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Bacteria that can tolerate and decontaminate cadmium and lead contaminated rice paddy soil

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Abstract One of the current issues in rice-growing regions is heavy metals contaminated rice paddy. The environment and the health of people, animals, plants, and crops are negatively impacted by high levels of cadmium (Cd) and lead (Pb). The present study isolated and identified potential microbial remediators from the rice growing area contaminated with heavy metals at Sitio Namangonan, Guiset Norte, San Manuel, Pangasinan, Luzon Island, Philippines. The bacterial isolates were molecularly identified using 16S ribosomal RNA gene sequencing, the Basic Local Alignment Search Tool (BLAST), and performed evolutionary analyses using MEGA 11. The soil sample contained 0.42 mg/kg cadmium and 57.80 mg/kg lead. Five species of bacteria (BI-1, BI-2, BI-3, BI-4, and BI-5) namely: *Priestia flexa* BI-1, *Priestia megaterium* BI-2, *Stenotrophomonas maltophilia* BI-3; *P. megaterium* BI-4; and *P. megaterium* BI-5 with 98.94%, 98.47%, 92.53%, 84.21%, and 99.67% similarity, respectively, were isolated from the soil contaminated with cadmium and lead. Furthermore, *P. megaterium* BI-2, *Stenotrophomonas maltophilia* BI-3, *Priestia megaterium* BI-4, and *P. megaterium* BI-5 are tolerant to up to 1000 mg/kg cadmium concentration while *P. flexa* BI-1 is identified as non-tolerant to cadmium contamination. Additionally, *Priestia megaterium* BI-2, *S. maltophilia* BI-3, *P. megaterium* BI-4, and *P. megaterium* BI-5 are resistant to lead concentrations of up to 1000 mg/kg. Therefore, in cadmium and lead-contaminated rice paddy soil, the bacterial isolates are resistant to heavy metals. Finally, these bacterial isolates could clean up lead and cadmium-contaminated rice paddy soil.

Keywords: Cadmium-resistant bacteria, Lead-resistant bacteria, Contaminated soil, Molecular identification

Introduction

Heavy metals and metalloid such as arsenic, cadmium, mercury, lead, and chromium are classified as environmental contaminants because they are

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hazardous, persist in the atmosphere, and accumulate in the human body through bioaccumulation. Pollution from toxic heavy metals both in terrestrial and aquatic ecosystems is a major environmental concern that will affect public health. Due to anthropogenic sources, some heavy metals become pollutants in the environment, however, most heavy metals occur naturally (Mitra *et al.*, 2022). In addition, the use of heavy metals in a variety of industrial processes and/or materials, such as color pigments and alloys, is known to expose workers to these metals at work that can cause exposure to the toxic heavy metals (Rehman *et al.*, 2018).

Apart from being environmental pollutant, cadmium is toxic to human and can cause several diseases. Exposure to cadmium may be related to cancer, including breast, lung, prostate, nasopharynx, pancreas, and kidney cancers. The extremely sensitive part of the human's body to the exposure of cadmium is the liver and kidneys (Genchi *et al.*, 2020; Charkiewicz *et al.*, 2023). It has also been implicated in the development of hypertension and various types of cancer (Bernard and Lauwerys, 1986). Due to environmental exposure and the consumption of tainted food, Cd builds up and biomagnifies in human bodies. Human health issues include diarrhea, stomachaches, bone fractures, infertility and possibly other reproductive failures, immune system and central nervous system damage, psychological disorders, and more. By preventing the creation of proteins, Cd can potentially turn healthy epithelial cells into cancerous ones (Sharma *et al.*, 2015).

The toxic pollutant lead (Pb) can come from soil in a variety of ways. There are several forms of lead, including: (1) surface assimilation to minerals from soil and carbon-based segments or changes as different complexes; (2) Pb is an independent ion in the groundwater under pressure; and (3) Pb complexed with different carbon and non-carbon ligands. Pb is the most accessible ion from soil and exists independently in soil composites (Meers *et al.*, 2009). Because of lead poisoning, the community is at risk from eating staple crops grown near the mine, particularly rice and vegetables (Zhuang *et al.*, 2009). Plants, animals, and the ecosystem are all at risk from lead (Pb). This element's contamination of soils can lead to warm temperatures, which can have a significant impact on people, domesticated and wild aquatic life, the growth of cities and rural areas, and natural ecosystems. Low pH or acidity of the air, water, and soil can lead to human-caused pollution, wetness, and a seasonally higher temperature. Pb is more readily available and mobile in environments that are acidic, increasing exposure to people, animals, and plants (Levin *et al.*, 2019).

