

Characterization of Pb-tolerant Plant-growth-promoting Endophytic Bacteria for Biosorption Potential, Isolated from Roots of Pb Excluders Grown in Different Habitats

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ABSTRACT

Bioremediation using metal-tolerant plant-growth-promoting endophytic bacteria has been studied. The biosorption potential of endophytic bacteria isolated from roots of non-metalliferous Pb excluders (*Acacia mangium* and *Eucalyptus camaldulensis*), and a metalliferous Pb excluder (*Pityrogramma calomelanos*) was evaluated. Five isolates were selected and designated as "Pc", "Pe", "Ai", "Aj", and "El". Phylogenetic reconstruction suggested that strain Ai was closely related to *Serratia proteamaculans*, Aj to *Pseudomonas* sp., El to *Bacillus cereus*, Pc to *Pseudomonas psychrophila*, and Pe to *Pseudomonas veronii*. They could equally tolerate Pb. Most of them had the capacity to produce siderophores and solubilize phosphate, except *B. cereus*. However, *B. cereus* showed high capacity of Pb uptake (4.54 ± 0.38 mg/g) and removal ($8.36 \pm 0.70\%$) with no significant difference ($p > 0.05$) from the other strains, except *P. psychrophila* (1.36 ± 0.23 mg/g of Pb uptake, and $2.60 \pm 0.44\%$ Pb removal). The results suggest that biosorption capacity may not involve the habitat of a plant host. Plant-growth-promoting traits were not the only factor for biosorption by endophytic bacteria. *S. proteamaculans*, *B. cereus*, and *P. veronii* showed the same Pb biosorption. Strains closely related to *P. veronii* could be promoted as candidates for the removal of Pb in polluted environments.

1. INTRODUCTION

Lead (Pb) toxicity can occur to all organisms living in the world (Bano et al., 2018). However, it is valuable for many industries, and it is also necessary for modern life (Sharma and Dubey, 2005; Gillani et al., 2017; Tseveendorj et al., 2017). Pb is used continuously, and the environment has Pb contamination unavoidably. Effective alternative methods for Pb removal are needed. Conventional methods are used to remediate and stabilize metals (including Pb) in the environment. These methods are precipitation, reverse osmosis, ion exchange, filtration, electrochemical treatment, membrane technologies, solvent extraction, adsorption, etc. (Gillani et al., 2017; Tseveendorj et al., 2017). However, these methods have their own disadvantage

in application such as being too expensive or inefficient, and they release toxic waste (Tseveendorj et al., 2017).

Bioremediation strategies using microorganisms (e.g., bacteria, yeast, algae, and fungi) to remediate Pb are becoming more attractive in contrast to the conventional methods. Bioremediation methods are eco-friendly and less expensive (Bhatnagar and Kumari, 2013; Govarthanan et al., 2016; Kumar and Fulekar, 2018). Although microorganisms cannot degrade and destroy heavy metals, they can transform them to less toxic substance to reduce their toxicity (Gupta et al., 2016). Biosorption using living and dead microorganisms as biosorbents can be used to remove heavy metals via a passive adsorption mechanism (Coelho et al.,

2015). It is fast, occurring in a few minutes, and reversible. Biosorption occurs under normal conditions (e.g., pressure and temperature) (Aslam et al., 2010; Coelho et al., 2015). In addition, there are many factors (i.e. pH, ionic strength, temperature, biosorbent concentration, and other ions in the solution) that influence the biosorption of metals (Coelho et al., 2015). Among microorganisms, bacteria are important candidates that are largely studied for their bioremediation potential (Gupta et al., 2016).

