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Isolation and identification of salt-tolerant, phosphorus-solubilizing bacterial strains from rice soil in rice-shrimp farming systems in Tien Giang Province, Vietnam

TRUONG QUOC TAT
Faculty of Technology and Engineering, Dong Thap University. 783 Pham Huu Lau Street, Ward 6, Cao Lanh City 870000, Vietnam.
Tel.: +84-778-739392, email: tqtat@dthu.edu.vn

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Abstract. *Tat TQ. 2024. Isolation and identification of salt-tolerant, phosphorus-solubilizing bacterial strains from rice soil in rice-shrimp farming systems in Tien Giang Province, Vietnam. Biodiversitas 25: 3868-3875.* This study aimed to isolate, select, and identify the phosphate-soluble bacterial (PSB) pathogens from eight salt-affected soil samples collected from paddy rice fields in the Go Cong Dong and Tan Phu Dong districts of Tien Giang Province, Vietnam. Bacteria were isolated on Pikovskaya's agar media supplemented with 1% NaCl, and the amount of phosphorus dissolved in liquid NBRIP was supplemented with various concentrations of NaCl via molybdate coloration to evaluate the salt tolerance and phosphorus solubility of the isolated bacterial strains. The result showed that from 8 saline soil samples, a total of 15 strains of phosphorus solubilizing bacteria were isolated and 2 (code 1.7 and 6.1) of them showed good phosphorus solubilization. In addition, isolates 1.7 and 6.1 exhibited good growth and phosphorus solubilization in liquid NBRIP media supplemented with NaCl at concentrations ranging from 1.0% to 5.0%. Molecular analysis results showed that strains 1.7 and 6.1 were identified as *Burkholderia vietnamiensis* 1.7 and *Priestia aryabhattai* 6.1, respectively. These results show that *B. vietnamiensis* 1.7 and *P. aryabhattai* 6.1 can be used as biofertilizers for rice cultivation in salty soils in Tien Giang Province, reducing the cost and use of chemical fertilizers.

Keywords: *Burkholderia vietnamiensis*, phosphate-solubilizing bacteria, *Priestia aryabhattai*, rice-shrimp soil, saline soil

INTRODUCTION

Phosphorus (P) is one of three essential macronutrients for plants (Malhotra et al. 2018). However, the amount of naturally available phosphorus in the soil is typically very low, primarily in the form of insoluble rocks, minerals, and ores (de Boer et al. 2019). This is because phosphorus is often immobilized by soil colloids and clay minerals (Chen and Arai 2023). As a result, it is necessary to fertilize plants with phosphorus and increase their solubility to support agricultural production. In recent years, there has been a rapid increase in agricultural cultivation on saline soil (Negacz et al. 2022). Like cultivation on fertile land, intensive cultivation of salt-resistant crops can lead to severe deficiencies in easily digestible phosphorus in saline soil (Liu et al. 2024). To address this issue, phosphorus is typically added to the soil in the form of fertilizer to supplement the amount of soluble phosphorus that plants can directly use (Johnston et al. 2014). However, when phosphorus is applied to soil, negatively charged phosphate groups can easily become fixed through precipitation with cations such as Ca^{2+}, Mg^{2+}, Fe^{3+}, and Al^{3+}. This results in a large portion of the phosphate fertilizer becoming indigestible and becoming unavailable by plant absorption.

In soil, microorganisms play a crucial role in converting immobilized P into a form that is available for plants to use. Therefore, the development of methods to improve the bioavailability of immobilized P in agricultural soils is critical for mitigating the continued overapplication of P beyond what is required by plants (Yu et al. 2019). The utilization of PSB has shown potential as a management strategy for enhancing P use efficiency. PSB can convert insoluble P into soluble forms that can be readily absorbed by plants (Paulucci et al. 2015; Khan et al. 2021). Numerous studies have focused on isolating, identifying, and utilizing bacteria with the ability to dissolve fixed phosphorus in the soil, resulting in significant increases in crop productivity. Examples of such bacteria include *Pseudomonas*, *Bacillus*, *Rhizobium*, and *Enterobacter* (Etesami and Alikhani 2019; Waithaisong 2024). The use of PSB in agricultural settings has been shown to have positive effects on the growth of various crops, including maize, wheat, soybean, barley, sesame, century plants, and wild mint (Pereira et al. 2014; Bautista-Cruz et al. 2019; Prakash and Arora 2019; Chouyia 2020; Kusale et al. 2021; Nithyapriya et al. 2021). However, these previously studied PSB bacteria are sensitive to saline environments and therefore not suitable for use in saline farmlands. As a result, there is a need to isolate bacterial strains that can tolerate salt and effectively dissolve phosphorus, particularly for use in agricultural cultivation, such as rice cultivation on saline soils.