One important alternative answer to problems is the use of microorganisms. Because of their incredible metabolic activity, microorganisms can survive anywhere on the biosphere and can emerge in a wide variety of

environmental settings. Because microorganisms have a wide range of nutritional capacities, they are exploited in environmental pollution bioremediation. Through the comprehensive action of microorganisms, bioremediation plays a significant role in the degradation, eradication, immobilization, or detoxification of various chemical wastes and physical harmful elements from the environment (Abatenh *et al.*, 2017). Utilizing biological agents like microorganisms, bioremediation eliminates or mitigates the impacts of environmental contaminants. Because of their rapid growth and ease of manipulation, which enhances their potential as bioremediation agents, microorganisms are used more frequently than the other. Many bacterial species have been employed to eliminate various environmental pollutants (Hakeem *et al.*, 2015; Ayilara and Babalola, 2023).

Agricultural land area contaminated with heavy metals, specifically the Cd and Pb, is one of the main ecological problems of the rice farmers. The stunted growth and low yield of rice are the main constraints of the contaminated soils with heavy metals. One of the best solutions is the application of bacterial bioremediators that thrive in the contaminated soils with heavy metals. These bacteria convert the toxic heavy metals into a non-toxic form. Moreover, the aim of this study was to isolate and identify the bacteria present in the soils contaminated with Cd and Pb as potential bioremediators. Thus, knowing the bacteria are capable of degrading and eliminating the environmental pollutants in the rice-growing area. This can provide safer agricultural production for the farmers, including consumers.

Materials and methods

Collection of soil samples

During the dry season of 2024, soil samples were taken from Sitio Namangonan, Guiset Norte, San Manuel, Pangasinan. Approximately 9,000 grams of composite samples were created by combining collected soil samples from nine sampling location points at a depth of 20 cm. After being put in a sterile plastic tub, the composite soil samples were sent right away to the laboratory for Cd- and Pb-analysis and isolation of soil-borne bacteria.

Analysis of cadmium and lead contents of soil samples

Aqua regia, or a 1:3 mixture of concentrated HNO_3 and concentrated HCl, was used to extract soil samples. Concentration of heavy metals specifically

cadmium and lead in the soil samples were evaluated through analytical methods of Shimadzu atomic absorption spectrophotometry (AAS).

Isolation of soil-borne bacteria as bioremediators

The soil-borne bacteria in the area were isolated using the serial dilution approach. Ninety milliliters (90 mL) of sterile distilled water (dH_2O) were mixed with ten grams (10 g) of air-dried soil sample. For half an hour (30 min), the suspension was vortexed. Nine milliliters of sterile dH_2O were mixed with one milliliter of suspension using a sterile pipette. A milliliter (1 mL) of the prior suspension was diluted five times in nine milliliters of sterile dH_2O. The values of the serial dilutions ranged from 10^{-1} to 10^{-5}.

The soil-borne bacteria were isolated from the freshly collected soil sample. The one hundred micro milliliters (0.1 mL) of each of the five suspensions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5}) was spread in sterile Petri plates. Warmed and melted sterile nutrient agar (NA) was dispensed in sterilized Petri plates with proper labels. The lid of the Petri plates was sealed with parafilm. The NA plates was incubated to allow the exponential growth of bacterial colonies in ambient room conditions (27°C) for 18 to 24 of incubation. The inoculating needle was used to pick individually the distinct bacterial colonies and aseptically transfer them to the test tubes with NA broth containing 100 mg (0.1%) peptone and 400 mg (0.4%) beef extract/L. These test tubes were served as sources of bacterial isolates and are known as stock cultures.

Purification of bacterial isolates

Bacterial suspension from stock culture was transferred using the streak-plating method. The sterile wire loop was dipped in the stock culture and streak by continuously stroking the wire loop in zigzag motion (back and forth) touching the main streak on the surface of sterile NA. The NA plates was incubated to allow the exponential growth of bacterial colonies at ambient room conditions (27°C) for 18 to 24 hours of incubation. The pure culture of bacterial isolates was expected that the colonies have with similar appearance. The pure culture of bacterial isolates was photographed for documentation.

