority of the cadmium recovered from the medium was approximately 80% in insoluble form which did not desorb with EDTA. Thus, it was assumed that cadmium was stably existing on the cell surface as tightly bound to the membrane or as inorganic cadmium precipitates. Other studies also supported the biosorbent capability of magnetotactic bacteria (MTB). One such study was on biomagnetic recovery of selenium conducted by Tanaka et al. (2016) which showed that cell growth was negatively affected by the increase of SeO_3^{2-} concentration and that no cell growth was observed at $\geq$ 250 μM. Likewise, biomagnetic recovery using *Magnetotacticum magneticum* was $3.6x10^8$ Se atoms per cell recovery. However, some Se atoms were lost during the process as indicated by a reduced value of $3.0x10^8$ Se atoms. Huiping et al. (2007), in a study on biosorption equilibrium and kinetics of Au (III) and Cu (II) on magnetotactic bacteria, found that pH level was the most important factor during the biosorption process as this strongly influences the overall solution chemistry of the heavy metals, which includes, hydrolysis, complexation by organic and/or inorganic ligands, redox reactions, and precipitation. Likewise, it was shown that Cu (II) has a "catalyzing" effect on Au (III) sorption with MTB, in which the maximum adsorption capacity for Au (III) nearly doubled as that in the single system while that for Cu (II) sharply dropped. The study underscored the biosorbent capability of MTB and the simple, effective and environment-friendly method of recovery through magnetic separation.

Generally, magnetotactic bacterial strain UPB-MAG05, purified and grown at 1.84 ppm, exhibited robust uptake of Cd resulting in a 48.81% decrease in Cd concentration after 24 hours. This concentration, apparently the highest tolerated by UPB-MAG05, is 360-fold higher than the toxic level in humans. This strain also tolerated other high concentrations of Cd tested in this study. Such tolerance is due to the propensity of magnetotactic bacteria to assimilate large amounts of iron and to the heavy metal-contaminated nature of the open pit mine environment from where it was isolated.

The result obtained from 16S rRNA gene sequencing when compared to the NCBI gene bank database using BLAST search program (https://www.ncbi.nlm.nih.gov/) showed a match closest and with 96% identity to *Acinetobacter johnsonii* which is affiliated with Class Gammaproteobacteria of the Proteobacteria, a phylum consisting of 250 genera. To date, there have been only 2 reports of magnetotactic bacteria that belong to this group (Simmons et al. 2004, Lefevre et al. 2012).

Phylogenetic analysis showed that strain UPB-MAG05 clustered with other *Acinetobacter* spp. (Figure 3). Iriwati et al. (2016) reported *Acinetobacter* sp. IrC2 to be copper-resistant. Strain UPB-MAG05 may possibly be the latest magnetotactic bacterial addition to this class. It is recommended that other markers, such as *mam* genes located in the magnetosome island (MAI) which is a cluster of genes not found in non-magnetic cells, be used to confirm the identity of the strain. The present work of the bacterium, isolated from the sediments of a mine-tailings lake and which consistently exhibited magnetotaxis and robust uptake of high concentrations of heavy metal, is a pioneering study on the characteristic traits of magnetotactic bacteria.

Mine tailings remain one of the major threats to the environment as they destroy natural flora and fauna and pose major health hazards to people in the immediate communities, as well as pollute surrounding bodies of waters such as rivers. Novel sources of toxic heavy metal biosorbent or bioremediating agents, such as metal- or iron-loving bacteria particularly the magnetotactic bacteria thriving in the sediments of mine tailing water bodies, are excellent candidates as they have already acclimatized and adapted to the toxic environment. The results

of the present study revealed the novelty and robust potential of isolated Philippine magnetotactic bacterial strains as efficient bioremediating agents for Cd-polluted waters.

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CONFLICTS OF INTEREST

RJ Calugay, AIJ Aguilar, JR Liwag, PS Ramos, and OD Giron declare that they have no conflicts of interest.

CONTRIBUTIONS OF INDIVIDUAL AUTHORS

The conceptual framework and experimental set-ups were prepared by RJ Calugay and OD Giron. Laboratory work, data analyses, and manuscript preparation were done by RJ Calugay, AIJ Aguilar, JR Liwag and PS Ramos.

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