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Characterization of arsenic-resistant endophytic *Priestia megaterium* R2.5.2 isolated from ferns in an arsenic-contaminated multi-metal mine in Vietnam

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ABSTRACT

Bioremediation is a biological process to remove or neutralize environmental pollutants. This study was carried out to investing at the efficacy of arsenic resistant endophytic bacteria isolated from *Pteris vittata*, *Pityrogramma calomelanos*, *Blenchum orientale*, and *Nephrolepis exaltata*, which grow in a highly arsenic (As) contamination mining site in Vietnam. Their segmented roots, stems, and leaves were homogenized separately and inoculated on LB agar plates containing 5mM As(III) and As(V). A total of 31 arsenic resistant endophytic strains were selected, in which strain R2.5.2 isolated from the root of *P. calomelanos* had the highest arsenic resistant capability. Strain R2.5.2 tolerated up to 320 mM and 160 mM of arsenate and arsenite, respectively. The strain developed well on a media of 0.1−5% NaCl, at 20-40ºC and pH 5−9, and actively utilized most of the sugar sources. It had a high IAA biosynthesis capacity with an average concentration of 19.14 mg/L, tolerated to 0.5-16 mM concentration of Ag^{+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Cr^{4+}, and reduced As(V). Based on 16s rDNA, R2.5.2 was identified as *Priestia megaterium*. The *ars C* gene coding for arsenate reductase catalyzing reduction of As(V) was successfully amplified in *P. megaterium* R2.5.2. The selected strain may have potential use for bioremediation practice.

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1 Introduction

Arsenic (As) is a naturally occurring mineral that is widely distributed in the Earth's crust and ubiquitous in the environment with origins from both geogenic and anthropogenic sources such as excessive usage of herbicides and pesticides, mining, burning fossil fuels, etc. As a consequence, arsenic concentration in air, soil, and groundwater has increased worldwide and raises public concern. According to World Health Organization (WHO) and International Agency for Research on Cancer (IARC), arsenic and arsenic compounds have been classified as Group 1 human carcinogens (IARC 2022). Additionally, it ranks top on the agency for toxic substances and disease registry's priority list of hazardous substances (Yang and Rosen 2016).

Although the biggest threat to human health comes from As-contaminated drinking water (Chung et al. 2014), As-polluted soil should not be overlooked. The average amount of arsenic in soil throughout the world is 5 mg/kg (Stafilov et al. 2010) and the permissible limit of arsenic in soil is 24 mg/kg according to the U.S. Environmental Protection Agency (Singh et al. 2015). However, in many countries, soil can be contaminated with concentrations of much arsenic higher than the permissible limit, for example, 18,100 mg/kg in an Au-enriched metallogenic of lower Silesia-Southwestern Poland, 200–860 mg/kg in Southeastern Brazil, 660 mg/kg in Simav plain-Turkey and 489 mg/kg in Esquiña-Chile (Singh et al. 2015). In the Nui Phao mine of Vietnam, the total concentrations of arsenic in the soil vary from 34–3,390 mg/kg, with a mean value of 50.93-55.44 mg/kg which exceeds the maximum allowable limit of the EPA by a factor of 1.4-141.25 (Nguyen et al. 2020) and directly or indirectly affected the health of local communities.

To remediate As-contaminated soil, mechanical or physio-chemical techniques such as soil incineration, excavation and landfill, soil washing, solidification, and electric field application have been effectively used (Lim et al. 2014), but along with their benefits, these methods might adversely affect soil fertility due to removal of basic cations. On the contrary, phytoremediation attracts great interest as a better option because of its environmentally friendly and cost-effective approach to treating two predominant inorganic oxidation states of As, arsenate (As(V)) and arsenite (As(III)) (Bali and Sidhu 2021). Although arsenic is non-essential and generally toxic to plants (Finnegan and Chen 2012), some As hyperaccumulators are capable of absorbing a large amount of As and translocating it to their aboveground biomass. The Chinese brake fern (*Pteris vittata L.*) was the first As hyperaccumulator to be reported in 2001. Since then, other As hyperaccumulators have been reported, but most of them are known to be *Pteris* ferns and *Pteris vittata* are the most promising models for phytoremediation. Extensive work has explored arsenic phytoextraction with the fern *P. vittata* as an *in situ* alternative to soil excavation-based arsenic remediation methods. Arsenic accumulated by ferns can be found in a range of soil physicochemical conditions (Danh et al. 2014). However, phytoextraction rates are slow even at a moderate arsenic concentration in soil (~100 mg As/kg soil), and the remediation time could take several decades to reduce arsenic contamination (Chen et al. 2002). Furthermore, the efficiency of phytoremediation is influenced by several factors such as plant growth rate, phytotoxicity, plant nutrition, and root exudation (Yang and Rosen 2016). Therefore, manipulations to increase soil arsenic availability, fern biomass, and fern arsenic uptake have been investigated to increase phytoextraction rates (Matzen et al. 2021).

The interaction between plants and endophytes that colonize in the plant's internal tissue are beneficial in the accumulation of heavy metals. The plant endosphere provides nutrients and serves as a habitat for the endophytes, while the endophytic microorganisms contribute in the plant growth by secreting plant growth-promoting substances such as organic acids, ACC deaminase, indole-3-acetic acid (IAA) and siderophores in turn (Titah et al. 2018). They share all the important characteristics to promote the growth of host plants found in rhizobacteria. Nevertheless, the valuable effects of the endophytic bacteria to host plants are usually greater than those provided by many rhizospheric bacteria, especially when plants are challenged by stress conditions (Afzal et al. 2019). Therefore, with the beneficial properties of endophytes isolated from arsenic-accumulating plants, their application in phytoremediation may also show great potential.