Currently, the biosorption of heavy metals by metal-tolerant endophytic bacteria (MTEB) with plant-growth-promoting traits (PGPT) is of great interest (Govarthanan et al., 2016; Ma et al., 2016). Bacteria colonizing in the healthy plant's tissues with little negative effects on the host are known as endophytic bacteria (EB). They are widely found in many plant species (Govarthanan et al., 2016). Endophytic bacteria (used for Pb removal) can transform Pb via various mechanisms (e.g., methylation, demethylation, and redox reactions). The bacterial cell surface contains many functional groups (e.g., carbonyl, carboxyl, hydroxyl, sulfhydryl, and phosphodiester groups), and they have key roles in the biosorption mechanism (Abdia and Kazemi, 2015). Pb-tolerant EB can be found in plants that accumulate a high Pb content in their tissues. Normally, they can be isolated from hyperaccumulators (e.g., *Pteris vittata* L. and *Pteris multifida* Poir.). Endophytic bacteria extracted from these hyperaccumulator ferns had PGPT leading to significant metal remediation (Zhu et al., 2014). However, biosorption by Pb-tolerant EB, especially when isolated from the roots of a Pb-excluder grown in diverse environmental habitats (between normal and contaminated sites), are not much investigated. The bacterial endophyte of phytostabilizer growing in the soil contaminated with high concentration of Pb and accumulating high levels of Pb in the root tissues, can tolerate a high amount of Pb due to the stabilizing ability of its host. It is considered as a novel biosorbent for Pb. Naturally, Pb polluted soil is rare, so the non-metalliferous Pb phytostabilizer is an alternative plant host to discover Pb-tolerant endophytic bacteria with plant-growth-promoting traits for the removal of Pb.

The criterion used to select phytostabilizer plants in this study was their ability to accumulate high Pb concentration in root tissue. Plants reported as Pb phytostabilizers with a bioconcentration factor

greater than 1 and translocation factor less than 1 were: *Pityrogramma calomelanos* (L.) Link, grown in a Pb contaminated site; and *Acacia mangium* Willd. and *Eucalyptus camaldulensis* Dehnh., grown in non-contaminated sites (Soongsombat et al., 2009; Meeinkuirt et al., 2012; Yongpisanphop et al., 2019). *P. calomelanos* (L.) Link belongs to the Pteridaceae family, and it is widely grown in various parts of Thailand. *P. calomelanos* is an arsenic hyperaccumulator (Francesconi et al., 2002), and Pb phytostabilizer (Soongsombat et al., 2009). *A. mangium* belongs to the Fabaceae family, while *E. camaldulensis* belongs to the Myrtaceae family. They are fast-growing trees, and have phytoremediation potential for Pb remediation (Yongpisanphop et al., 2017). For Pb contaminated areas, *P. calomelanos* was selected based on having the highest Pb concentration in root tissue amongst the plants collected. For plants grown in non-contaminated area, *A. mangium* and *E. camaldulensis* were selected based on the highest Pb concentration in root tissue according to hydroponic experiments.

This study screened Pb tolerant EB from *Acacia mangium*, *Eucalyptus camaldulensis*, and *Pityrogramma calomelanos*, and evaluated the Pb biosorption potential of isolates under *in vitro* conditions.

2. METHODOLOGY

2.1 Screen of Pb-tolerant EB

Six healthy *P. calomelanos* plants were collected from Pb contaminated soil, while those of *A. mangium* and *E. camaldulensis* were obtained from the Chatuchak market, Bangkok, Thailand. The roots were cleaned using a surface disinfection technique. The roots were cleaned with 70% ethanol for 40 seconds, followed by 2.5% sodium hypochlorite plus a droplet of polyoxyethylene 80, with gentle shaking for 15 min for surface disinfection before extraction (Luo et al., 2011). The endophytic bacteria were isolated, characterized, and identified according to Yongpisanphop et al. (2019). The obtained sequences were aligned by the BLAST tool on the NCBI website. The phylogenetic tree of partial 16S rRNA gene sequences was reconstructed by the Neighbor-Joining method (Saitou and Nei, 1987) based on 1,000 bootstrap replicates (Felsenstein, 1985), and implemented by MEGA7 (Kumar et al., 2016). Kimura 2-parameter model (Kimura, 1980) was used to estimate the evolutionary distances of the tree.