Molecular identification of bacterial isolates

The NA plates with bacterial isolates, underwent colony PCR method using 16S ribosomal RNA gene sequencing. Before the dilution of the final component, the single colony of the bacterial isolate was transferred to a 1.5 mL tube. For the

25 µl final component concentration for each sample was composed of 1.0 µl of DNA extract, 0.25 µl 27Forward (5'-AGA GTT TGA TCM TGG CTC AG-3') primer (Lane, 1991), 0.25 µl 1492Reverse (5'-CGG TTA CCT TGT TAC GAC TT-3') primer (Lane, 1991), 5.0 µl 5x PCR Buffer (KAPA), 18.0 µl sterilized distilled water and 0.5 Taq Polymerase (KAPA). To amplify the gene, the PCR profile was made up of 34 cycles with initial denaturation at 95°C for 1 min, denaturation at 95°C for 15 seconds, annealing at 51.5 for 15 seconds, extension at 72°C for 10 seconds, final extension 72°C for 5 min and final hold at 12°C. One (1) µl of amplification products and the 1kb DNA ladder stained with 1 µl gel red (Biotium) was run for 30 min at 100 V on 1.0% agarose gel (prepared in 1x TAE) and was analyzed using gel photo documentation system (Labnet GDS-1302 Enduro Imaging System).

Sequencing analyses for bacterial isolates

After the confirmation of the anticipated size of amplified fragments, the purification and sequencing of PCR products was done by Apical Scientific Sequencing in Malaysia as a service provider. The chromatogram data was assessed using 4Peaks sequencing analysis and run to The Basic Local Alignment Search Tool – National Center for Biotechnology Information (BLAST - NBCI).

Phylogenetic analysis for bacterial isolates

The evolutionary history of the bacterial isolates was determined using the Tamura-Nei model and the Maximum Likelihood approach (Tamaru and Neil, 1993). 500 replicates were used to infer the bootstrap consensus tree (Felsenstein, 1985). Neighbor-Join and BioNJ algorithms were used to determine the starting tree or trees and highest log probability for heuristic search. The Tamura-Nei model was used to estimate the matrix of pairwise distances and choose the topology with the highest log likelihood value. The number of substitutions per site in the phylogenetic tree was calculated and estimated using the draw scale with branch lengths. The final data set of the phylogenetic tree's nucleotide sequences and locations were identified and quantified. MEGA11 was used for evolutionary analysis (Tamura *et al*., 2021).

Effect of cadmium and lead concentration on bacterial growth

The center of NA culture media containing varying concentrations of Cd and Pb (0, 10, 100, and 1000 mg/kg) was inoculated with an inoculum (10 mm

disk) from the two-day-old culture of the bacterial isolates, with four replications. Every plate was incubated at either ambient temperature or 27°C. After 24 and 48 hours of incubation, the bacterial growth was seen. Tolerant species are bacteria that can survive in concentrations of heavy metals between 100 and 1000 mg/kg of Cd and Pb.

Results

Heavy metals concentration of soil

Lead and cadmium levels were measured to confirm the presence of these two dangerous heavy metals in the soil samples. The soil sample contains 0.42 mg/kg of cadmium and 57.80 mg/kg of lead.

Molecular Identification of the bacterial isolates

Five bacterial isolates were molecularly identified namely: *Priestia flexa* BI-1, *Priestia megaterium* BI-2, *Stenotrophomonas maltophilia* BI-3, *Priestia megaterium* BI-4, and *Priestia megaterium* BI-5 with 98.94%, 98.47%, 92.53.21%, 84.21%, and 99.67% similarity, respectively and compared to the available accessions in BLAST-NCBI (Table 1).

Table 1. Molecular identification of bacterial isolates from contaminated soil with heavy metals

Code	Species of Bacterial Isolates	Query Cover	Identity	Accession No.
BI-1	*Priestia flexa*	100%	98.94%	KY818996.1
BI-2	*Priestia megaterium*	100%	98.47%	MZ700082.1
BI-3	*Stenotrophomonas maltophilia*	90%	92.53%	EU442189.1
BI-4	*Priestia megaterium*	83%	84.21%	MT145964.1
BI-5	*Priestia megaterium*	100%	99.67%	MF974591.1

Phylogenetic analysis of the bacterial isolates

The phylogenetic tree analysis with bootstrap values was used to identify the five bacterial isolates (BI-1, BI-2, BI-3, BI-4, and BI-5) from contaminated rice paddy soil. Based on the phylogenetic tree analysis, the five bacterial isolates were identified as *Priestia flexa* BI-1, *Priestia megaterium* BI-2, *Stenotrophomonas maltophilia* BI-3, *Priestia megaterium* BI-4, and *P.* BI-5 with boostrap values of 98%, 81%, 71%, 99%, and 92%, respectively (Figure 1).