Some microorganisms have made necessary genetic adaptations to create resistance toward As, allowing them to survive and thrive in environments containing arsenic concentrations that are toxic to most other organisms. For bacteria, resistance to arsenite and arsenate compounds comes from the arsenical resistance (ars) operon. Bacterial *ars* operons consist of three to five genes (*ars* R, D, A, B, and C), which are located in the plasmids (Owolabi and Rosen 1990), or in the chromosomes (Diorio et al. 1995). *Ars* R and *ars* D are regulatory genes, while the complex of *ArsA* and *ArsB* forms an anion-translocating ATPase that catalyzes the extrusion of arsenite (As(III)) from the cytoplasm, lowering the intracellular concentration of the toxic arsenic (Mukhopadhyay et al. 2002, Owolabi and Rosen 1990). Arsenate (As(V)) is enzymatically reduced to arsenite (As(III)) by small cytoplasmic arsenate reductase, which is the product of the ars C gene. However, there are limited researches on endophytes associated with ferns and their roles in As tolerance and during the critical phase of reduction in roots before the translocation process (Gu et al. 2018).

In this study, endophytic bacteria were isolated from four common fern species *Pteris vittata*, *Pityrogramma calomelanos*, *Blenchum*

orientale, and *Nephrolepis exaltata* grown naturally on Nui Phao multi-metal mine located in Thai Nguyen province-Northern Vietnam, where the soil was highly contaminated with As due to mining activities. This study aimed to select the best endophyte and to evaluate its biological characteristics. The study suggests a potential strain for bioremediation.

2 Materials and Methods

2.1 Isolation and selection of highly arsenic-resistant endophytic bacteria

Four arsenic hyperaccumulating fern species, *Pteris vittata*, *Pityrogramma calomelanos*, *Blenchum orientale,* and *Nephrolepis exaltata* were collected at three locations including S1 - hamlet 2; S2 - hamlet 4, S3 - hamlet 11 around Nui Phao mine, Thai Nguyen province, Vietnam (21º38'15"N-21º38'54" N and 105º40'35"E-105º41'4" E).

Roots, stems, and leaves of each species were prepared separately and surface sterilized after Shutsrirung et al. (2013) and Phan et al. (2016). Next, plant samples were washed under running water for 10 min to remove all soil and dirt, and then the leaves, stem, and roots were cut into small pieces (1 × 1 cm for leave and 1cm segments for others) and soaked in 1% sodium hypochlorite for 1 min, followed by 70% ethanol for 5 min. The samples were further washed four to five times with sterile water. They were homogenized in a sterile laboratory porcelain mortar with a small amount of sterile distilled water. The obtained solutions were diluted to appropriate concentrations and inoculated on LB agar plates containing 5mM As(III) and As(V). After an incubation period of 3-5 days at 27-30°C, colonies with different morphotypes were selected and purified via multiple subcultures on LB agar.

The minimum inhibitory concentrations (MICs), minimum bactericidal concentrations (MBCs) of arsenate [As(V)] and arsenite [As(III)]-resistant endophytic bacteria were determined to evaluate their tolerance level to As as per Andrews (2001). For this, isolated bacterial strains were cultured in the shake cultural flasks containing LB medium supplemented with 5-320 mM arsenite ($NaAsO_2$) and arsenate ($Na_2HAsO_4.7H_2O$), at 30ºC and 200 rpm for 24 h. In all experiments, bacterial growth in shake flask cultures was assayed by measuring the optical density of the culture broth at 600nm (OD_{600}) using a Shimadzu UV-2550 UV/VIS spectrophotometer. The endophytic bacterium showing the highest As-tolerance was selected for further study.

2.2 Determination of IAA biosynthesis and As transformation capacity

The concentration of IAA in the culture broth was determined by Bric et al.(1991). The production of IAA was carried out in LB medium supplemented with 2 g/L tryptophan. The isolate was incubated at 30ºC, 200 rpm for 24 hours, and the supernatant was collected by centrifugation at 10,000 g for 10 min. For 1 mL of supernatant, 2 mL of Salkowski reagent (50 mL of 35% $HClO_4$ + 1 mL of 0.5 M $FeCl_3$) was added and the mixture was incubated at room temperature in the dark for 30 minutes, then light absorbance was measured at 530 nm and IAA concentration was calculated.

The ability of the bacteria to reduce As (V) or to oxidize As (III) was evaluated using the silver nitrate method after Simeonova et al. (2004). Endophytic bacteria were cultured on LB agar supplemented with 5 mM As(III) and As(V), respectively, at 30ºC for 72 h. The plates were then flooded with 0.1M silver nitrate solution. The appearance of a light yellow halo around the colony would indicate precipitation of silver ortho-arsenite (Ag_3AsO_3), while a light brown-red halo would relate to silver-ortho-arsenate (Ag_3AsO_4).

2.3 Characteristics of isolated strains

After 48 hours of bacterial isolates cultivation on LB medium plates at 37ºC, colony and cell morphology were investigated under optical microscopy. Photography of the cultured bacterial isolates was carried out using a scanning electron microscope JSM-5410LV (Jeol - Japan), at the voltage of 15 kV and under a high vacuum.

2.3.1 Determination of salt tolerance

The selected isolate was cultivated in LB medium supplemented with different concentrations (0.1-10%) of NaCl at 37ºC and 200 rpm for 48 hours.

2.3.2 Determination of favorable pH and temperatures for growth

The cultivation conditions were similar to that mentioned above, except that the initial pH of the LB medium was adjusted to different values from 3 to 12. In another set of experiments, the bacterial isolate was cultivated at different temperatures ranging from 10-55ºC.

All experiments had three replications. The growth, expressed in OD_{600}, was recorded and compared to find the range of salt tolerance and suitable pH and temperature conditions.

