J. Microbiol. Biotechnol. (2010), **20**(11), 1577–1584
doi: 10.4014/jmb.1007.07011
First published online 15 September 2010

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

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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Received: July 7, 2010 / Revised: July 28, 2010 / Accepted: July 29, 2010

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

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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 semiarid 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 approximatly 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 PGP 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 PGP 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 km^2 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 selected 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% $Ca_3(PO_4)_2$ 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'. 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-aminocyclopropane-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

according to the method of Glick [16], with modifications. The bacteria were first cultured in a TSA medium with 0.85 M NaCl. A solution of ACC (0.5 M) (Sigma Chemical Co., U.S.A.) was filter-sterilized (0.2 μm) and frozen at −20°C [34]. Halotolerant bacterial strains were streaked onto NFb medium supplemented with 3.0 mM ACC as a nitrogen source. Plates were incubated at 30°C for 4 days. The ability of a strain to utilize ACC was verified by maintaining the same strain in a control in the absence of any nitrogen source.

Quantification of ACC Deaminase Activity

ACC deaminase activity was assayed according to the method of Penrose and Glick [34], which measures the amount of α-ketobutyrate produced when the enzyme ACC deaminase cleaves ACC. The μmole quantity of α-ketobutyrate (Sigma-Aldrich Co., U.S.A.) produced by this reaction was determined by comparing the absorbance of a sample to a standard curve of α-ketobutyrate ranging between 0.1 and 1.0 nmol at 540 nm. A stock solution of α-ketobutyrate was prepared in 0.1 M Tris-HCl (pH 8.5) and stored at 4°C. In order to measure the specific activity of the cultures, protein estimation was carried out according to Lowry *et al*. [29].

Evaluation of Halotolerant Bacteria Inoculation Effects on Canola Growth Under Salt Stress in Gnotobiotic Conditions

The halotolerant bacterial strains *Br. epidermidis* RS15, *Br. iodinum* RS16, *P. rifietoensis* RS18, *E. acetylicum* RS19, *Z. alba* RS111, *M. yunnanensis* RS222, *O. smirnovii* RS231, *B. stratosphericus* ES445, *Br. iodinum* RS451, *B. stratosphericus* RS616, *B. licheniformis* RS656, *C. variabile* RS665, *B. aryabhattai* ES341, and *A. nicotianae* RSA68 were selected for the testing of root elongation and growth promotion under salt stress in gnotobiotic conditions, on the basis of the presence or absence of ACC deaminase activity and IAA production. According to Cheng *et al.* [8], the growth of canola seedlings was reduced by approximately 50% with 150 mM NaCl at 20°C. Hence, this concentration was used to check the bacterial inoculation effects on the growth promotion of canola. Seed treatment and gnotobiotic growth pouch assays were performed in accordance with Penrose and Glick [34], with slight modifications. Briefly, halotolerant bacterial strains were grown in TSA supplemented with 0.85 M NaCl. The cells were then collected and resuspended in a N-free medium containing 0.85 M NaCl and supplemented with 5 mM ACC as the sole nitrogen source, and subsequently incubated for 24 h at 30°C with shaking (120 rpm) in order to induce ACC deaminase activity. After that, the cells were harvested, and washed by resuspension in sterile 0.03 M $MgSO_4$. Canola seeds, *Brassica campestris*, (Hungnong Seed Co. Ltd., Korea and Seminis Korea Inc., Korea), were surface sterilized by immersion in 70% ethanol for 1 min and 2% NaCl for 30 s, followed by a thorough rinsing in sterile distilled water (6−7 times). Then these surface-sterilized seeds were soaked in sterile distilled water, or bacterial suspension (1×10^8 CFU/ml), for 24 h. Following on from this, 20 ml of water, or water containing 150 mM NaCl, was added to CYG seed germination pouches (Mega International Manufacturer Inc., U.S.A.) and autoclaved at 121°C for 15 min. Sprouted seeds were transferred aseptically to growth pouches and incubated in a DS 54 GLP growth chamber (DASOL Scientific Co., Ltd., Korea) and maintained at 20±1°C, with a relative humidity of 70% and a dark/light cycle beginning with 12 h of dark, followed by 12 h of light (18 μmol m^{-2} s^{-1}). Seven seeds were placed in each pouch for each treatment, and each treatment had three replicates. Seeds in growth pouches treated only with water were used as the negative control, and seeds in growth pouches treated only with salt solution were used as the positive control. In theory, the positive controls should be found to reduce growth in a manner very similar to the actual salt stress effects on plants in the field, whereas negative controls should provide the standard for any changes from the normal growth process of the plant. Data were collected regarding root length and the total dry weight of the plants from 7-day-old canola seedlings.

