Isolation of halo-tolerant bacteria with plant growth-promoting traits

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Abstract. Over the last decades, world agriculture has suffered from soil salinity problems such as nutrient deficiency and salt toxicity. The use of plant growth-promoting bacteria (PGPR) has been widely reported to alleviate salt stress and promote plant growth via various mechanisms including the decrease in ethylene levels and the increase of bioavailability of nutrients. We have tested 39 bacteria stains for their plant growth-promoting traits such as 1-aminocylopropane-1-carboxylate (ACC) deaminase, indole acetic acid (IAA) production as well as phosphate solubilization. The results showed that 33 isolates produced ACC deaminase, ranging from 0.3 to 35.0 µmol α-KA/mg/h and 27 isolates produced IAA from 1.4-59.9 µg/mL. Sixteen isolates showed the ability to solubilize bio-unavailable phosphate, resulting in halo-zones in the size of 1.5 to 13.3 mm, with 7 isolates of phosphate solubilization index above 2.0. To conclude, isolates with great potential for plant growth promotion have been isolated. The application of these isolates can be an option to replace chemicals used in current agriculture practices and help the development of sustainable agriculture.

1. Introduction
Over the last decades, world agriculture has suffered from soil salinity problems. Soil salinity inhibits plant growth by affecting physiological, biochemical and metabolic processes, eventually resulting in crop yield loss. Approaches such as traditional breeding and genetic engineering have been explored to increase the resistance to salt stress; however, these methods meet obstacles such as the great amount of time required, regulation restrictions and higher cost. One alternative to mitigate the soil salinity problem is the use of plant growth-promoting rhizobacteria (PGPR) to improve plant growth under high salinity conditions. PGPR is known to promote plant growth and improve nutrition either directly and indirectly. Direct mechanisms include ACC (1-aminocyclopropane-1-carboxylic acid) deaminase activity, phytohormones such as auxin, cytokine and gibberellin, siderophore production, and phosphate solubilization [1-5]. Bacterial strains containing ACC-deaminase plays role in the regulation of a plant hormone, ethylene and thus, growth and development of plant are modified [6]. On other hand also had function as a sink for ACC, the immediately precursor of C₂H₄ in higher plants, by hydrolyzing into α-ketobutyrate (KA) and ammonia, and consequently promote plant growth. Interestingly, plants that are inoculated with rhizobacteria having ACC-deaminase are more resistant to injurious of stress environment such as salinity, drought, flooding and heavy metal [6-9].

Indole-3-acetic acid is a phytohormone which plays a central role in plant growth and development as a regulator of numerous biological processes. Bacterial IAA producers have the potential to interfere with any of these processes by input of IAA into the auxin pool of plants [10, 11]. Phosphate soluble microorganism improving phosphate uptake by solubilizing phosphate complexes into plant absorbable and make it being usable forms. Major problem in salinity soil are generally low in fertility, particularly lacking phosphorus (P) and nitrogen (N) [12]. Since nutrient deficient and salt-stressed soil are known to severely suppress plant growth and crop productivity. P is one of important macronutrients also a component of key molecules such as nucleic acid, phospholipids and ATP [13]. Indirect mechanisms include those such as protection against phytopathogens (i.e., biocontrol) by
producing antifungal compounds [14-16]. As a result, PGPR can alleviate salt stress and improve plant growth in many crop plants such as avocado, cotton, groundnut, alfalfa, canola, chilli peppers, wheat, and oats by mitigating salt stress [8, 17-24]. The purpose of the current study was to isolate halo-tolerant rhizobacteria from multiple sampling locations in Taiwan and characterize their plant growth-promoting traits.

2. Material and Method

2.1. Isolation of rhizobacterial isolates

Twelve rhizosphere soil samples were collected from different grassland fields with high soil salinity in Taiwan. Isolation was conducted according to Penrose and Glick [25]. Briefly, one gram of soil from each sample was transferred into 50 ml of King’s B broth sterile media amendment 2 % of NaCl in a 250 ml flask. The flask was incubated at 200 rpm at room temperature. A 1 mL aliquot was transferred in DF salt liquid medium contain nitrogen and carbon sources and incubated with shaking at 200 rpm for 24 hours at room temperature. A 1 mL aliquot of this culture was washed two-times using DF salt medium without nitrogen source. An aliquot of 100 µL of the cell suspension was transferred into 5 ml of DF salt medium contains 30 µL ACC without nitrogen source and incubated at 200 rpm for 24 hours at room temperature. A loopful of each culture was streaked onto1.8 % agar DF minimum salt agar medium containing 3mM ACC as the sole nitrogen source and incubated for 3 days. Individual colonies were then selected. The purified isolates were kept at -80 °C in King’s B broth medium containing 40 % (w/v) glycerol before they were used in further experiments.

