Isolation, Characterization, and Use for Plant Growth Promotion Under Salt Stress, of ACC Deaminase-Producing Halotolerant Bacteria Derived from Coastal Soil

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In total, 140 halotolerant bacterial strains were isolated from both the soil of barren fields and the rhizosphere of six naturally growing halophytic plants in the vicinity of the Yellow Sea, near the city of Incheon in the Republic of Korea. All of these strains were characterized for multiple plant growth promoting traits, such as the production of indole acetic acid (IAA), nitrogen fixation, phosphorus (P) and zinc (Zn) solubilization, thiosulfate ($S_2O_3$) oxidation, the production of ammonia ($NH_3$), and the production of extracellular hydrolytic enzymes such as protease, chitinase, pectinase, cellulase, and lipase under in vitro conditions. From the original 140 strains tested, on the basis of the latter tests for plant growth promotional activity, 36 were selected for further examination. These 36 halotolerant bacterial strains were then tested for 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity. Twenty-five of these were found to be positive, and to be exhibiting significantly varying levels of activity. 16S rRNA gene sequencing analyses of the 36 halotolerant strains showed that they belong to 10 different bacterial genera: *Bacillus*, *Brevibacterium*, *Planococcus*, *Zhihengliuella*, *Halomonas*, *Exiguobacterium*, *Oceanimonas*, *Corynebacterium*, *Arthrobacter*, and *Micrococcus*. Inoculation of the 14 halotolerant bacterial strains to ameliorate salt stress (150 mM NaCl) in canola plants produced an increase in root length of between 5.2% and 47.8%, and dry weight of between 16.2% and 43%, in comparison with the uninoculated positive controls. In particular, three of the bacteria, *Brevibacterium epidermidis* RS15, *Micrococcus yunnanensis* RS222, and *Bacillus aryabhattai* RS341, all showed more than 40% increase in root elongation and dry weight when compared with uninoculated salt-stressed canola seedlings. These results indicate that certain halotolerant bacteria, isolated from coastal soils, have a real potential to enhance plant growth under saline stress, through the reduction of ethylene production via ACC deaminase activity.

Keywords: ACC deaminase, plant growth promoting rhizobacteria, halotolerant bacteria, root elongation, canola, salt stress

Plant growth promoting rhizobacteria (PGPR) comprise a group of beneficial bacteria that can be found in the rhizoplane and rhizosphere, the phyllosphere, or the inside of plant tissues as endophytes [16]. Different ecological niches have been explored for the isolation and characterization of PGPR and include the rhizosphere soil of different crop plants [18, 19], arable saline soil [32, 42], polluted or contaminated soils [5], composted municipal solid waste [25], milk [9], cow dung [40], and insect gut [21]. PGPR are able to promote plant growth via direct or indirect mechanisms, or a combination of both [15, 16]. Indirect mechanisms include the suppression of pathogens through the action of siderophores, and the production of antibiotics and extracellular hydrolytic enzymes [16, 40]. Direct mechanisms include an altered nutrition through the provision of fixed nitrogen; iron through siderophores; soluble phosphate (P) and zinc (Zn) [18, 19, 22]; the production of phytohormones such as indole acetic acid (IAA), cytokinin, and gibberellins [23, 31]; or by the activity of 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, an enzyme that can lower plant ethylene levels that are typically increased by a wide variety of environmental stresses such as flooding, drought, heavy metals, organic contaminants, pathogen attacks, and salt stress [5, 8, 17, 20, 32, 36, 45].

Salinity is a natural feature of ecosystems in arid and semi-arid regions and can also be induced by anthropogenic
activities such as irrigation [1]. Nearly 20% of the world’s cultivated land and nearly half of all irrigated lands are affected by salinity [46]. Salt stress has previously been reported to cause an increased production of ethylene in plants, thereby accelerating leaf and petal abscission and organ senescence, leading to premature death [8, 32, 45]. Reducing the stress-induced ethylene level can alleviate some of the effects of stresses on plants [14]. In fact, a high plant loss, of approximately 40% of photosynthates, is through root exudates [30], and it has been postulated that much of the ACC, which is a precursor of ethylene produced under stress conditions, may be exuded from plant roots [4] and then hydrolyzed by the enzyme ACC deaminase into ammonia and α-ketobutyrate. This means that more ACC is exuded by the plant root and drawn away from the ethylene synthesis pathway [15], and that quantities of ACC become lower as ACC oxidase is converted into ethylene. Thus, PGPR, with ACC deaminase activity, can be used to reduce the negative effects of salinity stress [8, 32, 45]. PGPR efficiency is determined by various environmental factors such as the climate, weather conditions, soil characteristics, and interaction with other indigenous microbial flora in the soil [12]. Mayak et al. [32] reported that ACC deaminase-producing salt-tolerant bacteria can survive well in a saline environment and that their beneficial properties help plants to overcome stress effects. Halotolerant bacteria are a group of microorganisms able to grow in media containing a wide range of NaCl (1–33%) or in the absence of NaCl [27]. Hence, it was hypothesized that the use of ACC deaminase-producing PGPR halotolerant bacteria could ameliorate the saline stress effect on plants by reducing ethylene levels.