The Mekong Delta is a crucial area for rice production in Vietnam, with nearly 4.0 million ha dedicated to rice cultivation, making up 80% of the region's agricultural land (Vu et al. 2022). However, currently, coastal provinces in the Mekong Delta, including Tien Giang Province in Go Cong Dong and Tan Phu Dong Districts, which have 32 km

coastlines, are facing increasingly severe saltwater intrusion as a result of climate change. In the dry season, saline water can encroach up to 30-40 km inland, significantly impacting agricultural production in this area. Saltwater intrusion is particularly detrimental to rice cultivation, as it is the primary food crop of local people (Linh and Bleys 2024). This challenge has attracted the attention of authorities and scientists, leading to the exploration of potential solutions for rice production development in the area. One such solution is the use of microbial technology, specifically salt-tolerant bacteria, which can also dissolve immobilized phosphorus in the soil. This approach not only reduces production costs but also minimizes environmental pollution, making it a necessary and urgent solution for Tien Giang Province, as well as other provinces of the Mekong Delta region. Despite the high potential of utilizing indigenous bacteria from saline soil to increase crop productivity, these bacteria have not been fully utilized. Therefore, the goal of this study was to isolate, select, and identify salt-tolerant bacterial strains that can simultaneously dissolve insoluble phosphorus from saline rice soil in a rice-shrimp farming (RSF) system.

MATERIALS AND METHODS

Chemicals

Pikovskaya's agar media contained the following components per liter: glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_47H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4H_2O$, 0.002 g; and $FeSO_47H_2O$, 0.002 g; NBRIP (National Botanical Research Institute's Phosphate) agar plates contained 10 g glucose per liter, 5.0 g $MgCl_2.6H_2O$, 0.25 g $MgSO_4.7H_2O$, 0.1 g $(NH_4)_2SO_4$, 0.2 g KCl, 5.0 g $Ca_3(PO_4)_2$, and 15 g agar, with the pH adjusted to 7.5-8.0; TSA (tryptone soy agar) medium included Tryptone Soya Broth 30 g/L and 15 g/L agar; NaCl, Na_2HPO_4, NaH_2PO_4, and ammonium molybdate ($[NH_4]_6MoO_{24}.4H_2O$. All chemicals used were of high purity (>90%), including CMC-Na, agar, and peptone, and were supplied by Merck (Darmstadt, Germany); other chemicals were obtained from Xilong (Shantou, China).

Saline soil sample collection method

Sampling was conducted in the current situation of rice cultivation in RSFs on saline soil in the Tan Phu Dong and Go Cong Dong districts, Tien Giang Province, Vietnam with 4 soil samples for each site. The rice shrimp fields chosen for soil sampling to isolate PSB were those with high rice yields during the cultivation season. Saline soil samples were taken at depths ranging from 0 to 20 cm via a 3 cm diameter chamber at the end of the rice crop. Ten points were collected in a zig zag line from each rice field. After collection, the soil was mixed to create a composite sample, which was subsequently sealed in a sterile self-sealing bag. The sample was then stored in a refrigerator at 4°C until use.

