

Postprint: Phosphate-Solubilizing Activity and Mechanism of Two Phosphate-Solubilizing Bacterial Strains

Authors: Chen Danyang, Li Hanquan, Zhang Binghuo, Cha Daiming, Yang Jianyuan

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Abstract

Phosphate-solubilizing bacteria play an important role in increasing soil soluble phosphorus content and improving phosphorus fertilizer utilization efficiency. To screen for highly efficient phosphate-solubilizing bacteria and explore their phosphate-solubilizing mechanisms, this study utilized the plate halo zone method to screen phosphate-solubilizing bacteria, employed the molybdenum-antimony anti-colorimetric method to investigate their phosphate-solubilizing activity, used the disodium phenyl phosphate method to study their phosphatase activity, applied thin-layer chromatography to analyze the organic acids they produce, and determined their taxonomic status based on physiological and biochemical characteristics and phylogenetic analysis of 16S rRNA gene sequences. The results showed that strains JXJ-11 and JXJ-15 exhibited strong degradation activity against calcium phytate, with soluble phosphorus concentrations in the culture medium increasing by 219 mg · L⁻¹ and 216 mg · L⁻¹, respectively, after 3 days; their degradation activity against calcium phosphate was relatively weak, with the maximum soluble phosphorus concentration being only 21.79%~30.37% of that for calcium phytate; phosphate-solubilizing bacteria could secrete acidic, neutral, and alkaline phosphatases to degrade insoluble phosphorus, and could produce organic acids such as propionic acid and succinic acid, thereby decreasing the culture medium pH and increasing soluble phosphorus concentration. Both strains were Gram-negative bacilli, non-sporulating, and produced hydrogen sulfide. The 16S rRNA gene sequence of strain JXJ-11 showed the highest similarity (99.79%) to *Sphingomonas melonis* DAPP-PG 224T and *S. aquatilis* JSS7T, while that of strain JXJ-15 exhibited the highest similarity (99.73%) to *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104T. Based on this information, strains JXJ-11 and JXJ-15 were identified as members of the genera *Sphingomonas* and *Klebsiella*, respectively. The phosphate-solubilizing mechanisms of strains JXJ-11 and JXJ-15 include the secretion of or-

ganic acids and phosphatases, with JXJ-11 showing potential application value in the development of microbial phosphorus fertilizers.

Full Text

Phosphate Solubilization Activities and Action Mechanisms of Two Phosphate-Solubilizing Bacteria

CHEN Danyang, LI Hanquan, ZHANG Binghuo, ZHA Daiming, YANG Jianyuan
(College of Pharmacy and Life Sciences, Jiujiang University, Jiujiang 332000, China)

Abstract: Phosphate-solubilizing bacteria play an important role in increasing soluble phosphorus content in soils and improving phosphorus fertilizer utilization efficiency. To screen highly efficient phosphate-solubilizing bacteria and explore their mechanisms, this study employed the halo zone method on agar plates for bacterial screening, the molybdenum-antimony colorimetric method to investigate phosphate-solubilizing activity, the disodium phenyl phosphate method to assess phosphatase activity, thin-layer chromatography (TLC) to analyze organic acids produced, and determined their taxonomic status based on physiological and biochemical characteristics combined with 16S rRNA gene sequence phylogenetic analysis. The results showed that strains JXJ-11 and JXJ-15 exhibited strong degradation activity against calcium phytate, with soluble phosphorus concentrations in the culture broth increasing by $219 \text{ mg} \cdot \text{L}^{-1}$ and $216 \text{ mg} \cdot \text{L}^{-1}$, respectively, after three days. Their activity against calcium phosphate was relatively weak, with maximum soluble phosphorus concentrations reaching only 21.79%-30.37% of those observed with calcium phytate. Both strains secreted acidic, neutral, and alkaline phosphatases to degrade insoluble phosphorus and likely produced organic acids such as propionic acid and succinic acid, which reduced culture broth pH and increased soluble phosphorus concentrations. Both strains were Gram-negative rods without spores and produced hydrogen sulfide. The 16S rRNA gene sequence of strain JXJ-11 showed highest similarity (99.79%) to *Sphingomonas melonis* DAPP-PG 224 and *S. aquatilis* JSS7, while strain JXJ-15 showed highest similarity (99.73%) to *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104. Based on these results, strains JXJ-11 and JXJ-15 were identified as members of the genera *Sphingomonas* and *Klebsiella*, respectively. The phosphate-solubilizing mechanisms of both strains included secretion of organic acids and phosphatases, with JXJ-11 showing potential application value in developing microbial phosphorus fertilizers.