Statistical Analysis

Data on the growth parameters of canola were subjected to analysis of variance (ANOVA). Significance at the 5% level was tested by Duncan's multiple range test (DMRT) using the SAS package, Version 9.1.3 (SAS, 2010).

Nucleotide Sequence Accession Numbers

The National Center for Biotechnology Information GenBank accession numbers for the sequences of halotolerant bacterial strains are from GU968455 through to GU968490.

RESULTS

Functional and Biochemical Characterization of Halotolerant Bacteria

One hundred and forty halotolerant bacterial stains, isolated from seven soil samples collected from coastal soil, were able to grow on medium containing 1.75 M (10%) NaCl. Of these, Table 1 illustrates the 36 strains that possessed multiple PGP traits, and were selected for further study.

16S rRNA Gene Sequencing and Phylogenetic Classification

The selected halotolerant bacterial strains were identified by 16S rRNA gene sequencing analysis to ascertain their taxonomic positions. The nucleotide sequences recovered from these bacterial strains were subjected to homology in the NCBI database, which showed that the representative 36 halotolerant bacterial strains belong to 3 phyla, 4 orders, 7 families, and 10 different genera (Fig. 1). Out of the 36 strains, 12 strains (RS652, RS616, RS654, ES445, ES446, RS233, RS340, RS447, RS114, RS656, RS341, and ES667) showed a 99−100% similarity with the 16S rRNA gene sequences of the genus *Bacillus*, 8 strains (RS263, RS212, RS449, RS451, RS448, RS16, RS15, and RS361) showed a 98−99.2% similarity to *Brevibacterium*, 5 strains (RS18, RS224, RS344, RS338, and RS112) exhibited likeness at ≤99% to *Planococcus*, 3 strains (RS234, RS236, and RS111) at ≤98% to *Zhihengliuella*, 2 strains (RS343 and RS19) at ≤99% to *Exiguobacterium*, 2 strains (ES11E and RS229) at 96−99% to *Halomonas*, 1 strain (RS231) at ≤98% to *Oceanimonas*, 1 strain (ES665) at ≤98% to *Corynebacterium*, 1 strain (RSA68) at ≤99% to *Arthrobacter*, and 1 strain (RS222) at ≤99% to *Micrococcus* (Table 1). The first phylum under Firmicutes had a close

Table 1. Identification and characteristics of halotolerant bacterial strains isolated from coastal soil.