Isolation of salt-tolerant bacteria with phosphorus-dissolving ability

Ten grams of the soil was suspended in 90 mL of sterile phosphate buffer in a 250 mL Erlenmeyer flask. The flasks were shaken horizontally at 150 rpm and 30°C for 1 hour, followed by 15-minute rest period. The bacterial extract was serially diluted to 10^{-2}-10^{-6} fold, and 50 µL of each dilution was plated on Pikovskaya's agar media supplemented with 1% NaCl. Experiment was performed in triplicates. The agar plates were then incubated at 30°C for 7 days. The growth of colonies on the plates was observed, with particular attention given to the presence of a clear halo around the colony, and the colonies were streaked onto new Pikovskaya agar media supplemented with 1% NaCl. The ability of isolated bacterial strain to dissolve phosphorus was assessed via the following formula: phosphate solubilization index (PSI) = clear zone diameter (including colony diameter)/colony diameter (Pathak et al. 2018). Colonies of bacterial strains were cultures in TSA slants for further research. The purity of the bacterial strains was confirmed via the pressed drop method, and the strains were observed under a microscope.

Evaluation of the salt tolerance and phosphorus solubility of isolated bacterial strains

Selection and enrichment of bacterial strains

Two bacterial strains were selected from 15 isolated strains with the greatest tolerance to salt and the greatest ability to dissolve phosphorus. The strains were cultured on agar plates containing TSA (40 g of TSA in 1 L of demineralized water), and all biomass was collected in a flask containing sterilized distilled water. The biomass of each bacterial strain was subsequently collected separately by transferring the entire culture mixture into a sterile 50 mL falcon tube and centrifuged for 3 minutes at 6,000 rpm. After centrifugation, supernatant was removed, and the bacterial biomass below was retained. After that, 20 mL of sterile demineralized water was added to the falcon tube containing bacterial biomass, and was vortexed for 2 minutes and then centrifuged. The process was repeated twice to completely remove the remaining phosphorus from the TSA nutrient-rich solution. Next, turbidity of the bacterial mixture was calibrated with sterile demineralized water via a spectrophotometer to an OD_{600nm}=0.7 turbidity.

Experimental setup

An aliquot of 1 mL of the microbial solution was added into a 100 mL Erlenmeyer flask containing 49 mL NBRIP liquid medium supplemented with 1%, 3%, and 5% NaCl which was heated, sterilized and cooled. Each bacterial strain was arranged with 3 replicates. The Erlenmeyer flasks containing samples were shaken at 120 rpm at 30°C. The control treatment was performed similarly, except without bacterial inoculation. The amount of dissolved phosphorus and the bacterial population were measured at 0, 1, 7, 14, and 21 days. The concentration of solubilized phosphate in the supernatant was determined via the ascorbate method (Ames 1966).

Identification of selected bacteria

The two bacterial strains were subsequently grown on TSA to extract 3% of the DNA with CTAB (Ihrmark et al. 2012). Afterwards, the DNA products were amplified via PCR with 27F/1492R primers (Justé et al. 2008). The forward primer used was 5' AGA GTT TGA TCC TGG CTC AG 3', and the reverse primer used was 5' TAC GGT TAC CTT GTT ACG ACT 3'. The PCR cycle included following steps: pre-denaturation at 95°C for 5 minutes; 30 cycles of denaturation at 95°C for 1 minute, annealing at 53°C for 30 seconds, extension at 72°C for 90 seconds, and a final extension at 72°C for 5 minutes; and subsequent use of the sequence in a BLAST search limited to a bacterial database. The unknown bacteria were identified by examining the top-scoring sequences from the BLAST search results. Sequence homology analysis of the 16S rRNA gene was conducted using GenBank data. A phylogenetic analysis with the neighbor-joining model was performed using the MEGA version 11 program. The reliability of the branching and clustering patterns was estimated with 1,000 bootstrap replicates.

Statistical analysis and data processing

All the experiments were performed in triplicates. All the data were entered, calculated and graphed via Microsoft Excel 2019 software. One-way analysis of variance was performed via Minitab 16 software to determine the significance of differences at $p<0.05$, and Tukey's test was applied.