2.2. Characterization of Rhizobacterial Isolates

Rhizobacterial strains were characterized for different traits including ACC-deaminase activity, IAA production, and phosphate production.

2.2.1 Production of ACC deaminase

The ACC deaminase activity assay is based on the method in Penrose and Glick [25, 26]. Briefly, α-ketobutyrate (KA) amount was measured by comparing the absorbance at 540 nm of sample to a standard curve ranging between 0.1 and 1.0 µmol. The bacterial cell pellets were prepared of each suspension in 5 ml of DF containing 60 µl of ACC as sole nitrogen source followed by incubation at 25°C for 24 h at 200 rpm. The pellets were harvested by centrifugation at 4,000 xg for 10 min followed washed use 1 ml of 0.1 M Tris-HCl (pH 7.8) and transferred to a 1.5 ml microcentrifuge tube. The pellet was suspended in 600 µl of 0.1 M Tris- HCl (pH 8.5). Thirty microliters of toluene was added to the cell suspension and vortexes vigorously for 30 s, and 100 ml aliquot of “toluened cells” was set aside and stored at 4°C for protein assay at a later time. The remaining toluenized cell suspension was used for assay of ACC deaminase activity. For ACC deaminase assay all samples were measured and carried out in triplicates. Two hundred microliters of the toluenized cells were placed in a new 1.5 ml microcentrifuge and 20 µl of 0.5M ACC were added to the suspension, briefly vortexes and incubated at 30°C for 15 min. Following the addition of 1 ml of 0.56 M HCl 35%, the mixture was vortexes and centrifuged for 5 min at 13,000 xg at room temperature. Then, 300 µl of the 2.4-dinitrophenyl hydrazine reagent (0.2 % 2.4 dinitrophenylhydrazine in 2 M HCl) was added to the glass tube, vortexes and incubated for 30 min at 30°C. Following the addition and mixing of 2 ml of 2 N NaOH, the absorbance was measures at 540 nm. The absorbance of the reaction including the substrate, ACC, and the bacterial extract was determined with spectrophotometer. After the indicated incubations, the absorbance at 540 nm of the assay reagent in the presence of ACC was used as a reference for the spectrophotometric reading; it was substrate from the absorbance value calculated above.

2.2.2 Production of indole acetic acid
Production of IAA was done by method described by Gordon and Weber [27]. Bacterial culture was grown in King’s B broth and 0.5 ml overnight culture were inoculated in DF salt liquid medium and incubated for 48 hours at 30 °C. An aliquot of 20 µl were transferred into 5 ml of DF salt minimal media supplemented with 500 µg/ml L-tryptophan (from a filter-sterilized 2-mg/ml stock prepared in warm water; Sigma-Aldrich). After 48 h period time of incubation, the density of each culture was measured using spectrophotometer 600 nm for determine optical density each isolate followed 1.5 ml of growth culture was centrifuged at 5500 ×g for 10 minutes. A 1 mL aliquot of the supernatant was mixed vigorously with 2 mL of Salkowski’s reagent (250 ml, 60% of H₂SO₄, 7.5 ml of 0.5 M FeCl₃·6H₂O solution) and allowed to stand at room temperature for 25 min. Absorbance was measured at 535 nm by spectrophotometry. The concentration of IAA in each culture medium was determined by a standard curve [2, 28].

2.2.3 Phosphate production

The ability of rhizobacterial isolates to solubilize bio-unavailable phosphate was tested by using tricalcium phosphate in the PVK agar plate and NBRIP broth [29]. The solubilization of insoluble tricalcium phosphate salt in the agar was indicated by a clear zone around the colony. The solubilization index (SI) was determined with measuring scale and calculation using the formula: solubilization index = (colony diameter + zone halo)/ colony diameter [30]. For quantitative measurement of the phosphate solubilization ability, the vanado-molybdate method was used with the absorbance at a wavelength of 410 nm. The concentration of soluble phosphorus in each culture medium was determined by comparison with a standard curve [31].

3. Result and Discussion

3.1 Isolation of Rhizobacteria

The purpose of this study was to isolation and characterized the bacteria have the plant growth-promoting traits from 12 locations of a variety of soil properties as well as vegetation. A total of 39 isolates of rhizobacteria were isolated and characterized.