2.3.3 Biosynthesis of extracellular enzymes

The bacterial isolate was cultured on LB medium supplemented with 1% of one of the following substrates: soluble starch, casein, cellulose, chitin, xylan, and CMC (carboxymethylcellulose). Respective enzyme activity was assayed as per Phan et al. (2021).

2.3.4 Carbon source utilization

The isolate was grown on ISP9 mineral medium supplemented with 1% of one of the following carbon sources: D-Glucose, L-Arabinose, D-Xylose, D-Manitol, D-Fructose, D-Cellulose, D-Rafinose, and Sucrose. The sugars were sterilized by the Tyndall method. Glucose was used as a positive control and ISP9 medium as a negative control (Nonomura 1974).

2.4 Investigation of metal tolerance

The ability of isolates to tolerate other heavy metals including Ag^{+}, Hg^{+2}, Cr^{4+}, Co^{+2}, $Ni^{+2,}$ and Cu^{2+} was investigated by adding $AgNO_3$, $HgCl_{2,,}$ $K_2Cr_2O_7$, $CoCl_2.6H_2O$, $Ni(NO_3)_2.6H_2O$ and $CuSO_4.5H_2O$, respectively was also checked at a concentration ranging from 0.5 to 16mM to LB medium. Shake flask cultivation was carried out at 200 rpm at 37°C. Bacterial growth (OD_{600}) was recorded after 48 hours. All experiments were performed in triplicate. The sterile medium was used as a reference.

2.5 Strain identification and phylogeny analysis

The DNA was extracted after Sambrook (2001)and subjected to PCR to amplify the 16S rDNA gene using two primers: 27f (5'-TAACACATGCAAGTCGAACG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The thermal cycling program was initially denaturation at 94°C for 5 min, followed by 35 cycles: 94°C for 60 sec, 60°C for 60 sec, and 72°C for 90 sec, with a final step of 72°C for 10 min before keeping the sample at 4°C. The PCR products were analyzed using ABI PRISM 3100 Avant Genetic Analyzer sequence reader, processed with SeqAssem version 01/2005 and Sequencher version 4.0.5 software. The 16S rDNA nucleotide sequences were analyzed according to NCBI Gene Bank data. Sequence similarity was determined and compared with other sequences compared on the GenBank using BLAST (www.ncbi.nlm.nih.gov). The genetic similarity of the strains was constructed using CLC DNA workbench 6.6 software. The phylogenetic tree was created with Mega 6.0.

2.6 Arsenic-resistance gene amplification

The primer set BmegaarsC was used for PCR amplification of the Arsenic-resistant gene (Table 1). The PCR reaction mixture consisted of 1 µl of 10 pM of each primer, 2 µl of the DNA template, 10 µl of PCR buffer, and deionized water to a final volume of 20 µl. PCR amplification conditions were set as follows: 94ºC for 5 min, followed by 35 cycles of denaturation at 94ºC for 30 s, incubation at 53ºC for 30 s and elongation at 72ºC for 30 s, and extension at 72ºC for 5 minutes. PCR products were determined by gel electrophoresis using 1% agarose (Sambrook 2001).

2.7 Statistical analyses

All treatment data were presented as the mean of three replicates ± standard deviation (SD) using Microsoft Excel 2010. Duncan's multiple range test was employed to analyze differences between means using IBM SPSS Statistics for Windows, Version 20.0 (Armonk, NY: IBM Corp.).

3 Results

3.1 Isolation and selection of arsenic-resistant endophytes

The study showed that arsenic accumulation in the rhizosphere (0 – 20 cm deep) at this region varied from 316-1606 mg/kg of soil. From the 20 collected fern plants at the Nui Phao mine, 26 As(V) tolerant and 5 As(III) tolerant endophytic bacterial strains were isolated. Among 31 strains obtained from the incubation, isolate R2.5.2 from the root of *P.calomelanos* had the highest tolerance to arsenate (320 mM) and arsenite (160 mM). Therefore, this strain was selected for further examination of its characteristics.

3.2 Biological characteristics and growth analysis of selected strain

Bacterial isolate R2.5.2 was gram-positive with rod-shaped smooth cells and had a size of 0.9 x 3.6 µm (from SEM images of this isolate at 10000 x magnification, Figure 1). The strain had opaque white and non-convex spherical colonies of 1 mm diameter and produced brown pigment.

Furthermore, the medium with 0.1% to 2% NaCl did not have any significant effect on the growth of isolate R2.5.2 (Figure 2). Higher NaCl concentrations decreased the growth of the strain and its growth rate stopped at 10% NaCl. pH values between 5-9 were suitable for isolating R2.5.2. Similar to pH, temperature also affected the growth of isolate R2.5.2, and a temperature range of 20-40ºC was suitable for the growth of isolate R2.5.2 (Figure 2).

The isolate R2.5.2 was able to produce endospores and synthesized enzymes such as cellulase, chitinase, CMCase, xylanase, and protease with hydrolysis zone diameters of 8, 12, 15, 23, and 35 mm, respectively (Table 2). This isolate actively utilized most of the sugar sources and grew well on agar plates containing D-fructose, D-manitol, D-cellulose, sucrose, and D-rafinose (Table 2).