RESULTS AND DISCUSSION

Isolation of PSB from saline rice-shrimp farming system soil

From a total of 8 samples of saline soil collected from rice-shrimp farming fields in 2 different coastal districts of Tien Giang province, Vietnam, 15 PSBs were isolated and cultured on NBRIP agar medium containing 1% NaCl. The colonial and cell morphologies, as well as the bacterial Gram stain, were described for each of these bacteria. The results showed a high diversity in colony morphology, including variations in color, size, shape, surface, and bacterial cell shapes (Figures 1 and 2). Interestingly, the majority of PSBs were found in 4 out of the 8 samples collected from Tan Phu Dong district, accounting for 66.67% of the total isolated bacteria. In contrast, a lower number of PSBs were found in the soil samples from Go Cong Dong district (33.33%). This suggests that although all soil samples collected from the same rice-shrimp farming system were affected by salinity, significant differences were also observed in the number and diversity of phosphorus solubilizing bacteria. These findings indicate that salinity levels and geography may play a crucial role in regulating the abundance and diversity of phosphorus solubilizing bacteria in soil samples. Similar results on the occurrence and isolation of PSB were found by El-Komy (2005) and Neelam and Meenu (2003). In addition, the study of Nguyen et al. (2023) also showed that salinity and geographical location also affect the diversity of salt-tolerant bacteria that solubilize phosphorus in the soil.

The bacterial strains with codes of 1.7, 4.1 and 6.1 showed the highest ability to solubilize phosphorus, with a statistically significant difference compared with the other strains. Additionally, these three strains also demonstrated strong growth in the presence of varying concentrations of NaCl. Specifically, strains 1.7 and 6.1 exhibited both high phosphorus solubility and good growth at NaCl concentrations (Table 1).

Rice-shrimp farming is practiced by farmers in the coastal area of the Vietnamese Mekong Delta, where saline intrusion during the dry season limits rice production but creates conditions suitable for shrimp production. Salinity is a major abiotic stress that significantly reduces plant growth and yields in various regions of the world (El Sabagh et al. 2020). It is estimated that 20% of cultivated land worldwide is negatively impacted by high salt concentrations, which inhibit plant growth and yield (Mustafa 2019). The presence of excessive soluble salts in the soil can lead to osmotic stress, specific ion toxicity, and ionic imbalances (Pessarakli and Szabolcs 2019), ultimately resulting in plant death or yield losses (Zörb et al. 2019).

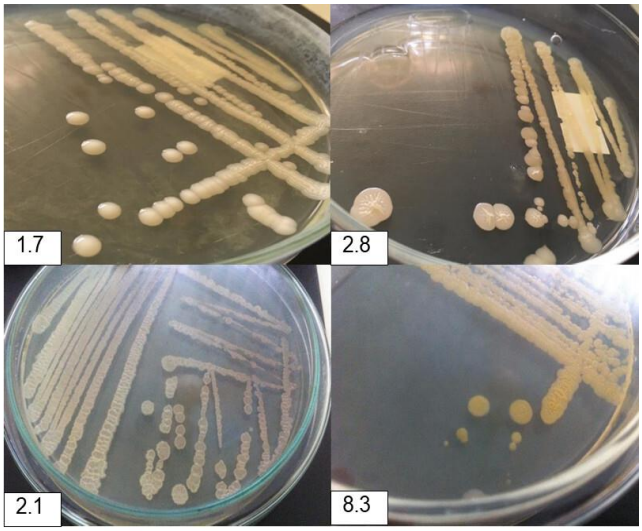

Figure 1. Colonies of soil bacteria on agar media after 48 hours of culture

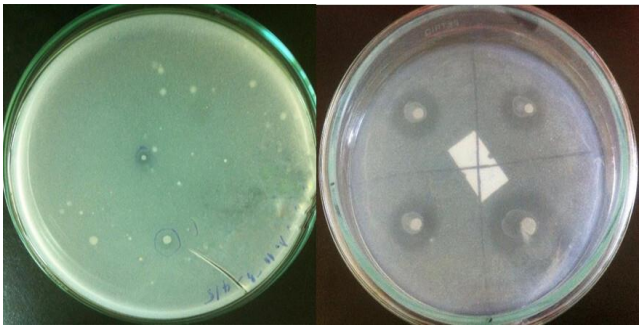

Figure 2. Colony morphology characteristics of bacterial strains 1.7, 2.8, 2.1 and 8.3