Keywords: Phosphate-solubilizing bacteria; Phosphate solubilization activity; Organic phosphate; Inorganic phosphate; *Sphingomonas*; *Klebsiella*; 16S rRNA gene sequence

Phosphorus is an essential element for normal plant growth and development. More than 95% of phosphorus in soil exists as insoluble forms that plants cannot directly absorb [1], and approximately 74% of cultivated land in China suffers from phosphorus deficiency [1]. The application of organic and chemical phosphorus fertilizers constitutes the primary source of soil phosphorus [2]. However, soil minerals exhibit strong adsorption and fixation of phosphorus, often forming insoluble and unavailable phosphates [1-2], resulting in a utilization efficiency of only 5%-25% for applied phosphorus fertilizers in the current season [1]. Phosphorus fertilizers are primarily derived from phosphate rock, a non-renewable resource that will be depleted within 50-100 years [3]. Therefore, increasing soluble phosphorus content in soil and improving phosphorus fertilizer utilization efficiency are urgent issues in agriculture.

Soil contains numerous phosphate-solubilizing microorganisms, including bacteria, fungi, and actinomycetes. Through their metabolic activities, these microorganisms convert insoluble phosphorus into soluble forms for plant uptake and enhance the efficiency of applied phosphorus fertilizers. Culturable phosphate-solubilizing microorganisms account for 27.1%-82.1% of the total culturable microbial community in China's dryland soils, with phosphate-solubilizing bacteria being the most abundant [4], indicating substantial potential for screening highly efficient strains. Chen et al. [5] isolated 36 calcium phosphate-dissolving bacteria from a soil sample in central Taiwan, belonging to the genera *Bacillus*, *Rhodococcus*, *Arthrobacter*, *Serratia*, *Chryseobacterium*, *Delftia*, *Gordonia*, and *Phyllobacterium*, which released soluble phosphorus concentrations ranging from 31.5 to 519.7 mg · L⁻¹ from calcium phosphate. In recent years, Chinese researchers have conducted extensive studies on phosphate-solubilizing bacteria, identifying many strains with good solubilizing activity, primarily including *Burkholderia* [6], *Pseudomonas*, *Enterobacter* [7-9], *Acinetobacter*, *Serratia* [8], *Bacillus* [9-11], and *Pantoea* [12]. These bacteria demonstrate good solubilizing activity against calcium phosphate, with soluble phosphorus concentrations reaching 190-663 mg · L⁻¹ after inoculation.

Current research in China has primarily focused on inorganic phosphorus (mainly calcium phosphate), with fewer studies on organic phosphorus solubilizing activity. Moreover, identified phosphate-solubilizing bacteria generally exhibit weaker activity against organic phosphorus than inorganic phosphorus, with soluble phosphorus concentrations below 26 mg · L⁻¹ in lecithin medium [10,12] and below 100 mg · L⁻¹ in calcium phytate medium [11] after inoculation. Organic phosphorus accounts for 20%-50% of total phosphorus in soil [1-2] and is particularly important for crop growth [1]. Therefore, research on organic phosphorus-solubilizing bacteria should be strengthened alongside studies on inorganic phosphorus-solubilizing bacteria. Using calcium phosphate and calcium phytate as phosphorus sources, this study investigated the phosphate-solubilizing activity and mechanisms of bacteria isolated from forest soils in Jiangxi Province, aiming to screen strains with good degradation activity against both insoluble inorganic and organic phosphorus and to preliminarily understand their solubilizing mechanisms, thereby providing new materials and

theoretical foundations for developing highly efficient microbial phosphorus fertilizers.