Strain code	Closest species (% similarity)	Accession number in NCBI	ACC deaminase activity[a]	Plant growth promoting traits[b]								
				NF	IAA	S_2O_3	NH_3	Protease	Pectinase	Chitinase	Cellulase	Lipase
RS15	*Brevibacterium epidermidis* (99.1)	GU968456	2.37±0.48	+	+	+	+	+	-	-	+	+
RS16	*Brevibacterium iodinum* (99.0)	GU968457	4.13±1.05	+	+	+	+	+	-	-	-	-
RS18	*Planococcus rifietoensis* (99.8)	GU968458	-	+	-	+	+	-	-	-	+	-
RS19	*Exiguobacterium acetylicum* (99.5)	GU968459	-	+	-	+	+	-	-	-	+	+
RS111	*Zhihengliuella alba* (98.3)	GU968460	1.38±0.86	+	+	+	+	-	+	+	-	-
RS112	*Planococcus rifietoensis* (99.7)	GU968461	-	+	+	+	+	-	-	+	+	+
RS114	*Bacillus pumilus* (99.7)	GU968462	-	+	-	+	+	+	-	-	-	-
ES11E	*Halomonas neptunia* (99.7)	GU968455	0.93±0.09	+	-	+	+	-	+	-	+	+
RS222	*Micrococcus yunnanensis* (99.7)	GU968463	0.82±0.09	+	+	+	+	-	+	-	+	-
RS212	*Brevibacterium epidermidis* (99.1)	GU968464	2.25±0.51	+	+	+	+	-	+	+	-	-
RS224	*Planococcus rifietoensis* (99.9)	GU968465	0.96±0.06	+	-	+	+	-	-	+	-	-
RS229	*Halomonas korlensis* (96.2)	GU968466	-	+	+	-	+	-	-	-	-	-
RS231	*Oceanimonas smirnovii* (98.9)	GU968467	1.40±0.51	+	+	+	+	-	-	-	-	+
RS233	*Bacillus stratosphericus* (100)	GU968468	1.93±0.54	+	+	+	+	-	-	+	+	+
RS234	*Zhihengliuella alba* (98.3)	GU968469	-	+	+	+	+	-	-	-	-	-
RS236	*Zhihengliuella alba* (98.3)	GU968470	0.73±0.06	+	+	+	+	-	-	+	-	-
RS263	*Brevibacterium epidermidis* (99.2)	GU968486	-	+	+	+	+	-	+	-	+	-
RS338	*Planococcus rifietoensis* (99.9)	GU968471	-	+	+	+	+	+	-	+	-	-
RS340	*Bacillus stratosphericus* (100)	GU968472	-	+	-	+	+	+	+	-	-	-
RS341	*Bacillus aryabhattai* (99.9)	GU968473	1.11±0.42	+	-	+	+	-	+	-	-	-
RS343	*Exiguobacterium acetylicum* (99.5)	GU968474	0.99±0.09	+	+	+	+	-	-	+	-	+
RS344	*Planococcus rifietoensis* (99.9)	GU968475	-	+	-	+	+	-	+	-	-	-
RS361	*Brevibacterium epidermidis* (98.9)	GU968485	1.27±0.81	+	+	+	+	-	+	+	-	-
ES445	*Bacillus stratosphericus* (100)	GU968476	1.19±0.18	+	-	+	+	-	-	+	+	-
ES446	*Bacillus stratosphericus* (100)	GU968477	2.89±0.87	+	-	+	+	+	-	-	+	-
RS447	*Bacillus pumilus* (99.7)	GU968478	0.89±0.06	+	-	+	+	+	-	+	-	-
RS448	*Brevibacterium epidermidis* (99)	GU968479	4.90±1.0	+	+	+	+	-	-	-	-	-
RS449	*Brevibacterium epidermidis* (99.1)	GU968480	0.69±0.27	+	+	+	+	-	-	-	-	-
RS451	*Brevibacterium iodinum* (98.6)	GU968481	1.11±0.12	+	+	+	+	-	-	-	-	-
RS652	*Bacillus stratosphericus* (99.7)	GU968482	2.01±0.63	+	+	+	+	+	-	-	-	-
RS616	*Bacillus stratosphericus* (99.7)	GU968483	-	+	+	+	+	-	-	-	-	-
RS654	*Bacillus stratosphericus* (99.9)	GU968484	1.63±0.21	+	+	+	+	+	-	-	+	+
RS656	*Bacillus licheniformis* (99.7)	GU968490	3.06±1.14	+	-	+	+	-	-	-	-	-
RS665	*Corynebacterium variabile* (98.3)	GU968487	1.17±0.45	+	-	+	+	+	-	-	-	-
ES667	*Bacillus aryabhattai* (100)	GU968488	1.37±0.15	+	-	+	+	+	+	-	-	-
RSA68	*Arthrobacter nicotianae* (99.2)	GU968489	0.70±0.06	+	+	+	+	+	-	-	-	-

[a]ACC deaminase activity: μmol α-ketobutyrate mg $protein^{-1}$ h^{-1}; values are means ± SD of three replications.

[b]Plant growth promoting traits: NF=Nitrogen fixation; IAA=Indole acetic acid production; S_2O_3=Thiosulfate oxidation; NH_3=Ammonia production; + (plus) indicates presence; - (minus) indicates absence of respective traits tested.