In the present study, a preliminary screening of native bacterial populations from saline soils in Taiwan was carried out to investigate their plant growth-promoting attributes. To ensure that isolated bacteria strains are indeed halo-tolerant so that they can be applied in saline soil in the field, culture medium was amended with 2% NaCl during the initial isolation process. Thirty-nine of halo-tolerant rhizobacteria were screened for their plant growth-promoting traits. The results obtained corroborate with the fact that isolates from saline soil have more plant growth-promoting properties [15, 21, 32, 33]. Many isolates showed great ACC-deaminase activity to ameliorate stress because strains that have an enzyme activity more than 20 µmol α-KA/mg/h could influence plant growth under salt stress by decreasing stress-induced ethylene production [34]. Furthermore, α-KA and ammonia breakdown products of ACC, serve as sources of nitrogen and energy respectively for the associated bacteria [34].

3.2 Characterization of Rhizobacteria for Plant Growth Traits

The bacteria isolates were tested for different plant growth promoting traits. The isolates showed different PGPR traits such as ACC-deaminase activity, IAA production, and phosphate solubilization (Table 1).
Table 1. Semi-quantitative comparison of ACC deaminase, IAA production and phosphate solubilization ability of isolated strains.

| Isolate | ACC deaminase $^a$ | IAA production $^b$ | Phosphate solubilization $^c$ |
|---------|-------------------|-------------------|---------------------|
| A1      | +                 | -                 | +                   |
| A2      | +                 | +                 | -                   |
| B1      | +                 | +                 | +                   |
| B2      | +                 | -                 | ++                  |
| C1      | +                 | +                 | -                   |
| C2      | +                 | ++                | -                   |
| C3      | -                 | -                 | -                   |
| D1      | +                 | -                 | -                   |
| D2      | +                 | -                 | -                   |
| D3      | +                 | +++               | -                   |
| E1      | +                 | +                 | +++                 |
| E2      | -                 | -                 | ++                  |
| E3      | +                 | -                 | -                   |
| F1      | +                 | -                 | -                   |
| F2      | +                 | ++                | -                   |
| F3      | +                 | +                 | -                   |
| F4      | +                 | -                 | -                   |
| G1      | +                 | -                 | +                   |
| G2      | +                 | -                 | -                   |
| G3      | +                 | ++                | -                   |
| H1      | +                 | +++               | +                   |
| H2      | -                 | -                 | ++                  |
| H3      | -                 | +++               | +                   |
| H4      | +                 | -                 | +                   |
| I1      | +                 | +                 | -                   |
| I2      | -                 | -                 | -                   |
| I3      | -                 | +                 | +                   |
| J1      | +                 | +                 | ++                  |
| J2      | +                 | +                 | +                   |
| J3      | +                 | +                 | +                   |
| K1      | +                 | -                 | -                   |
| K2      | +                 | +                 | -                   |
| K3      | +                 | -                 | -                   |
| L1      | +                 | ++                | +                   |
| L2      | +                 | -                 | -                   |
| L3      | +                 | -                 | -                   |
| L4      | ++++              | -                 | -                   |
| L5      | ++++              | +                 | +                   |
| L6      | ++++              | +                 | +++                 |

$^a$ (-) not detected, (+) production of ACC-deaminase under 20 α-KA/mg/h, (+++) production of ACC-deaminase 40-60 α-KA/mg/h, (+++++) production of ACC-deaminase above 80 α-KA/mg/h.

$^b$ (-) not detected, (+) production of IAA under 20 µg/mL, (+++) production of IAA 40-60 µg/mL.

$^c$ Phosphate solubilization: (-) no halo zone observed; (+) solubilization index under 2, (+++) solubilization index 2-4, (++++) solubilization index 4-6, (+++++) solubilization index above 6.

3.2.1 Production of ACC deaminase

Rhizobacteria were characterized for ACC-deaminase activity. In the present study, 33 out of 39 rhizobacteria isolates were able to produce ACC-deaminase in the presence of the substrate ACC (Figure 1). The highest ACC-deaminase activity was exhibited by PGPR isolates L4 (140 µmole α-KA/mg/h) followed by L5 (57.1 µmole α-KA/mg/h) and L6 (46 µmole α-KA/mg/h).
3.2.2 Production of Indole Acetic Acid

Screening of 39 bacterial isolates for IAA production revealed that 27 isolates showed IAA production in the presence of the precursor L-tryptophan (Figure 2). Production of indole-3-acetic acid (IAA) by rhizosphere-associated bacteria isolates. The IAA production ability varied greatly among isolates, ranging from 1.4-59.9 µg/mL.

Figure 2. Production of indole-3-acetic acid (IAA) by rhizosphere-associated bacteria isolates. The bars represent the standard deviation of mean (n = 3).