2.2 Pb biosorption

The biosorption experiment was set to investigate the bioremediation potential of the EB isolates. This experiment was carried out under the optimum conditions reported for the maximum Pb biosorption by living bacterial cells: 10 min, pH ranging from 5 to 6, 100 mg/L of initial concentration (Wierzba and Latala, 2010). Pre-culture of each EB isolate was prepared by transferring a loop of each (pure) fresh colony into LB broth, and incubated on a shaker under optimal conditions (100 rpm, 30±2°C, and 48 h). Pre-culture (100 µL) was added to LB medium broth (30 mL), and incubated on a rotary shaker (150 rpm, 30±2°C, and 16 h). Cultured medium was centrifuged (20 min, 3500 rcf, and 4°C) to collect the EB cells, and suspended in 30 mL sterile 0.85% NaCl. The biomass in the cell suspensions was harvested using an Eppendorf tube and determined as the fresh weight of living bacterial cells. Living bacterial cells (50 mg) were re-suspended in a 125 mL Erlenmeyer flask containing 30 mL of 100 mg/L of Pb solution as Pb(CH₃COO)₂·3H₂O, pH about 5. Non-inoculated Pb solution was used as the control. All flasks were incubated on a rotary incubator (150 rpm, 30±2°C, and 10 min). The solution (10 mL) was filtered through a 0.2-µm Millipore filter, and Pb concentrations were determined using AAS (SpectrAA 553, Varian). The biosorption efficiency (Tseveendorj et al., 2017) and specific metal uptake (Wierzba and Latala, 2010) were calculated as in Equations (1) and (2), respectively.

$$\text{Biosorption efficiency (\%)} = [(C_{ini} - C_{fin}) / C_{ini}] \times 100 \quad (1)$$

$$\text{Specific metal uptake (mg/g)} = [(C_{ini} - C_{fin}) / M] \times V \quad (2)$$

Where; C_{ini} and C_{fin} are the initial and final concentrations of Pb in the solution (mg/L), respectively, V is the volume of the metal solution (L), and m is the fresh weight (g).

2.3 Statistical analysis

All data were expressed as the mean and standard deviation (mean±S.D.) of the three replicates. The data were analyzed by one-way analysis of variance (ANOVA). The means were compared using the LSD with a significant difference at p≤0.05. All the statistical analyses were carried out using the SPSS trial version for Windows.

3. RESULTS AND DISCUSSION

3.1 Screen of Pb-tolerant endophytic bacteria

In the present study, five EB isolates were discovered. Isolates were designated as “Pc” and “Pe” (isolated from *P. calomelanos*), “Ai” and “Aj” (isolated from *A. mangium*), and “Ei” (isolated from *E. camaldulensis*). The strains of these EB were indicated via the results of the phylogenetic tree (Figure 1). Isolate Ai was identified as a strain closely related to *S. proteamaculans* (Gram-negative bacteria), belonging to Yersiniaceae. Isolate Aj was identified as a strain closely related to *Pseudomonas* sp. (Gram-negative bacteria), belonging to Pseudomonaceae. Isolate Ei was identified as a strain closely related to *B. cereus* (Gram-positive bacteria), belonging to Bacillaceae. Isolates Pc and Pe were identified as strains closely related to *P. psychrophila* and *P. veronii*, respectively.

As expected, bacterial endophytes found in this study were members of γ-Proteobacteria (*Pseudomonas* and *Serratia*) and Firmicutes (*Bacillus*), which are a common class of EB. All genera in this study are also cultivable EB (Liotti et al., 2018). Our EB were both Gram-positive and negative corresponding with the variation of EB, which span a significant range of Gram-positive and negative bacteria (Lodewyckx et al., 2002). Moreover, *Pseudomonas* sp. was found in plants with different habitats. This is consistent with Li et al. (2012) who reported that EB with the same genus can be isolated from metal-polluted and non-metal-polluted plants.