1 Materials and Methods

1.1 Bacterial Strains

The tested strains comprised over 30 bacterial isolates obtained from forest soil samples collected in Jiujiang City, Jiangxi Province.

1.2 Culture Media

Solid phosphate-solubilizing medium: Prepared according to the formula specified in the Agricultural Industry Standard of the People' s Republic of China NY/T 1847–2010 [13], containing glucose 10 g, (NH) SO 0.5 g, NaCl 0.3 g, MgSO · 7H O 0.3 g, KCl 0.3 g, FeSO · 4H O 0.036 g, MnSO · 4H O 0.03 g, organic phosphorus source calcium phytate 2.0 g [or inorganic phosphorus source Ca (PO) 10 g], deionized water 1000 mL, agar 15 g, pH 7.0, sterilized at 121 °C for 30 min.

Liquid phosphate-solubilizing medium: Same composition as the solid medium but without agar.

Bacterial culture medium: Contained glucose 4 g, yeast extract powder 4 g, malt extract powder 5 g, agar 20 g (omitted for liquid medium), deionized water 1000 mL, pH 7.4, sterilized at 121 °C for 30 min.

1.3 Primary Screening on Solid Phosphate-Solubilizing Medium

Pure cultures of bacterial strains were spot-inoculated onto solid phosphate-solubilizing medium plates and incubated at 28 °C for 3–6 days. The presence and size of halo zones were observed, and the ratio of halo zone diameter to colony diameter was used to evaluate phosphate-solubilizing activity.

1.4 Phosphate Solubilization and Phosphatase Activity in Liquid Culture

Strains were first cultured in bacterial medium for 48 h. The cultures were then centrifuged under sterile conditions, and the cell pellets were resuspended in sterile water. The bacterial suspensions were inoculated into liquid phosphate-solubilizing medium (control groups received equal volumes of sterile water) to a final cell concentration of 5×10^8 CFU · mL⁻¹. Cultures were incubated at 28 °C with shaking at 180 r · min⁻¹. Samples were collected every 24 h to determine soluble phosphorus concentration, pH, organic acid types, and phosphatase activity.

According to the Agricultural Industry Standard NY/T 1847–2010 issued by the Ministry of Agriculture of China [13], soluble phosphorus concentration in culture broth was measured using spectrophotometry with the control group

reaction solution as the reference for zero adjustment. Culture broth pH was measured using a pH meter. For organic acid analysis, culture samples were spotted on silica gel GF plates alongside standard solutions of organic acids including tartaric acid, propionic acid, lactic acid, succinic acid, malic acid, and oxalic acid. After drying, plates were developed using solvent systems of methanol/chloroform (3:4, 5:4, 3:2, v/v) and observed under 254 nm ultraviolet light. Organic acid types were preliminarily identified based on UV absorption and migration rate (Rf).

Phosphatase activity was determined following the method of Zhao et al. [14]. The procedure was as follows: 1 mL of culture broth was mixed with 0.2 mL toluene, 4.5 mL disodium phenyl phosphate solution (0.676%), and 4.5 mL of appropriate buffer (acetate buffer pH 5.0 for acidic phosphatase, citrate buffer pH 7.0 for neutral phosphatase, or borate buffer pH 10.0 for alkaline phosphatase). The mixture was sealed and incubated at 37 °C for 12 h. Control groups used equal volumes of distilled water instead of disodium phenyl phosphate solution under identical conditions. After incubation, 1 mL of reaction solution was sequentially mixed with 5 mL borate buffer (pH 9.0), 3 mL 2.5% potassium ferricyanide, and 3 mL 0.5% 4-aminoantipyrine, then diluted to 50 mL with distilled water. After 30 min, absorbance was measured at 570 nm using the control group as the reference, and phenol content was calculated from a standard curve. One unit of phosphatase activity was defined as the amount of enzyme that catalyzed the production of 0.1 mg phenol from disodium phenyl phosphate in 1 mL of culture broth within 12 h.