Table 1 Primer set used for PCR amplification

Primer	Primer sequence (5' to 3')	T_m(ºC)
Bmega-arsC-F	GGAATTCCATATGTCTAAAAAAACACTTTATTTC	53
Bmega-arsC-R	CGCGGATCCTTAGTGATGGTGATGGTGATGTTTACCTGTTTCAGCAAAACG	

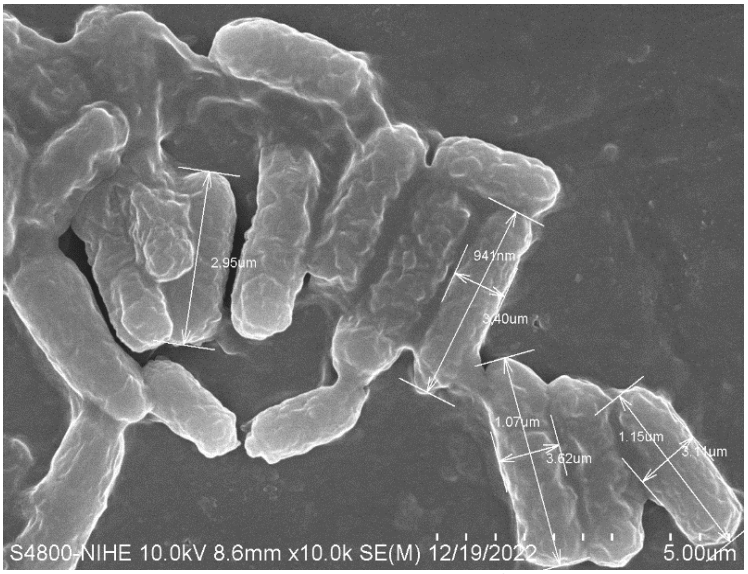


Figure 1 Scanning Electron Microscopy (SEM) image of isolate R2.5.2 (x10,000)

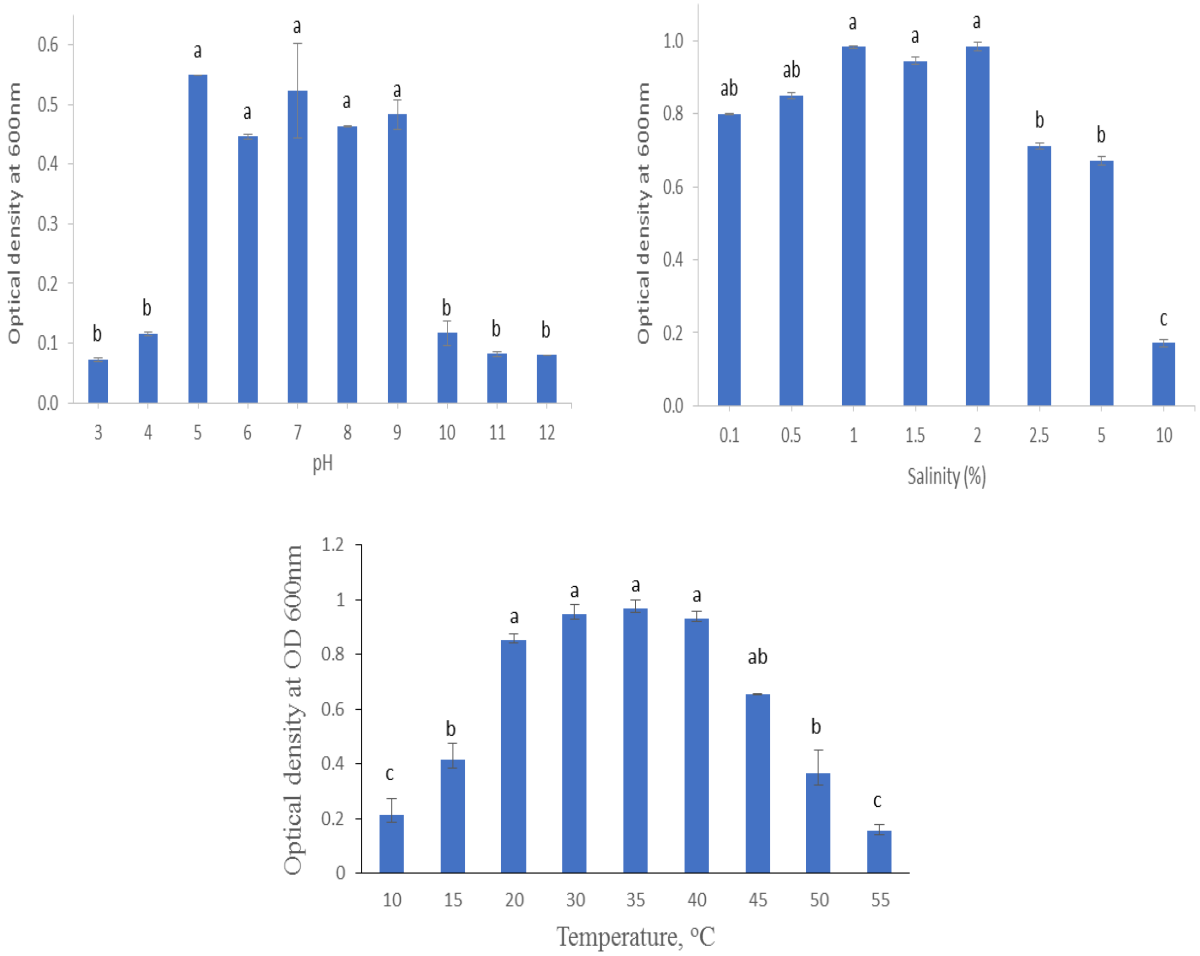


Figure 2 Effects of pH, NaCl, and temperature on the growth of strain R2.5.2. The bars stand for the standard error of three replicates. Means with different letters are significantly different at $p < 0.05$ (Duncan's test).