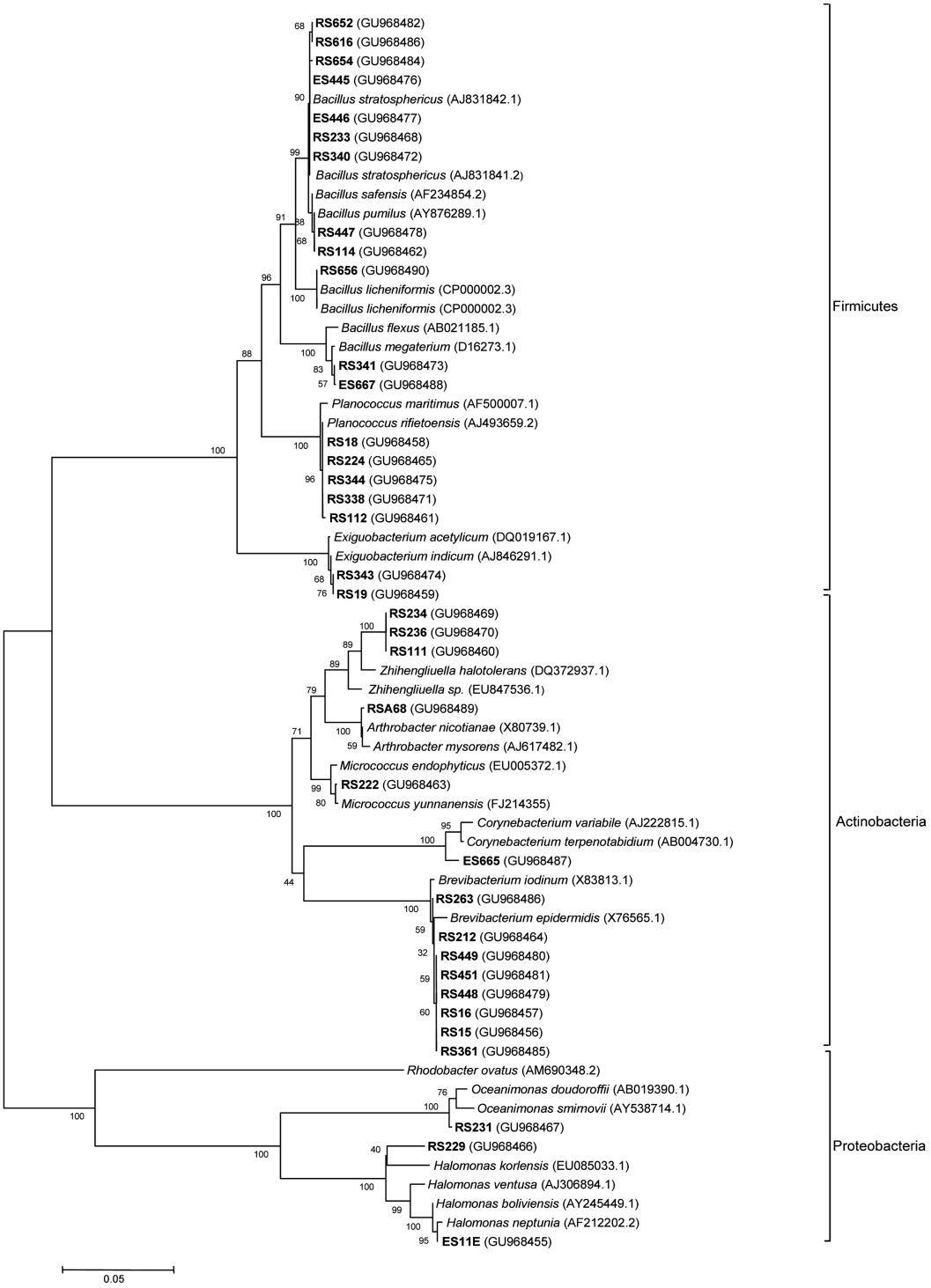

Fig. 1. Phylogenetic tree based on a 16S rRNA gene sequence analysis.
The halotolerant bacterial sequence is shown in bold font. The scale bar represents the expected number of substitutions.

sequence similarity, with a low DNA G+C content, to genera *Bacillus*, *Planococcus*, and *Exiguobacterium*; the second phyla under Actinobacteria, with a high DNA G+C content, was composed of the genera *Brevibacterium*, *Zhihengliuella*, *Arthrobacter*, *Micrococcus*, and *Corynebacterium*; and the third phyla Proteobacteria comprised the genera *Halomonas* and *Oceanimonas* (Fig. 1). Strain RS229 showed a 96% sequence homology with *Halomonas korlensis* in the database, indicating that it may belong to a new species of *Halomonas*.