1.5 Strain Identification

Physiological and biochemical characteristics were determined following the methods of Shen et al. [15], including Gram staining, spore staining, capsule staining, sugar fermentation tests, hydrogen sulfide production tests, methyl red test, Voges-Proskauer (V-P) reaction, and starch and lipid hydrolysis tests.

Bacterial genomic DNA was extracted using the Chelex 100 method. The 16S rRNA gene was amplified using universal bacterial primers 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'-TACGGCTACCTTGTTACGACTT-3') [16]. Amplification products were sequenced by Shanghai Sangon Biotech and compared with sequences in the EzTaxon database (<http://www.ezbiocloud.net/eztaxon>). Highly similar sequences were retrieved, multiple sequence alignment was performed using CLUSTAL_X 1.83, and a phylogenetic tree was constructed using MEGA software with the neighbor-joining method.

1.6 Statistical Analysis

Data were processed using SPSS 19.0 statistical software. Mean values and standard deviations (SD) were calculated from three replicates. Graphical data represent the mean values of three experiments. Changes in soluble phospho-

rus concentration, pH, and phosphatase activity were analyzed using one-way ANOVA. Differences were considered significant at $P < 0.05$ and highly significant at $P < 0.01$.

2 Results

2.1 Phosphate-Solubilizing Activity on Solid Medium

The results showed that over 30 bacterial strains grew well on both calcium phytate and calcium phosphate solid media, indicating their ability to solubilize insoluble phosphorus for growth. However, only strains JXJ-11 and JXJ-15 produced obvious halo zones on both media [Figure 1: see original paper], with halo zone diameter/colony diameter ratios of 2.75 ± 0.17 and 1.48 ± 0.03 on calcium phytate medium, and 2.42 ± 0.14 and 1.80 ± 0.08 on calcium phosphate medium, respectively. This demonstrated their strong capacity to solubilize insoluble phosphorus, particularly against calcium phytate, leading to their selection for further study.

2.2.1 Soluble Phosphorus Content

As shown in [Figure 2: see original paper], in liquid medium containing calcium phosphate, soluble phosphorus concentrations peaked at 2 days and 1 day after inoculation with strains JXJ-11 and JXJ-15, reaching $(47.28 \pm 0.51) \text{ mg} \cdot \text{L}^{-1}$ and $(67.37 \pm 1.21) \text{ mg} \cdot \text{L}^{-1}$, respectively, before declining rapidly. In contrast, in calcium phytate medium, soluble phosphorus concentrations increased rapidly after bacterial inoculation. For strain JXJ-11, the concentration reached $(219.69 \pm 3.38) \text{ mg} \cdot \text{L}^{-1}$ after 3 days, accounting for 42.54% of the total phosphorus in calcium phytate, with little change thereafter. For strain JXJ-15, the maximum concentration of $(216.96 \pm 11.25) \text{ mg} \cdot \text{L}^{-1}$ was achieved after 3 days, representing 42.02% of the total phosphorus in calcium phytate, followed by a slight decrease.

2.2.2 pH and Phosphatase Activity

As shown in [Figure 3: see original paper], one day after inoculation, the pH of calcium phytate medium decreased sharply from approximately 7.07 to 3.1–3.2, remaining relatively stable thereafter, while the uninoculated control maintained a pH around 7.0. In calcium phosphate medium, pH decreased from approximately 7.3 to 4.3–4.4 one day after inoculation, but rebounded to 5.4–5.5 after 2 days, with a slower recovery rate subsequently. After 5 days, the pH of cultures of strains JXJ-11 and JXJ-15 had recovered to 5.81 ± 0.08 and 6.15 ± 0.11 , respectively, while the control remained at approximately pH 7.3. TLC analysis revealed that culture broths of both strains grown on calcium phytate and calcium phosphate media contained substances with Rf values similar to propionic acid and succinic acid, suggesting that these organic acids may be produced by the two strains.