Table 2 Biological characteristics of strain R2.5.2

Experiment	Carbon source	Growth
Carbon source utilization	D-Glucose	+
	L-Arabinose	+
	D-Xylose	+
	D-Manitol	++
	D-Fructose	++
	D-Cellulose	++
	D-Rafinose	++
	Sucrose	++
	Mineral	+-
	Extracellular enzyme	Diameter (D-d) mm
Extracellular enzyme	Amylase	-
	Protease	35
	CMCase	15
	Chitinase	12
	Xylanase	23
	Cellulase	8

3.3 IAA biosynthesis and As transformation capacity

In this study, the strain R2.5.2 possessed high IAA biosynthesis capacity with an average concentration of 19.14 mg/L (Figure 3). The test results indicated that strain R2.5.2 reduced As(V) and As(III) due to the formation of light yellow color and light brown-red color halo around the colony, respectively, in LB agar medium supplemented with As(V) and As(III) (Figure 4).

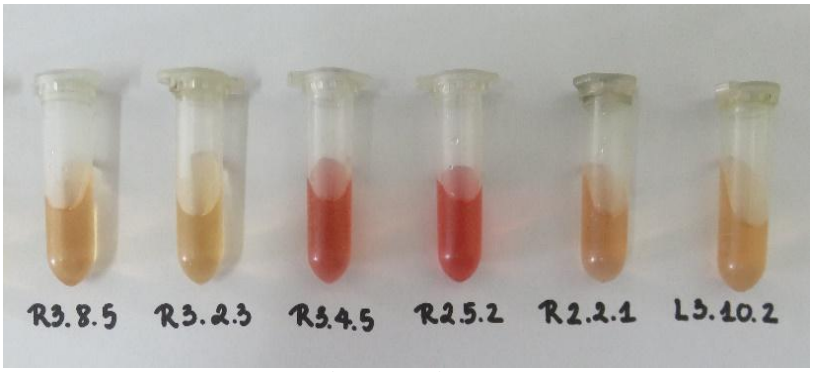
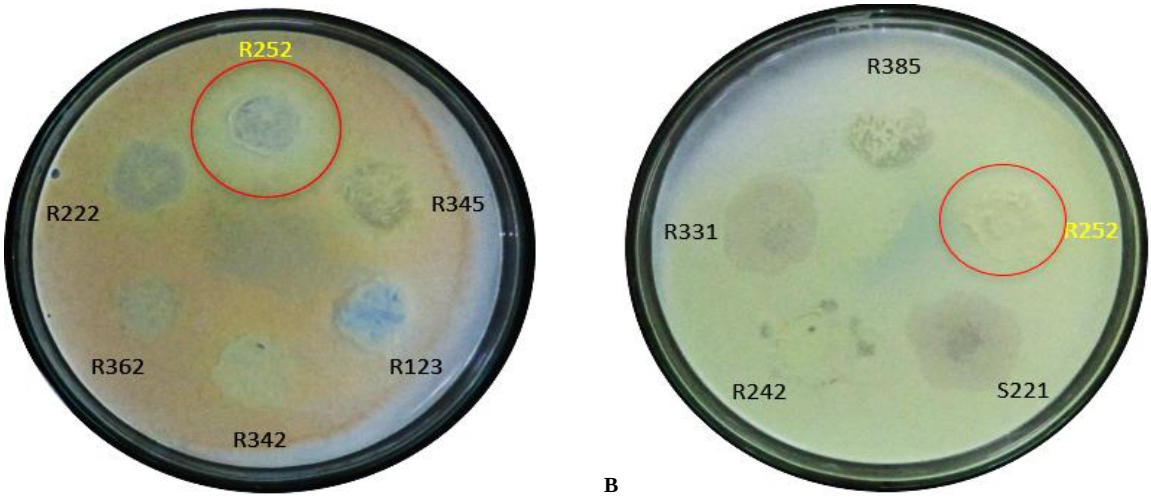


Figure 3 The ability of synthesis of IAA by the R2.5.2 strain



A **B**

Figure 4 The ability of the R2.5.2 strain to reduce As (V) or to oxidize As (III); LB agar supplemented with 5 mM As(V) (A) and As(III) (B)

Isolated strain R2.5.2 was tested for its ability to grow under the presence of some heavy metals and the results revealed that strain R2.5.2 effectively grew in the presence of other toxic heavy metals. Strain R2.5.2 was resistant to Ag^{+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}và Cr^{4+} heavy metal in the range of 0.5-16 mM, but at 16 mM concentration, strain R2.5.2 showed little or no positive sign of growth (Figure 5).

3.4 Strain identification of the isolate R2.5.2

The total DNA of the selected strain R2.5.2 was extracted using a total DNA extraction kit (NucleoSpin® Tissue extraction kit, Macherey-Nagel, Germany). The 16S rRNA gene of the bacterial strain was amplified by PCR using primer pair 27F-1492R (Figure 7a) and showed high homology of over 99% with the corresponding genes of several bacteria belonging to *Priestia megaterium* species (Figure 6). From the results of 16S rDNA gene sequencing combined with biological and physiological characteristics, strain R2.5.2 was named *P.megaterium* R2.5.2 and submitted to GenBank under accession number OL662937.1.

3.5 Arsenic-resistance gene of strain *Priestiamegaterium* R2.5.2

The *arsC* gene of strain *P. megaterium* R2.5.2 was amplified by PCR from a genomic DNA (gDNA) template using primer pairs