However, the ability to tolerate salt varies among crop varieties and microbial strains. Several studies have reported variations in salt tolerance among different crops and microbial inoculants, such as wheat (Al-Khaishany et al. 2018), rice (Ganie et al. 2019), and soil bacteria (Sharma et al. 2021). In various natural environments, PSBs play a significant ecological role by mobilizing ISIP in plants (Iftikhar et al. 2024). Currently, PSB isolated from soil are commonly utilized as plant growth promoters in agriculture (Ateş et al. 2020).

Effect of NaCl concentration on phosphorus solubility and population of two bacterial strains

The results presented in Figure 3 revealed that as the bacterial population increases, the ability to dissolve phosphorus also increases. Specifically, both bacterial strains 1.7 and 6.1 can tolerate 5% NaCl without inhibiting their ability to dissolve phosphorus. However, this concentration resulted in a decrease in the amount of phosphorus dissolved compared with other concentrations. The bacterial strain 1.7 was isolated from the Tan Phu Dong district. The highest amount of dissolved phosphorus and bacterial density of this strain were observed after 21 days of culture, with values of 58.12 P_2O_5 (mg/L) and 7.56 (CFU/mL), respectively. These values were not significantly different from those observed after 1 day of culture.

However, after 7, 14, and 21 days of culture, the amount of dissolved phosphorus gradually decreased at different NaCl concentrations. The bacterial strain 6.1 was isolated from the Go Cong Dong district. The highest amount of dissolved phosphorus and bacterial population of this strain were observed at a 1% NaCl concentration after 21 days of culture, with values of 132.09 P_2O_5 (mg/L) and 9.48 (CFU/mL), respectively. These values were significantly different from those observed at other concentrations. After 14 and 21 days of culture, the amount of dissolved phosphorus gradually decreased at concentrations of 3% and 5%, respectively.

Interestingly, at 1% NaCl concentration, bacterial strain 6.1 reached the highest amount of dissolved phosphorus after only 1 day of culture, with no significant difference compared with bacterial strain 1.7 isolated from the Tan Phu Dong district. After 7, 14, and 21 days of culture, the amount of dissolved phosphorus also gradually decreased. Thus, the phosphorus solubilities of 1.7 and 6.1 strains were 58.44 mg/L and 132.09 mg/L, respectively, after 1 day and 7 days of culture. This result shows that at a salt tolerance level of 2% NaCl, the phosphorus solubilities of the two lines were affected, and the phosphorus solubilities decrease above 4% NaCl (Figure 3.A). This shows that these two bacterial strains have potential for application in agricultural production in the Mekong Delta, especially in saline lands, helping to increase production costs and crop productivity.

Table 1. PSI and ability of 15 bacterial strains on four NaCl concentrations

Codes of bacteria	PSI	[NaCl] (%)			
		0.5	1.0	3.0	5.0
1.1	1.00^{d}	-	-	-	-
1.3	1.28^{bc}	+	+	+	+
1.4	1.13^{cd}	+	+	+	+
1.7	1.94^{a}	+	+	+	+
1.8	1.17^{cd}	+	+	+	+
2.1	1.05^{d}	+	+	+	+
2.3	1.09^{cd}	+	+	+	+
2.8	1.41^{b}	+	+	+	+
4.1	1.95^{a}	+	+	+	-
4.3	1.10^{cd}	-	-	-	-
5.3	1.19^{cd}	+	+	+	+
5.5	1.07^{d}	+	+	-	-
6.1	1.93^{a}	+	+	-	-
7.3	1.13^{cd}	-	-	-	-
8.3	1.09^{cd}	+	+	+	+

Notes: (+): live; (-): die; numbers with the same letter in the same column are not significantly different (p>0.05)