3.2.3 Phosphate Solubilization

Screening of 39 bacterial isolates for phosphate solubilization activity revealed that 16 of these showed good growth. Phosphate solubilizing bacteria were identified by the formation of visible halo zones around the colony on Pikovskaya’s agar plate amended with calcium triphosphates (Figure 3A). Isolate E1 showed outstanding phosphate solubilization ability of index 13.3, while isolates B2, E2, H2, H3, I3, and J1 have phosphate solubilization index around 2 (Figure 3B). The relative P-efficiency of 16 isolates in solubilizing calcium triphosphates was evaluating in vitro using calcium triphosphates in liquid medium as a function of time up to 7 days after incubation. The results showed that increasing amount of P was released by different isolates with increasing period of incubation.

Figure 1. The ACC deaminase activity by rhizosphere-associated bacteria isolates in DF salt liquid medium supplemented after 48 h of incubation. The bars represent the standard error of the mean (n = 3).
Figure 3. Qualification measurements of phosphate solubilization. A) Phosphate solubilization halo produced by one of the most promising strains, B) Halo-zone size of the ability of rhizosphere-associated bacteria to solubilize bio-unavailable phosphate on Pikovskaya’s agar plate up to 7 days after incubation. The bars represent the standard error of the mean (n = 3).

Figure 4. Quantitative measurements of phosphate solubilization and the change of pH over the 7-day culture period. A) Solublized phosphate concentrations in the culture supernatant. B) The pH values of bacterial culture in NBRIP liquid culture medium. Control means the culture without bacterial inoculation. Consortium means the culture that was inoculated with all the bacterial isolates). DAI means days after inoculation. The bars represent the standard error of the mean (n = 3).
The phosphate solubilization ranged from 39.3-712.7 µg/mL after 7 days of period incubation (Fig 4A). The bacterial isolate E1 (712.7 µg/mL) was the most efficient solubilize followed by B2 (700.2 µg/mL), E2 (689.1 µg/mL) and G1 (615.5 µg/mL). Strain E1 was a potent solubilize and showed maximal P-solubilization by 120 h of incubation. It was observed that isolates B1, B2, E2, G1, H1, H2, I3, J1, J2, K3, and L3 exhibited maximal phosphate solubilization potential on day 5 which then declined gradually, while the isolate JH3 and JL1 exhibiting the potential on six-day. To conclude, variability existed in the relative P-solubilization potential among rhizobacterial isolates, and the solubilized P concentrations reached the maximal level at day 3 or day 5, followed by a decline. One possible reason why the decrease in soluble P is that the bioavailable P in the culture were consumed by the bacteria themselves.

At the end of the incubation time in the NBRIP liquid culture medium, the pH dropped to around 4.0 – 5.0, compared to the control (pH 6.6) (Fig 4B). This result showed that phosphate solubilization of all 16 PGPR isolates were in an inverse relationship with the pH of the bacteria culture during the incubation period, suggesting that organic acid secreted by bacteria may not only decrease the overall pH but also play a role in phosphate solubilization. The pH optimum for production of phosphate solubilization is 4 to 5.5.

IAA production is a prominent feature of plant growth promoting activities because IAA modulated plant root growth, cell division and development [35]. IAA production by PGPR can vary among different species and strains, and also depend influenced by culture conditions, growth stage and substrate availability. Our work showed the amount of IAA produced by some isolates was higher than the production optimum of indole acetic acid that previous study [36-38]. It has been suggested in the literature that organic acids produced such as oxalic, malonic, fumaric, tartaric and succinic acid in media may play a role in P-solubilization [39, 40]. The increase in organic acids is generally followed by a sharp decrease in pH, which results in the dissolution of insoluble calcium-phosphate. However, the direct role of organic acids in P-solubilization has been questioned because some reports have mentioned other mechanisms such as the production of chelating compounds as the predominant drive for P solubilization as the pH variation had no consistent correlation with P-solubilization activity [41, 42]. Furthermore, one puzzling result is the lack of consistency of P-solubilization ability of bacterial strains when determined by measuring the halo-zone size and by measuring the soluble phosphate concentrations with spectrophotometry. This inconsistency could be attributed to the different culturing conditions, one on solid surface and the other in liquid, as literature has mentioned the difference in metabolites in liquid and solid media [40, 41, 43].

4. Conclusion
Our isolated halo-tolerant strains showed great variation in plant growth-promoting traits. Isolate L4 exhibited the highest level of ACC-deaminase activity, D3 produced the highest level of IAA concentration, and E1 had the best phosphate solubilization ability. Potential use these PGPR isolates needs further testing for their ability to enhance plant growth under growth chamber, greenhouse as well as field conditions.

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