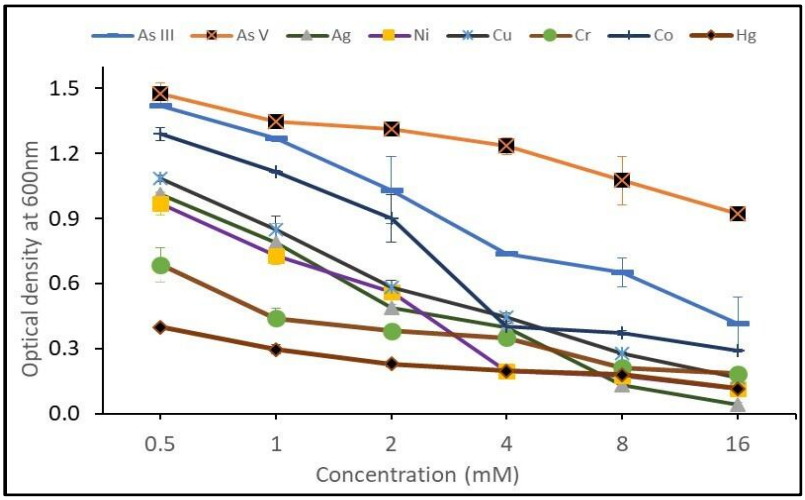


Figure 5 Influence of heavy metals on growth of strain R2.5.2.The bars stand for the standard error of three replicates

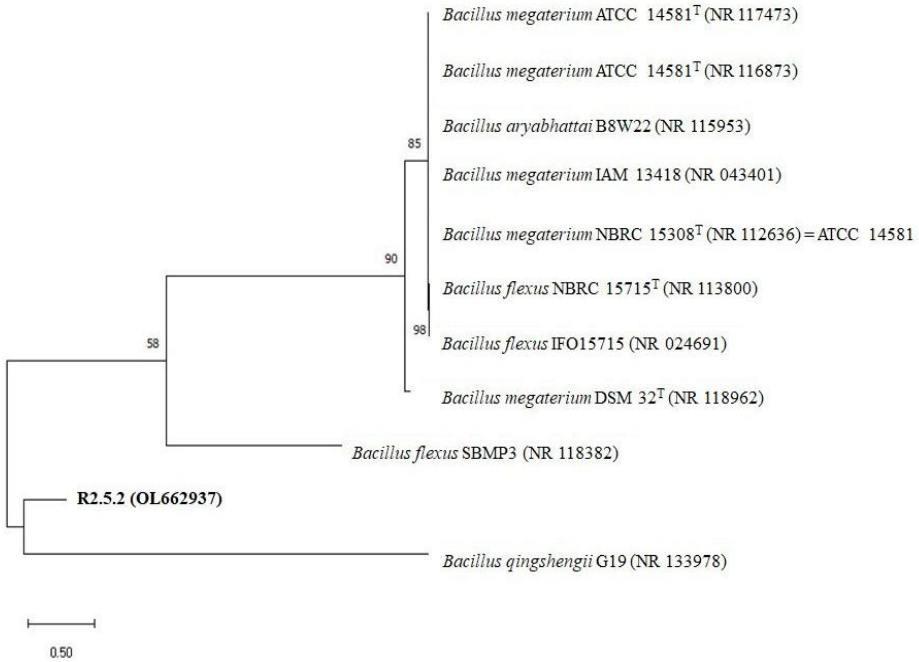


Figure 6 Neighbor-joining tree showing the phylogenetic relationships based on 16S rRNA gene sequence of the strain R2.5.2 and closest species. Numbers on branches correspond to bootstrap values obtained with 1000 replicates.

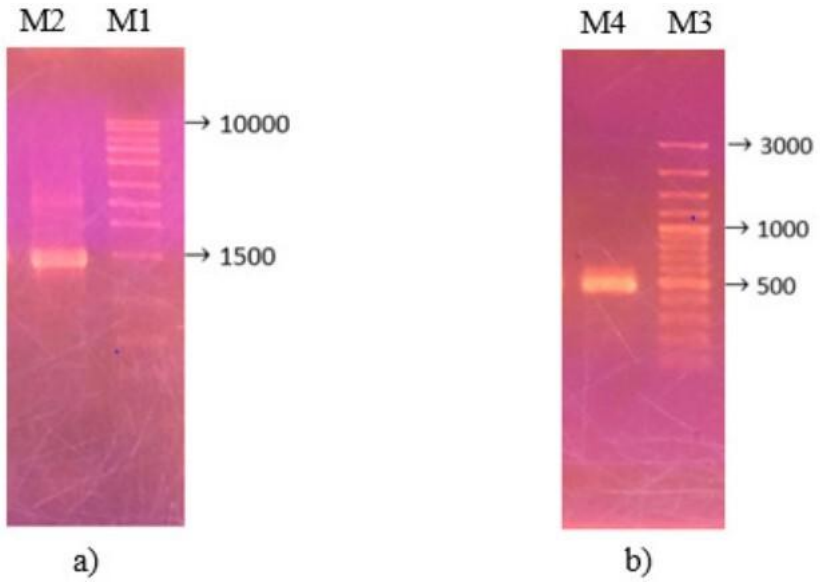


Figure 7 (a) PCR amplification of 16S rRNA gene (M1: 1 kb DNA Ladder, M2: 16S rRNA gene) and (b) arsC gene of strain R2.5.2 (M3: GeneRuler 100 bp DNA Ladder (G Biosciences, America), M4: ars C gene)