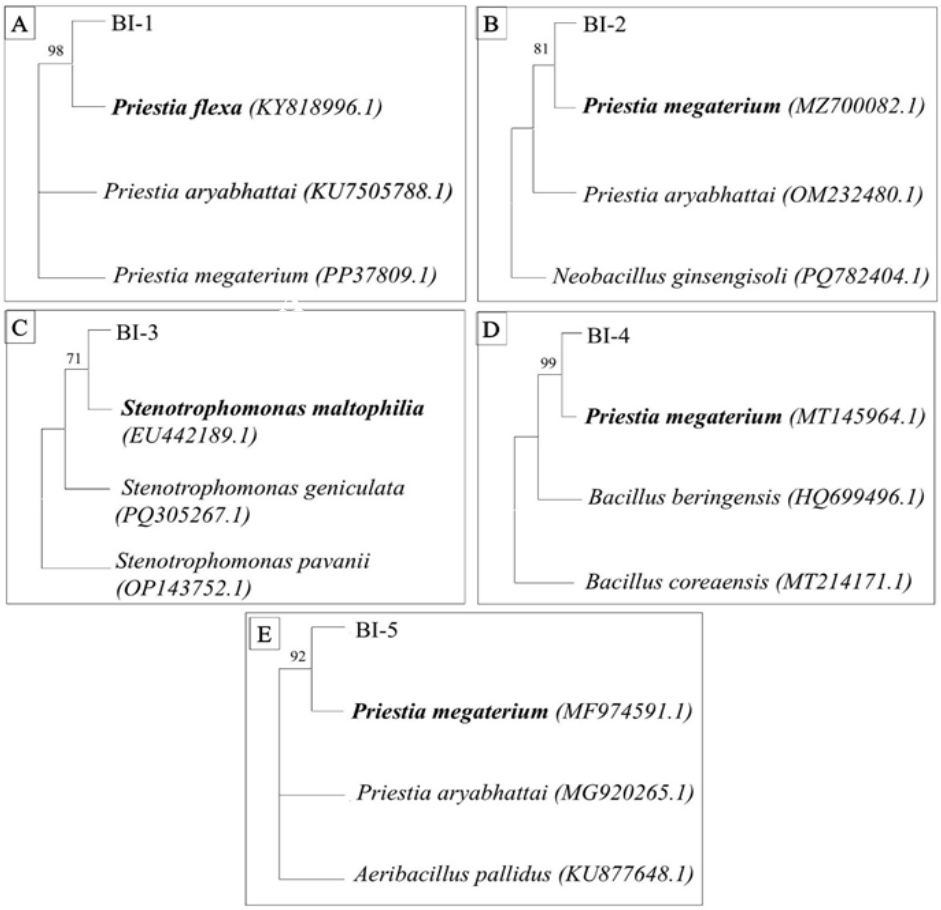

Figure 1. Phylogenetic analysis of the five bacterial isolates inferred using 16S RNA: **(A)** *Priestia flexa* BI-1, **(B)** *Priestia megaterium* BI-2, **(C)** *Stenotrophomonas maltophilia* BI-3, **(D)** *Priestia megaterium* BI-4, and **(E)** *Priestia megaterium* BI-5. This phylogenetic was done using the maximum likelihood method and Tamura-Nei model with a bootstrap analysis with 500 replicates.

Bacterial growth and density on different concentration of cadmium

To determine the potential of the isolated bacterial species from heavy metal-contaminated soil, they were cultured on nutrient agar (NA) containing different concentrations of cadmium (0, 10, 100, and 1000 mg/kg). At 24- and 48-hour incubations, the five bacterial isolates namely, *P. megaterium* BI-2, *S. maltophilia* BI-3, *P. megaterium* BI-4, and *P. megaterium* BI-5 were identified as tolerant to 10 to 1000 mg/kg of cadmium concentration. However, *P. flexa* BI-

1 was identified as non-tolerant at 10 to 1000 mg/kg of cadmium concentrations (Table 2).

Table 2. Bacterial tolerance of *Priestia* spp., and *Stenotrophomona* sp. from heavy metal contaminated soil on nutrient agar media with different cadmium concentration at 24- and 48-hour incubations

Cadmium concentration (mg/kg)	Incubation period (hours)	Bacterial Tolerance				
		P. flexa BI-1	*P. megaterium* BI-2	*S. maltophilia* BI-3	*P. megaterium* BI-4	*P. megaterium* BI-5
0	24	+	+	+	+	+
	48	+	+	+	+	+
10	24	-	+	+	+	+
	48	-	+	+	+	+
100	24	-	+	+	+	+
	48	-	+	+	+	+
1000	24	-	+	+	+	+
	48	-	+	+	+	+