ACC Deaminase Activity

The 36 strains possessing multiple PGP traits were tested for ACC deaminase activity, and 25 strains were found to be positive. Quantification of ACC deaminase activity showed wide variations (Table 1). The activity was highest in the cell-free extracts obtained from *Br. epidermidis* RS448 (4.9 μmol α-ketobutyrate mg protein^{-1} h^{-1}) followed by *Br. iodinum* RS16 (4.1 μmol α-ketobutyrate mg protein^{-1} h^{-1}), *B. licheniformis* RS656 (3 μmol α-ketobutyrate mg protein^{-1} h^{-1}), *B. stratosphericus* RS446 (2.9 μmol α-ketobutyrate mg protein^{-1} h^{-1}), *Br. epidermidis* RS15 (2.4 μmol α-ketobutyrate mg protein^{-1} h^{-1}), and *Br. epidermidis* RS212 (2.3 μmol α-ketobutyrate mg protein^{-1} h^{-1}) (Table 1).

Halotolerant Bacterial Inoculation Effects on Canola Growth Under Salt Stress in Gnotobiotic Conditions

Most of the tested strains were able to significantly promote the growth of canola seedlings in the presence of salt. Salt stress (150 mM NaCl) reduced root length by 37% and the plant total dry weight biomass by 40% in 7-day-old canola seedlings when compared with the negative control. Other than *B. stratosphericus* RS616, *P. rifietoensis* RS18, and *E. acetylicum* RS19, all of the 14 tested halotolerant bacterial strains were able to improve root length by between 29% and 47%, and dry weight by between 35% and 43% when under salt-stressed conditions in 7-day-old canola seedlings when compared with the positive control (Table 2).

Table 2. Halotolerant bacterial inoculation effects on canola growth under salt stress in gnotobiotic condition.

Treatment	Root length (cm plant^{-1})	Dry weight (mg plant^{-1})
Negative control	10.4±1.2^{bc}	1.05±0.03^{ab}
Positive control	6.5±0.24^{ef}	0.62±0.03^{e}
Brevibacterium epidermidis RS15	12.4±0.6^{a}	1.09±0.06^{a}
Brevibacterium iodinum RS16	11.4±0.9^{ab}	1.09±0.06^{a}
Planococcus rifietoensis RS18	5.9±0.21^{ef}	0.74±0.03^{d}
Exiguobacterium acetylicum RS19	7.7±0.6^{de}	0.81±0.03^{d}
Arthrobacter nicotianae RSA68	10.6±0.21^{abc}	1.01±0.06^{abc}
Zhihengliuella alba RS111	11.6±0.6^{ab}	1.03±0.03^{abc}
Micrococcus yunnanensis RS222	11.8±0.21^{ab}	1.06±0.03^{ab}
Oceanimonas smirnovii RS231	9.2±1.2^{dc}	0.99±0.09^{bc}
Bacillus licheniformis RS656	9.2±l.2 ^{dc}	0.97±0.06^{bc}
Bacillus stratosphericus ES445	10.3±0.2^{bc}	0.96±0.03^{c}
Bacillus aryabhattai RS341	11.2±0.9^{ab}	1.05±0.03^{ab}
Bacillus stratosphericus RS616	6.1±0.09^{ef}	0.76±0.03^{d}
Corynebacterium variabile RS665	11.7±1.5^{ab}	1.03±0.03^{abc}
Bacillus aryabhattai ES667	10.3±0.6^{bc}	1.02±0.03^{abc}

*Values (mean±SD *r=3) with the same letters are not significantly different at $p \leq 0.05$.

DISCUSSION

PGPR stimulate plant growth *via* both direct and indirect mechanisms with variable results depending on a number of environmental factors [12, 15, 16]. In the current study, a large number of halotolerant bacteria were characterized as being positive for nitrogen fixation, IAA production, P and Zn solubilization, S oxidation, and NH_3 production. Upadhyay *et al*. [42] found that only 18% (24 out of 130) of strains tested could tolerate up to 8% of NaCl, while maintaining the ability to produce IAA, fix nitrogen, and solubilize P. It is possible that extracellular hydrolytic enzymes may indirectly promote plant growth [16, 40], and Rohban *et al.* [37] reported that a large number of halotolerant bacteria were found positive for extracellular hydrolytic enzyme production. These findings agree with other studies [5, 42], where a number of halotolerant bacteria were found to have multiple PGP traits.