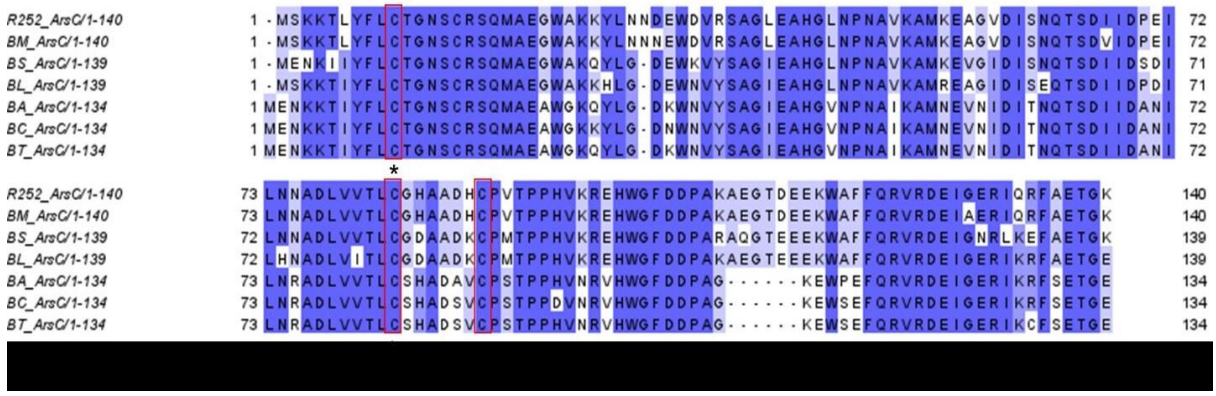


Figure 8 Comparison of protein sequences of ArsC gene from strain R2.5.2 with corresponding protein sequences of other *Bacillus* strains

Bmega-arsC-F and Bmega-arsC-R (Table 1), giving a single gene cassette with the size of 500 bp on 1% (w/v) agarose gel, which corresponded to the expected size when designing primers for amplifying the *arsC* gene from *Bacillus megaterium* (Figure 7b). The *arsC* gene of *P. megaterium* R2.5.2 was successfully amplified and the PCR product (Figure 7b) was purified and used for paired-end sequencing using primer pairs Bmega-arsC-F and Bmega-arsC-R. The *arsC* gene of *P. megaterium* R2.5.2 had a length of 423 bp, encoding a protein of 140 amino acids. The arsC gene sequence of strain *P. megaterium* R2.5.2 was submitted to the GenBank database under accession number OM055827.

A comparison between the amino acid sequence of *arsC* product from *P. megaterium* R2.5.2 with corresponding *arsC* products of other *Bacillus* strains (*B. megaterium*, *B. subtilis*, and *B. licheniformis*) revealed that the similarity was high (83% - 97%) (Figure 8).

ArsC of strain *P. megaterium* R2.5.2 was compared with that of *B. megaterium* DSM 319 (BMD_1727, 97.8%), *B. subtilis* 168 (P45947, 83.4%), *B. licheniformis* (Q65IV4, 87.05%), *B. anthracis* (Q81NJ6, 72.9%), *B. cereus* (A0A1Y6B0W9, 72.9%) and *B. thuringiensis* (Q6HGP0, 72.1%). The active site of ArsC was framed and marked with an asterisk (*). Protein sequence comparisons were performed with Clustal Omega and presented in Jalview. The intensity of the blue color gradient was encoded based on 50% sequence similarity.

4 Discussion

There are numerous arsenic-resistant bacteria have been collected from arsenic-rich environmental plants. In this study, 26 As(V) tolerant and 5 As(III) tolerant endophytic bacterial strains were isolated. Most of the As-resistant endophytic bacteria were found in the fern roots. Among them, *P. megaterium* R2.5.2 from the root

of *P. calomelanos* had the maximum tolerance to arsenate (320 mM) and arsenite (160 mM). Román-Ponce et al. (2018) reported that 27 strains isolated from roots of *Prosopis laevigata* and *Sphaerealcea angustifolia* were able to tolerate high concentrations of arsenic with MIC from 20 to over 100 mM for As(V) and 10–20 mM for As(III). According to Ghosh et al. (2018), *Bacillus aryabhattai* MCC3374 exhibited high resistance to arsenate (MIC: 100 mM) and arsenite (MIC: 20 mM). Furthermore, *K. palustris* NE1RL3 was an arsenic-resistant bacterium with MIC of 14.4 mM and 300 mM for As(III) and As(V), respectively, in the LB medium (Zacaria Vital et al. 2019). Similarly, Bermanec et al. (2021) isolated three bacterial strains from an arsenic-contaminated CrvenDol mine in North Macedonia with a MIC of 209 mM for arsenite and 564 mM for arsenate.

The results of this study showed that temperatures in the range of 10-50ºC was suitable for R2.5.2, and the optimal temperature is between 20 and 40ºC. A similar finding revealed that arsenite-oxidizing bacteria could also grow at a temperature between 40ºC and 50ºC (Kinegam et al. 2008).

Among the sugar sources that this isolate could actively consume, L-arabinose was considered as a major plant saccharide that could not be found in animals (Crozier et al. 2021). Hence, the ability to utilize L-arabinose was regarded as a trait contributing to an endophytic lifestyle, similar to the case of *Pseudomonas* endophytes of cucumber (Podolich et al. 2015). The capacity to consume a wide range of nutrient sources and produce numerous extracellular enzymes is likely to help this strain easily adapt to environmental conditions with diverse substrates ranging from sugars to complex organic compounds. In this study, isolate R2.5.2 could synthesize some extracellular enzymes, such as cellulase, chitinase, CMCase, xylanase, and protease. This isolate could actively utilize most of the sugar sources and grew well on agar plates containing D-fructose, D-mannitol, D-cellulose, sucrose, L-arabinose, and D-raffinose.

The biosynthesis of plant hormones such as IAA in endophytic bacteria is one of the vital mechanisms that can affect the growth of host plants, and increase the biomass of leaves, roots, and root length. Many studies have shown that metal-resistant endophytes were capable of producing IAA, which promoted plant growth. In this study, strain R2.5.2 possessed high IAA biosynthesis capacity with an average concentration of 19.14 mg/L. According to Luo et al. (2011), 30 Cd-resistant endophytes belonged to 4 groups i.e. *Actinobacteria* (43%), *Proteobacteria* (23%), *Bacteroidetes* (27%) and *Firmicut*es (7%), which produced 0.6-122 mg/LIAA. Similarly, a higher concentration of IAA biosynthesized by two Pb-resistant endophytes from the roots of *Alnus firma* was 15.8-27.9 mg/L was isolated by Sheng et al. (2008). According to Xu et al. (2016), bacterial strains isolated from *Pteris vittata* were also able to biosynthesize IAA ranging from 2.43-32.4 mg/L, which was higher than the findings reported by Zhu et al (2014), where IAA concentration was 0.2-10.8 mg/L. This is an important characteristic of endophytic bacteria, which increases the plant biomass to enhance arsenic resistance and accumulation.