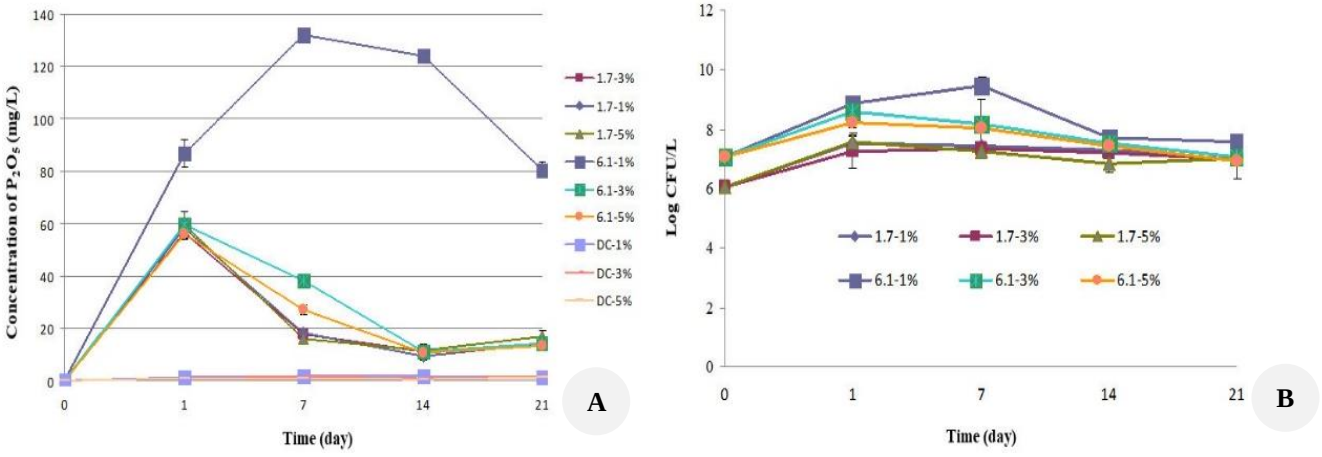

Figure 3. A. Phosphorus solubilization ability; B. Bacterial population of 2 bacterial strains, 1.7 and 6.1, in NBRIP medium at 3 different salt concentrations

The populations of the two bacterial strains at different NaCl concentrations throughout the culture process are shown in Figure 3.B. The results revealed that the populations of both bacterial strains increased and reached a maximum after 1 day of culture at different salt concentrations. At 7, 14 and 21 days of culture, the bacterial population at most concentrations decreased slightly, and the difference was not statistically significant. This result shows that 1.7 and 6.1 bacterial strains, can grow in environments with NaCl concentrations ranging from 1% to 5%. When bacterial strain 6.1 was cultured in NBRIP medium with a 1% salt concentration, the population gradually increased and reached its maximum after 7 days of culture. This finding is consistent with the results showing the ability of bacterial strain 6.1 to dissolve phosphorus, indicating that the greater the population is, the greater the ability to dissolve phosphorus.

Nguyen et al. (2012), reported that PSB belonging to the genera *Bacillus*, *Brevibacillus*, and *Acinetobacter soli* were able to dissolve phosphorus in the range of 40.0-86.0 P_2O_5 (mg/L) after 10 days of culture. Walpola et al. (2012) reported that bacterial strain *Burkholderia anthina* is able to dissolve phosphorus in the form of $Ca_3(PO_4)_2$ in NBRIP media, with the highest amount of dissolved phosphorus reached 665 mg/L P_2O_5 after 7 days of experimentation. Additionally, Cherif-Silini et al. (2013) studied two bacterial strains (D1 and D13) and reported that they were able to dissolve the highest amounts of phosphorus in NBRIP media after 10 years of testing, with levels reaching 173.28 and 146.57 mg/L P_2O_5, respectively. Moreover, these two bacterial strains were studied for their ability to dissolve phosphorus at different salinity concentrations, such as 0, 2, 4, 6, and 8% NaCl, for 5 days. The results showed that at a salinity level of 2% NaCl, the phosphorus solubilities of both were not affected and that the amount of soluble phosphorus began to decrease above 4% NaCl. So, bacterial strain 1.7 and 6.1 may be native bacteria that can have good phosphorus solubility and high salt tolerance.