Phylogenetic analysis of the halotolerant bacterial 16S rRNA gene sequence revealed that highly diverse bacterial populations exist in coastal soils, representing some 10 different genera (Table 1 and Fig. 1). From 16S rRNA analysis, one third of the halotolerant bacteria exhibited 98–100% sequence homology with known 16S rRNA of the cultivated *Bacillus* genus (Table 1 and Fig. 1). In the course of the characterization of microorganisms present in a tidal flat of the Yellow Sea in Korea, many moderately halotolerant/halophilic bacteria have been isolated and characterized taxonomically as belonging to *Bacillus* [44].

PGPR that have ACC deaminase activity help plants to withstand stress (biotic or abiotic) by reducing the level of stress ethylene [8, 32, 45]. In the present study, we screened halotolerant bacteria having ACC deaminase activity with multiple PGP traits, and found that 25 out of the screened 36 strains showed ACC deaminase activity. Variations in levels of ACC deaminase activity of the strains were noted, and these results are concurrent with earlier findings [5, 21]. All of the genera (except *Planococcus*) isolated exhibited ACC deaminase activity. Thus, this is consistent with previous observations that diverse groups of bacteria were found to exhibit ACC deaminase activity [5, 21, 34].

Several reports show that ACC deaminase-producing PGPR increase root elongation and plant growth by reducing the ethylene stress [8, 13–15, 32, 45]. In this study, 14 halotolerant bacterial strains were tested for their growth promoting activity, under axenic conditions at 150 mM NaCl, by conducting growth pouch experiments on canola. Halotolerant bacteria, having the ability to produce both ACC deaminase and IAA, were found to enhance root elongation and the dry weight of canola to a greater extent than strains that produced solely ACC deaminase. It is likely that auxin and ACC deaminase

stimulate root growth in a coordinated fashion [13, 28]. Since IAA secreted by a bacterium may promote root growth directly, by stimulating plant cell elongation or cell division [33], this observation is consistent with the model [15] that envisions a complex cross-talk between IAA and ethylene in the promotion of plant growth by PGPR. The fact that halotolerant bacterial strains, which produce IAA but not ACC deaminase, inhibit root growth rather than promote elongation in the presence of salt might reflect a higher synthesis rate of ACC under stress. Moreover, IAA stimulates ACC synthase transcription, which, in the absence of ACC deaminase, could result in enhanced ethylene biosynthesis. In concordance with the results of the present study, Cheng *et al.* [8] showed that a wild-type strain of *Pseudomonas putida* UW4 (which produces IAA and ACC deaminase), but not an ACC deaminase minus mutant of this bacterium, protected canola seedlings from growth inhibition by high levels of salt.

In conclusion, our work has demonstrated that different halotolerant bacteria, isolated from soils obtained in a barren field and from the rhizosphere of halophytes, are able to withstand high salt concentrations (1.75 M NaCl) and pH (6.5 to 8.5), and can facilitate plant growth promotion in the presence of growth inhibitory levels of salt. These results further suggest, given the variation in activity observed, that the selection and subsequent industrial use of ACC deaminase-producing halotolerant bacteria, with multiple PGP activities for the facilitation of plant growth in saline environments, will be a highly important area for future research. Hence, further evaluation of these halotolerant bacterial strains is needed to uncover their efficiency as plant growth promoting bacteria in soil–plant systems.

Acknowledgments

This work was supported by the Mid-Career Researcher Program through an NRF grant funded by the MEST (No. 2010-0000418). Md. Ashaduzzaman Siddikee would like to convey a special thanks to Brain Korea21 (BK21) for their award of a Ph.D. fellowship. All of the authors are also very grateful to Prof. Bernard R. Glick of the University of Waterloo in Canada, and Dr. M. Madhaiyan of the Temasek Life Sciences Laboratory at the National University of Singapore, for their critical review and helpful suggestions for this manuscript.

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