The present study was therefore conducted in an attempt to isolate and characterize the diverse group of halotolerant bacteria from coastal soil for their numerous PGPR traits. Selected strains were then checked for their ability to ameliorate saline stress under gnotobiotic conditions using canola plants.

**MATERIALS AND METHODS**

**Soil Sampling and Isolation of Halotolerant Bacteria**

The sampling sites were situated within 15 km² of the saline coastal region of the Yellow Sea near the city of Incheon in the Republic of Korea. A total of seven soil samples were randomly collected from either barren fields or the rhizosphere of six different naturally growing halophytic plants, during the later period of the winter of 2009 (designated as sites 1, 2, and 3 nearest to the coastline; site 4 and sites 5, 6, and 7 about 500 m and 1.5–2 km away from the coastline, respectively). Three samples were collected from each site, mixed together to make one composite sample for that site, sieved at 2 mm in order to separate plant debris and visible fauna, and then stored at 4°C.

Ten-fold serial dilutions of the samples were made by mixing the soil with sterile saline water (0.85% NaCl), shaking for 15 min at 150 rpm, and then plating on a tryptic soy agar medium (peptone, 15 g/l; tryptone, 5 g/l; dextrose, 2.5 g/l) modified with 1.75 M (−10%) NaCl, and adjusted to a pH of 8.5 [7]. The plates were incubated at 28°C for 3–4 days, and strains were isolated based on colony morphology, pigmentation, and growth rate. Pure cultures of the halotolerant bacterial strains were maintained in 30% glycerol at −80°C.

**Functional and Biochemical Characterization of Halotolerant Bacterial Strains**

The assay media for the functional and biochemical characterization of the isolated halotolerant bacterial strains were modified by the addition of 0.85 M (−5%) NaCl and adjusted to a pH of 7.2. Nitrogen fixing and sulfur-oxidizing potentials were tested by the methods described by Gothwal et al. [18] and Anandham et al. [2], respectively. The indole-3-acetic acid (IAA) and ammonia producing abilities of the halotolerant bacterial strains were tested by the method reported by Brick et al. [6] and Wani et al. [43], respectively. The phosphate (P) and zinc (Zn) solubilizing abilities of the halophiles were tested on Pikovskaya’s medium [35], which was supplemented with either 0.5% Ca₃(PO₄)₂, or 0.12% ZnO. Characterization for extracellular hydrolytic enzyme production was carried out using recommended media, modified by the addition of 0.85 M NaCl, and supplemented with the specific substrate for each enzyme: 1.5% (v/v) colloidal chitin for chitinase, methyl cellulose for cellulase, pectin for pectinase, tributyrin for lipase, casein for protease, and gelatine for gelatinase [3, 11]. Urease production was tested using Difco urea broth (Becton Dickinson Inc., U.S.A.).

**Characterization Based on 16S rRNA Gene Sequencing and Phylogenetic Analyses**

For molecular characterization, the selected halotolerant bacterial strains were subjected to 16S rRNA gene sequence analyses. The selected bacterial strains were grown in TSA and the DNA was extracted [38]. The 16S rRNA genes were amplified by a PCR using the forward primer 27F 5'-AGAGTTTGATCCTGGCTCAG-3' and the reverse primer 1492R 5'-GGTTACCTTGTTACGACTT-3' and the reverse primer 1492R 5'-GGTTACCTTGTTACGACTT-3'. The 16S rRNA gene sequences were identified by PCR-direct sequencing, using the fluorescent dye terminator method with ABI prism equipment and a BigDye Terminator cycle sequencing ready reaction kit V.3.1 (Applied Biosystems Inc., U.S.A.), and the products were purified with a Millipore-Montage dye removal kit (Millipore-Montage Inc., U.S.A.). Finally, the products were run in an ABI 3730XL capillary DNA sequencer (Applied Biosystems Inc., U.S.A.), with a 50 cm capillary.

The obtained 16S rRNA gene sequences were aligned and the affiliations deduced, using BLAST analysis. Phylogenetic analyses were performed using MEGA version 4.1 [26] after multiple alignments of the data by CLUSTAL W [41]. Distances were obtained using options according to the Kimura two-parameter model [24], and clustering was performed using the neighbor-joining method [39]. The statistical confidence of the nodes was estimated by bootstrapping using 1,000 replications [10].

**Qualitative Assay of Utilization of ACC**

The availability of 1-aminoacyclopropane-1-carboxylic acid (ACC) as a nitrogen source is as a consequence of the enzymatic activity of ACC deaminase (E.C. 4.1.99.4). ACC deaminase activity was checked...