[Figure 4: see original paper] shows that for strain JXJ-11 in calcium phytate medium, acidic phosphatase activity remained relatively stable at 1.10-1.67 U · mL⁻¹ from day 2 onward; neutral phosphatase activity was low during the first 3 days, increased rapidly to (12.04±0.57) U · mL⁻¹ on day 4, then decreased quickly; alkaline phosphatase activity remained consistently low, reaching only (0.75±0.05) U · mL⁻¹ at its peak on day 4 [Figure 4a: see original paper]. In calcium phosphate medium, all three phosphatase activities of strain JXJ-11 were relatively strong, with acidic phosphatase activity peaking at (4.78±0.14) U · mL⁻¹ after 4 days, and neutral and alkaline phosphatase activities reaching maxima of (6.34±0.36) U · mL⁻¹ and (8.20±0.28) U · mL⁻¹, respectively, after 3 days [Figure 4b: see original paper].

Phosphatase activities of strain JXJ-15 were similar to those of JXJ-11 [Figure 5: see original paper]. In calcium phytate medium, acidic phosphatase activity of strain JXJ-15 remained stable at 1.95-2.92 U · mL⁻¹ after day 1; neutral phosphatase activity was below 1 U · mL⁻¹ during the first 4 days, increasing rapidly to (6.26±0.05) U · mL⁻¹ on day 5; alkaline phosphatase activity remained consistently low, reaching only (0.91±0.09) U · mL⁻¹ at its maximum on day 4 [Figure 5a: see original paper]. In calcium phosphate medium, all three phosphatase activities of strain JXJ-15 increased, with acidic phosphatase activity peaking at (8.44±0.48) U · mL⁻¹ after 4 days, neutral phosphatase activity reaching (3.52±0.17) U · mL⁻¹ after 2 days (with maximum activity similar to that in calcium phytate medium), and alkaline phosphatase activity reaching a maximum of (3.04±0.12) U · mL⁻¹ after 4 days [Figure 5b: see original paper].

2.3 Taxonomic Status of Strains JXJ-11 and JXJ-15

Both JXJ-11 and JXJ-15 were rod-shaped, non-spore-forming, Gram-negative bacteria. Strain JXJ-11 could ferment lactose and glucose with acid and gas production, decompose sulfur-containing organic compounds to produce hydrogen sulfide, was positive for V-P test, negative for methyl red test, and negative for starch and lipid hydrolysis. Strain JXJ-15 could ferment lactose and glucose with acid but no gas production, decompose sulfur-containing organic compounds to produce hydrogen sulfide, was negative for V-P test, positive for methyl red test, and negative for starch and lipid hydrolysis.

16S rRNA gene sequence analysis revealed that strain JXJ-11 (1,560 bp) belonged to the genus *Sphingomonas*, showing 99.79% sequence similarity to both *Sphingomonas melonis* DAPP-PG 224 and *S. aquatilis* JSS7, clustering with these two strains in the neighbor-joining phylogenetic tree [Figure 6: see original paper] and showing 98.25% similarity to other validly published species. Strain JXJ-15 (1,625 bp) belonged to the genus *Klebsiella*, showing 99.73% sequence similarity to *Klebsiella pneumoniae* subsp. *pneumoniae* DSM 30104, clustering with this strain in the neighbor-joining phylogenetic tree [Figure 7: see original paper].

3 Discussion and Conclusion

The halo zone method on solid medium and liquid culture method are the two most commonly used approaches for studying microbial phosphate-solubilizing capacity. This study utilized these methods to screen phosphate-solubilizing bacteria JXJ-11 and JXJ-15, which demonstrated good degradation capabilities against both insoluble inorganic and organic phosphorus. 16S rRNA gene sequence analysis identified these strains as members of the genera *Sphingomonas* and *Klebsiella*, respectively, suggesting their potential application value in addressing the problem of available phosphorus deficiency despite severe phosphorus accumulation in cultivated soils.