Identification of selected bacteria

The results of agarose gel electrophoresis of the 16S rRNA gene sequences of strains 1.7 and 6.1 are shown in Figure 4. The DNA fragments amplified via PCR were single bands weighing approximately 1500 bp each. The PCR products of strains 1.7 and 6.1 were sequenced to obtain the full-length 1423 bp and 1386 bp gene sequences, respectively. The phylogenetic trees of the two isolates were constructed on the basis of their 16S rRNA gene sequences, as depicted in Figures 5 and 6. The results of the phylogenetic analysis revealed that isolate 1.7 most closely related to *Burkholderia* sp. was *Burkholderia vietnamiensis* strain 139, with 99.97% similarity. In contrast, isolate 6.1 associated with *Priestia* sp. was *Priestia aryabhattai* strain B8W22T.44, with 100% similarity. Combined with the physiological and biochemical results, strains 1.7 and 6.1 were identified as *B. vietnamiensis* strain 1.7 and *P. aryabhattai* strain 6.1, respectively.

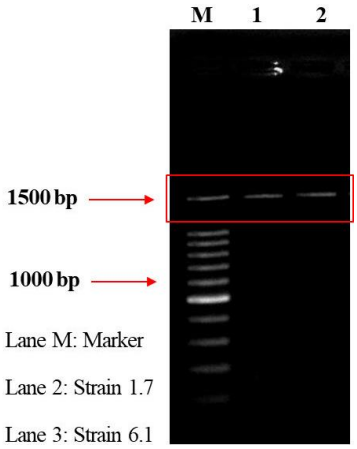

Figure 4. Agarose gel electrophoresis of the amplified products of the 16S rRNA gene