Colonial density: with growth and tolerant (+), no growth and non-tolerant (–)

Bacterial growth and density on different concentrations of lead

The influence of various concentrations of lead (0, 10, 100, and 1000 mg/kg) on the colonial growth of *Priestia flexa* BI-1, *Priestia megaterium* BI-2, *Stenotrophomonas maltophilia* BI-3, *P. megaterium* BI-4, and *P.* BI-5 was evaluated. At 24- and 48-hour incubations, the five bacterial isolates such as *P. flexa* BI-1, *P. megaterium* BI-2, *S. maltophilia* BI-3, *P. megaterium* BI-4, and *P. megaterium* BI-5 were identified as tolerant at 10 to 1000 mg/kg of lead concentrations (Table 3).

Table 3. Bacterial tolerance of *Priestia* spp., and *Stenotrophomona* sp. from heavy metal contaminated soil on nutrient agar media with different lead concentration at 24- and 48-hour incubations

Lead concentration (mg/kg)	Incubation period (hours)	Bacterial Tolerance				
		P. flexa BI-1	*P. megaterium* BI-2	*S. maltophilia* BI-3	*P. megaterium* BI-4	*P. megaterium* BI-5
0	24	+	+	+	+	+
	48	+	+	+	+	+
10	24	+	+	+	+	+
	48	+	+	+	+	+
100	24	+	+	+	+	+
	48	+	+	+	+	+
1000	24	+	+	+	+	+
	48	+	+	+	+	+

Colonial density: with growth and tolerant (+), no growth and non-tolerant (–)

Discussion

This study reports and documents the existence of the five species of bacterial isolates (*Priestia flexa* BI-1, *Priestia megaterium* BI-2, *Stenotrophomonas maltophilia* BI-3, *Priestia megaterium* BI-4, and *Priestia megaterium* BI-5) in rice paddy soil contaminated with lead and cadmium. This work supports previous findings showing soil contaminated with cadmium and lead contained various bacterial species (Kumari *et al.*, 2015; Liaquat *et al.*, 2020; Tam *et al.*, 2022). It was shown in this investigation that several bacterial isolates showed distinct tolerance to different concentrations of cadmium and lead. The tolerance of the bacterial isolates demonstrated their capacity to absorb the heavy metal-contaminated soil (Ramos *et al.*, 2020; Soto-Valera *et al.*, 2024). High cellular accumulation and molecular mechanisms of the bacterial isolates allow them to tolerate heavy metal-contaminated soil (Ikhimiukor and Adelowo, 2019). Their capacity to detoxify, break down, remove, and reduce the availability of heavy metals in soils is the most effective way to identify them as bioremediators (Ghosh and Saha, 2013; Liu *et al.*, 2024; Sevak *et al.*, 2024).

Previous research has documented and validated the potential use of bacteria in bioremediation. For example, according to Alzahrani *et al.* (2022), *P. megaterium* is an excellent choice for high cadmium concentrations in water remediation since it is cadmium-tolerant at concentrations of 10 to 1000 mg/kg. Furthermore, according to earlier work by Chie *et al.* (2007), *S. maltophilia* was shown to be a cadmium-resistant bacterium with inhibitory ability and coping mechanisms due to its fast growth and multiplication rates. However, according to Soto-Valera *et al.* (2024), *Priestia flexa* is not tolerant of high cadmium concentrations and is not tolerant of concentrations between 10 and 1000 mg/kg. Nonetheless, *Priestia flexa* was found to be resistant to elevated levels of mercury, lead, and chromium. Additionally, Zelaya-Molina *et al.* (2023) and Guzmán-Moreno *et al.* (2023) confirmed that *Priestia megaterium* is regarded as a heavy metal-resistant bacterium in addition to being a plant growth-promoting bacterium. Furthermore, Ojo *et al.* (2023) also explained that the *P. megaterium* can resist high concentrations of lead. This bacterium is identified as the best candidate for microbial remediation. Moreover, according to Wierzba (2015) and Ikhimiukor and Adelowo (2019), *S. maltophilia* is also known for the useful biosorption of lead and other heavy metals in industrial wastewater.

In summary, the study reported the successful isolation and molecular identification of bacterial isolates, namely, *Priestia flexa*, *Stenotrophomonas maltophilia*, and *Priestia megaterium*, from rice paddy soil contaminated with heavy metals, specifically cadmium and lead. It can suggest their potential as microbial remediators, which needs to be investigated in future studies.

Conflict of interest

No conflict of interest declares by the authors.

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