The Pb-tolerance and PGPT are shown in Table 1. The ability to tolerate heavy metals plays a significant role in heavy metal contaminated environments for bioremediation (Paul and Sinha, 2015). Our results found that Pb phytostabilizers grown in Pb-contaminated and non-contaminated soils could harbor Pb-tolerant EB. Pb-tolerance is a common ability of endophytic bacteria living in plants grown in Pb contaminated soil. Many studies showed that *Pseudomonas*, *Bacillus*, and *Serratia* can tolerate heavy metals, including Pb (Li et al., 2012; Alzubaidy, 2012; El Aafi et al., 2012). Li et al. (2012) reported that the same genus of EB extracted from different host species grown in different habitats (metal or non-metal contaminated soil) showed no difference in metal resistance as *Pseudomonas* sp. from this study.

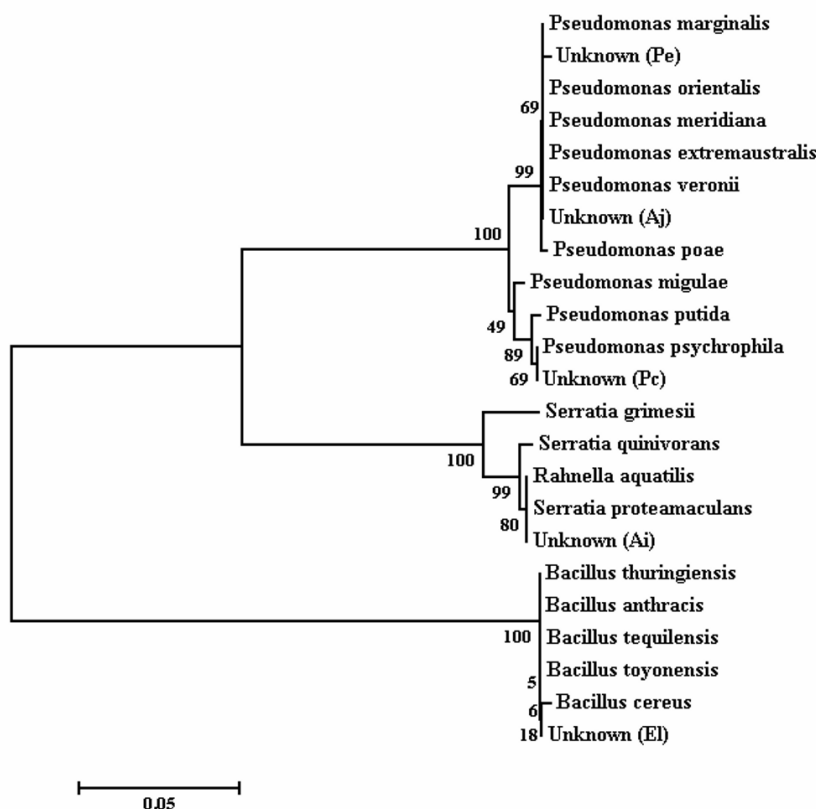


Figure 1. Phylogenetic tree, regenerated by the Neighbor-Joining method implemented in MEGA 7.