As for As-tolerant/non-hyperaccumulator plants, evaporation or efflux of arsenic in roots can decrease arsenic translocation to shoot. In their roots, the conversion of arsenic to less toxic organic forms or transportation to vacuoles as As(III) or As(III)-glutathione/phytochelatin complexes occurs to prevent arsenic translocation to shoots. In contrast, the translocation of arsenic to shoots and reduction of As(V) to As(III) in hyperaccumulators are reported to be highly efficient, and efflux levels are insignificant. As(III) has been identified as the principal form of arsenic transported from root to shoot, regardless of whether As(V) or As(III) was supplied to the plants, even though As(III) was more toxic than As(V) in their organic forms (Gupta 2018). The reduction of As(V) to As(III) in roots may be a reason for translocating high amounts of arsenic in hyperaccumulators such as *I. cappadocica* and *P. vittata*. They were able to accumulate 60–80% of arsenic in shoots, while only 5–10% of total arsenic is found in non-accumulating species such as *P. tremula* and rice. Additionally, the reduction of As(V) to As(III) via arsenate reductase was accepted as the first step in the detoxification of arsenic in hyperaccumulators (Souri et al. 2017). Consequently, endophytic bacterial strains capable of converting As(V) into As(III) will increase the arsenic accumulation capacity of the plant by translocating arsenic from root to shoot, the part with the highest proportion of biomass of the plant. This study confirmed that isolate R2.5.2 belonged to the *Priestia megaterium* species. In previous studies, many members of the genus *Priestia* have demonstrated their ability to stimulate plant growth as well as tolerance to high arsenic concentrations. Some other studies also concluded that *P. megaterium* YC4-R4 and TG1-E1 showed high salt tolerance as well as plant growth-promoting characteristics (Biedendieck et al. 2021), *Exiguobacterium auranticum* SV7, *Paenibacillus sp.* SV10 and *Priestia koreensis* LV19 possessed an extensive range of antifungal as well as plant growth promoting activities (Bashir et al. 2021). Further, Gupta et al. (2020) reported that *Priestia aryabhattai* is resistant to arsenic and UV radiation. Similarly, Titah et al (2018) isolated *Bacillus megaterium* species from the roots of *Ludwigia octovalvis* and reported that it was resistant to arsenic and was also capable of absorbing arsenic.

In As-resistant bacteria, arsenate reductase coded by gene *arsC* mediated the reduction of As(V) to As(III) in the cytoplasm, and then As(III) was expelled from the cell via an ATP-independent ArsB, which facilitated arsenic detoxification (Stolz et al. 2006; Rosen 2002). Thus, the higher the As(V) reduction capacity of endophytes was, the higher their resistance to As(V) was. Xu et al.

(2016) also concluded that As(V) reduction by *Pretis vittata* endophytes had a positive relationship with arsenic tolerance. From this study, strain R2.5.2 possessed the arsC gene, which showed its potential in bioremediation of As-polluted soil, when used with As-hyperaccumulator plants.

In addition to arsenic, endophytic bacteria have been recognized for their contribution to plant growth and tolerance towards a wide range of heavy metals (Rajkumar et al. 2009). Das and Barooah (2018) studied the MIC of *Staphylococcus sp*. TA6 strain for Hg^{+2}, Cd^{+2}, Co^{+2}, Ni^{+2}, Cr^{+2} and found that the strain could survive at 0.5mM, 0.8mM, 1.0mM, 4mM, and 6mM, respectively. According to Manzoor et al. (2019), *P. aeruginosa* had high tolerance against Cd (20 mM), Zn (28 mM); *P. fluorescens* JH 70-4 displayed high tolerance to As (8 mM), Cu (6 mM), Ni (6 mM), and Cd (0.9 mM); *Pseudomonas spp*. PG-12 was efficient in resisting up to 0.6 mM Cd. In this study, strain R2.5.2 was also resistant to Ag^{+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, andCr^{4+} in the range of 0.5-16 mM, but at 16 mM concentration, strain R2.5.2 showed little to no positive reaction for growth. In particular, strain R2.5.2's growth was healthy with an increase in the presence of As (V) and As (III) at both 0.5mM (17.74% and 13.07%, respectively) and 1mM (7.29% and 1.12% in turn). Karn and Pan (2016) also showed that *Bacillus sp*. XS2 grew better at low As (III) concentration.

Conclusion

The selected strain R2.5.2 isolated from the root of *P. calomelanos* showed tolerance to high levels of arsenate (320 mM) and arsenite (160 mM) and was identified as *P. megaterium* R2.5.2. The strain grew well within a wide range of salinity (0.1-5% NaCl), pH (5-9), and temperature (20-40ºC). Apart from its ability to reduce As(V), strain *P. megaterium* R2.5.2 was tolerant to 0.5-16 mM of several heavy metals, including Ag^{+}, Hg^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, and Cr^{4+}. In addition, strain *P. megaterium* R2.5.2 produced on average19.14 mg/L of indole-3-acetic acid, suggesting its ability to promote host plant growth. The findings of this study may contribute to further understanding of As-hyperaccumulator plants and their endophytic bacteria in bioremediation of As-polluted soil.

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Conflict of interest

The authors of this article declare no conflict of interest.

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