Secretion of organic acids to reduce pH and dissolve insoluble phosphorus, thereby increasing soluble phosphorus content, represents one of the important mechanisms of microbial phosphate solubilization. Organic acids secreted by phosphate-solubilizing microorganisms mainly include oxalic acid, acetic acid, lactic acid, tartaric acid, succinic acid, citric acid, butyric acid, propionic acid, gluconic acid, and malic acid [5,17-18]. This study found that strains JXJ-11 and JXJ-15 likely produced organic acids such as propionic acid and succinic acid, leading to decreased culture broth pH and increased soluble phosphorus content. However, different insoluble phosphorus sources affected organic acid secretion, with calcium phytate appearing to promote greater acid production than calcium phosphate. When utilizing calcium phosphate as the sole phosphorus source, lower pH correlated with higher soluble phosphorus concentrations in the culture broth, indicating a relationship between bacterial acid production and soluble phosphorus concentration, consistent with previously reported results [18-19].

Secretion of phosphatases represents another important mechanism for microbial degradation of insoluble phosphorus [2]. Bacteria JXJ-11 and JXJ-15 could secrete acidic, neutral, and alkaline phosphatases in liquid phosphate-solubilizing medium. The type of phosphorus source significantly affected the activities of the three phosphatases. In calcium phytate medium, only acidic phosphatase activity remained stable, neutral phosphatase activity increased rapidly after 4 days but remained high for only a short period, and alkaline phosphatase activity remained consistently low. This may be because the culture broth in calcium phytate medium maintained a pH around 3.1 for an extended period, leading to denaturation of neutral and alkaline phosphatases. In calcium phosphate medium, the pH decrease was smaller (to approximately pH 4.4) and began to recover after 1 day, eventually reaching approximately pH 6.0. Consequently, denaturation of all three phosphatases (particularly neutral and alkaline phosphatases) was substantially reduced, which should be the main reason why bacteria JXJ-11 and JXJ-15 exhibited good activities of acidic, neutral, and alkaline phosphatases in calcium phosphate liquid medium.

Guan et al. [6] reported that phosphatases have minimal effect on calcium phosphate degradation. Therefore, although strains JXJ-11 and JXJ-15 secreted rel-

atively strong activities of all three phosphatases in calcium phosphate medium, bacterial degradation of calcium phosphate may primarily depend on organic acid secretion. In contrast, their degradation of calcium phytate could proceed through both organic acid secretion and phosphatase activity, which should be the main reason why strains JXJ-11 and JXJ-15 showed much stronger degradation capacity against calcium phytate than against calcium phosphate.

Sphingomonas was first reported in 1990 [20]. These microorganisms exhibit strong degradation capabilities for high-molecular-weight organic pollutants, and current research has primarily focused on their ability to degrade various organic pollutants [21]. *Sphingomonas* can tolerate extremely oligotrophic conditions [21-22], possesses autonomous nitrogen-fixing activity [23], is a component of plant rhizosphere microbiota [24-25], and has multiple functions including phosphate solubilization and indole-3-acetic acid secretion, capable of alleviating crop continuous cropping obstacles [24]. Therefore, it holds certain value for microbial fertilizer development. This study found that *Sphingomonas* strain JXJ-11 exhibited strong solubilizing capacity against calcium phytate, releasing 42% of phosphorus from calcium phytate as soluble phosphorus within 3 days under the experimental conditions. Additionally, this strain showed some solubilizing effect on calcium phosphate. Thus, strain JXJ-11 has potential value for developing microbial preparations that increase soluble phosphorus content in soil. However, since a few *Sphingomonas* species are opportunistic pathogens and plant pathogens—for example, *S. paucimobilis* can infect postoperative and immunocompromised individuals [26], and *Sphingomonas melonis* can cause brown spots on Spanish melon (*Cucumis melo* var. *inodorus*) fruits [27]—further studies on the relevant characteristics of this strain are needed to determine whether it can be used for microbial fertilizer development.

Members of the genus *Klebsiella* are mostly opportunistic pathogens, particularly *Klebsiella pneumoniae*, which is highly pathogenic and prohibited for use in microbial fertilizers in China [28]. Therefore, although strain JXJ-15 showed strong phosphate-solubilizing activity, its identification as *Klebsiella* with 99.73% similarity to *Klebsiella pneumoniae* subsp. *pneumoniae* precludes its use in microbial fertilizer development due to safety concerns. However, its phosphate-solubilizing genes remain worthy of study and could provide target genes for constructing highly efficient engineered phosphate-solubilizing strains.

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