The results of our bacterial endophyte's PGPT (isolated from the plant grown in a non-contaminated soil) are in agreement with previous studies. Some bacterial endophytic isolates (RM and RM3) isolated from the roots of *Tridax procumbens* L. grown in non-contaminated soil could produce siderophores and solubilize phosphate like the Aj isolate. However, some isolates (RM1 and RM4) do not have both properties (Govarthanam et al., 2016). Endophytic bacteria, *Microbacterium trichothecenolyticum* and *Agrococcus terreus*, isolated from the roots of wild *Dodonaea viscosa* (L.) could produce siderophores but could not solubilize phosphate. *Pseudomonas geniculata* and *Pseudomonas taiwanensis* could produce siderophores and solubilize phosphate like Aj (*Pseudomonas extremaustralis*) (Afzal et al., 2017). In this study, *Pseudomonas* sp. isolated from the roots of a plant grown in Pb contaminated soil and *Pseudomonas chlororaphis* isolated from the root of *Robinia pseudoacacia* L. grown in a Pb-Zn mining area showed the same PGPT (siderophore production and phosphate solubilization) (Fan et al., 2018).

3.2 Pb biosorption

The results for biosorption capacity are shown in Table 2. The efficacy of this method of using living cells of Pb-tolerant EB (with and without plant growth-promoting traits) as biosorbents was compared in terms of the Pb uptake and removal. *P. psychrophila* showed the lowest biosorption capacity. The Pb biosorption efficacy of *S. proteamaculans*, *B. cereus*, and *P. veronii* showed no significant difference ($p > 0.05$). Their efficiency of Pb uptake and Pb removal was higher by 3.57-fold, and 3.12-fold, respectively, compared to *P. psychrophila*. The Pb-biosorption capacity from this study cannot be compared with other studies due to the different conditions that were used.

Table 1. Pb-tolerance and plant-growth-promoting traits (PGPT) of the isolates

Traits	Endophytic bacteria				
	Ai	Aj	EI	Pc	Pe
MIC Value (mg/L)	1,875				
Siderophore production	+	+	-	+	+
Phosphate solubilization	-	+	-	+	+

(+) indicates a positive result, having PGPT.

(-) indicates a negative result, lacking PGPT.

Table 2. Biosorption capacity of Pb-tolerant endophytic bacteria

Strains	Biosorption capacity	
	Pb uptake (mg/g)	Pb removal (%)
<i>Serratia proteamaculans</i> (Gram-negative)	4.68±0.54 ^{bc}	8.37±0.91 ^b
<i>Pseudomonas</i> sp. (Gram-negative)	3.41±1.35 ^b	6.57±1.91 ^b
<i>Bacillus cereus</i> (Gram-positive)	4.54±0.38 ^{bc}	8.36±0.70 ^b
<i>Pseudomonas psychrophila</i>	1.36±0.23 ^a	2.60±0.44 ^a
<i>Pseudomonas veronii</i>	5.35±0.61 ^c	7.61±0.74 ^b

Values are the mean±S.D. of triplicates. Mean values with a different letter within each column have a significant difference ($p \leq 0.05$), according to the LSD test.

Our study showed that endophytic bacteria (*S. proteamaculans*, *B. cereus*, and *P. veronii*) in different plants and grown in different habitats had the same biosorption capacity. This could be because they can use different mechanisms to remove Pb. Generally, bacterial cells use various mechanisms (e.g., complexation, coordination, physical adsorption, chelation, ion exchange, and precipitation) alone and/or combinations of them to remove heavy metals (Abdel-Ghani and El-Chaghaby, 2014). *B. cereus* without PGPT uses its cell wall to trap Pb (physical absorption), making its cell wall an important structure playing a key role in the Pb biosorption. On bacterial cell walls, there are many components that respond to metal biosorption, depending on the type of bacteria. In Gram-positive bacteria, cell walls composed of about 90% peptidoglycan component (together with teichoic and teichuronic acids) are responsible for Pb binding. In contrast, Gram-negative bacterial cell walls contain 20% of peptidoglycan (Shamim, 2018). This property can compensate for a lack of PGPT.