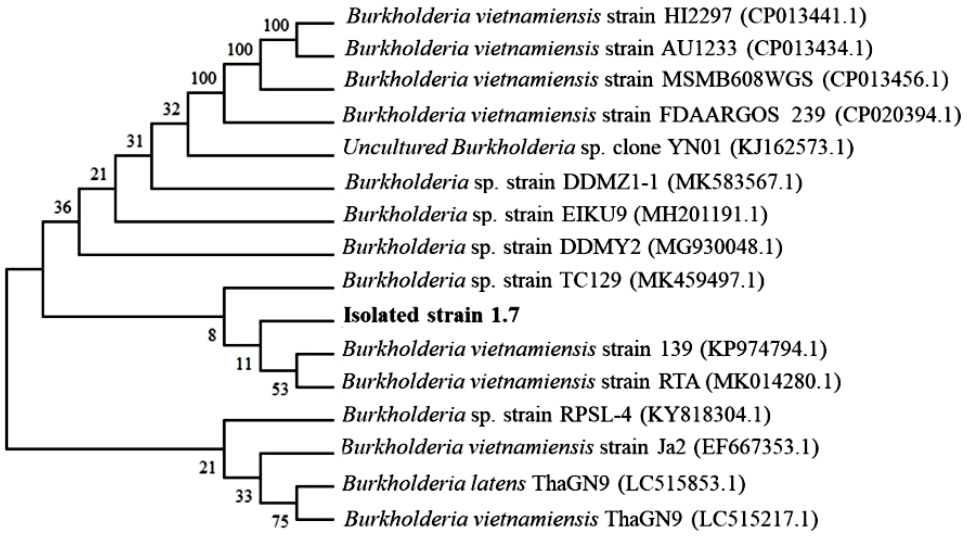

Figure 5. Phylogenetic analysis of the isolated strain 1.7

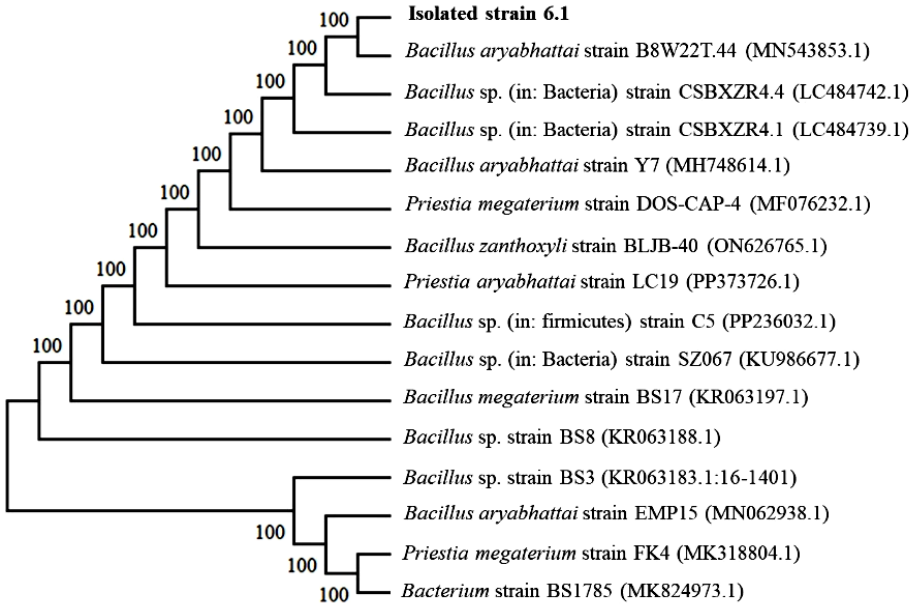

Figure 6. Phylogenetic analysis of the isolated strain 6.1

Research has shown that *Burkholderia* sp. and *Priestia* sp. (*Bacillus*) strains are classified as biosafety level 1 organisms and potential PSB strains (Reimer et al. 2019). These strains are found in agricultural soil and the root zones of plants and have been detected in soil samples under salt stress conditions (Park et al. 2010; Hwang et al. 2022). They are promoters of crop growth, improving plant productivity by converting insoluble forms of soil phosphorus (P) into soluble forms that plants can absorb (PO_4^{3-} ions) (Park et al. 2010; Ahmad et al. 2019; Hwang et al. 2022). Additionally, these strains can fix nitrogen, act as biological control agents, and stimulate plant growth (Damo et al. 2022; Phringpaen et al. 2023).

Burkholderia vietnamiensis strains, which are predominantly found in Vietnam (Gillis et al. 1995), fix nitrogen and rapidly solubilize insoluble phosphate under various environmental stresses, such as high salt, low and high pH levels, and low temperatures (Park et al. 2010; Ghosh and Mandal 2020). These strains produce gluconic and 2-keto-gluconic acids, with the highest concentrations of soluble phosphorus produced from $Ca_3(PO_4)_2$ and $CaHPO_4$ being 1,039 and 2,132 mg/L, respectively (Park et al. 2010). *Priestia aryabhattai* secretes organic acids and extracellular phosphatases at high levels, which can solubilize insoluble phosphates up to 388.62 µg/mL (Song et al. 2022; Wu et al. 2019). These factors increase soil available phosphorus and improve the growth and nutrition of maize crops and mung beans (Ahmad et al. 2019). As a result, isolated bacterial strains have the potential to be developed into biological products or biofertilizers for use in environmentally stressed soils. These factors could lead to increased crop yields, reduced use of chemical fertilizers and minimal negative impacts on the environment and soil quality in the Mekong Delta region, which are dangerous consequences of climate change (Kontgis et al. 2019).

In conclusion, from a total of 8 saline soil samples collected from 2 districts in Tien Giang province, Vietnam, 15 bacterial strains of salt-tolerant phosphorus solubilizing bacteria were isolated. Among these, two isolates (1.7 and 6.1) were found to be efficient phosphorus solubilizers, exhibiting strong growth and phosphorus solubilization in liquid NBRIP media supplemented with NaCl concentrations ranging from 1.0% to 5.0%. These isolates were molecularly identified as *Burkholderia vietnamiensis* 1.7 and *Priestia aryabhattai* 6.1. These two isolates have the potential to be used as microbial fertilizers to increase crop yields and reduce the need for chemical phosphate fertilizers, promoting safe and sustainable agricultural production.

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