S. proteamaculans and *P. veronii* use siderophores to bind with Pb in solution (chelation). Siderophores as low-molecular-mass iron chelators can form stable complexes with metals including Pb (Li et al., 2012). Microbial siderophores can solubilize large amounts of Cr and Pb in soil. This molecule reduces Pb phytotoxicity via enhancing iron uptake by plants (Shin et al., 2012). Phosphate solubilization is not a major role in Pb-biosorption since Gram-negative endophytic bacteria with and without this property showed the same Pb biosorption. Similarly, *S. proteamaculans* cannot convert insoluble inorganic tricalcium phosphate to soluble forms (Sgroy et al., 2009; Ngamau et al., 2012). Many *Pseudomonas* strains express this trait (Ahmad, 2015). However, solubilizing-phosphate bacteria can chelate Pb via secreting organic acids to solubilize it (Paul and Sinha, 2015). Moreover, many studies have shown that phosphate-solubilizing EB can

enhance the bioavailability of heavy metals, leading to increased plant uptake (Jeong et al., 2012).

To utilize in a contaminated site, the strain closely related to *P. veronii* could be an appropriate choice based on high Pb-tolerance, uptake, and removal including PGPT. To survive in the harsh Pb contaminated soil, this strain, as a Gram-negative bacteria, may adsorb Pb at phosphate functional groups of lipopolysaccharide as the binding site on the cell wall (Nalik and Dubey, 2013). Moreover, *Pseudomonas* sp. uses exopolysaccharides to bind with Pb (Jarosławiecka and Piotrowska-Seget, 2014). These mechanisms can protect the cellular component of the bacterial cell. Once Pb enters the cell, this strain may precipitate Pb (Jarosławiecka and Piotrowska-Seget, 2014), and accumulate Pb via binding with metallothionein protein to protect metabolisms of bacteria (Nalik and Dubey, 2013). If the Pb concentration in a cell is high, bacteria need to release Pb from the cell. This process may use efflux system via transmembrane transporters (P-type ATPase) to keep Pb homeostasis, leading to high Pb-tolerance (Jarosławiecka and Piotrowska-Seget, 2014). The strain closely related to *P. veronii* from this study can be used to remediate Pb by bioaugmentation and phytoremediation. Normally, Pb has the lowest bioavailability in soil, making it difficult to remove (Ali et al., 2013). Using this bacterium as a bio-inoculant can increase Pb bioavailability and promote sustainable technology for removal. Alternatively, this bacterium can be used to assist a plant for phytoremediation. Generally, EB increase phytoremediative ability using various processes (e.g., promoting plant growth, reducing phytotoxicity, distributing heavy metal in plants, increasing metal bioavailability in soil, and enhancing plant uptake) (Weyens et al., 2009; Rajkumar et al., 2010; Li et al., 2012, Ma et al., 2016). There are many *Pseudomonas* sp. that were tested for Pb removal, especially *Pseudomonas aeruginosa*. However, *P. aeruginosa* is a pathogen in plants,

animals, and humans (Wu et al., 2015). Therefore, *P. veronii* may be a better choice for Pb removal as it is non-pathogenic bacteria (Montes et al., 2016).

4. CONCLUSION

In this study, Pb-tolerant EB was isolated from roots of Pb phytostabilizers grown in Pb-contaminated and non-contaminated soils. The highest Pb removal capacities by living biomass of isolates Ai, El, and Pe were 4.68, 4.54 and 5.35 mg/g, respectively. Considering PGPT, only isolate Pe could produce siderophore and solubilize phosphate and it was identified as the strain closely related to *P. veronii* which is a non-pathogenic bacteria. These traits in the strain closely related to *P. veronii* could be used as inoculant for assisting phytoremediation and as sorbent media for bioaugmentation of Pb contaminated soil. Moreover, to the best of our knowledge, this is the first research paper reporting that the strain closely related to *P. veronii* can remove Pb. Besides, the results suggest that Pb-tolerant EB could reside in plants growing in various habitats. The potential source of Pb-tolerant EB with PGPT could be metalliferous plants grown in specific habitat as metalliferous soil. However, the optimum conditions influencing the sorption ability and the kinetic modeling of this strain should be studied further.

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