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REGULAR ARTICLE

The roots of the halophyte *Salicornia brachiata* are a source of new halotolerant diazotrophic bacteria with plant growth-promoting potential

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Abstract Soil salinity is the major cause limiting plant productivity worldwide. Nitrogen-fixing bacteria were enriched and characterised from roots of *Salicornia brachiata*, an extreme halophyte which has substantial economic value as a bioresource of diverse and valuable products. Nitrogen-free semisolid NFb medium with malate as carbon source and up to 4% NaCl were used for enrichment and isolation of diazotrophic bacteria. The isolates were tested for plant growth-promoting traits and 16S rRNA, *nifH* and *acdS* genes were analysed. For selected strains, plant growth-promoting activities were tested in axenically grown *Salicornia* seedlings at different NaCl concentrations (0–0.5M). New halotolerant diazotrophic bacteria were isolated from roots of *S. brachiata*. The isolates were identified as *Brachybacterium saurashtrense* sp. nov., *Zhihengliuella* sp., *Brevibacterium casei*, *Haererehalobacter* sp., *Halomonas* sp., *Vibrio* sp., *Cronobacter sakazakii*, *Pseudomonas* spp., *Rhizobium radiobacter*, and *Mesorhizobium* sp. Nitrogen fixation as well as plant growth-promoting traits such as indole acetic acid (IAA) production, phosphate solubilisation, and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase activity were demonstrated. For *Brachybacterium saurashtrense* and *Pseudomonas* sp., significant plant growth-promoting activities were observed in *Salicornia* in salt stress conditions. *Salicornia brachiata* is a useful source of new halotolerant diazotrophic bacteria with plant growth-promoting potential.

Keywords 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase · Acetylene reduction · Halotolerant · IAA production · Plant growth-promoting rhizobacteria (PGPR) · *Salicornia*

This communication is dedicated to Dr. Johanna Doebereiner in appreciation of her great impact on the research on diazotrophic bacteria that promote plant growth.

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Introduction

Salt-affected soils are a consistent feature of arid and semiarid areas. The salinisation of arable land is increasing each day and poses a serious threat to our expanding needs for fertile soils and food production. Over 800 million hectares of land throughout the world are affected by salt (FAO 2008; http://www.fao.org/ag/

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agl/agll/spush/). According to global change scenarios, rising sea level will threaten agricultural production in large areas of hitherto fertile lands by increasing the salinity of the soil. Agriculture under saline conditions already presents major challenges in many countries for different reasons. To tackle this problem, the use of traditional breeding, genetic engineering of halotolerant transgenic plants and application of halotolerant plant growth promoting rhizobacteria (PGPR) are the major strategies by which cultivation in saline soils can be improved (Mayak et al. 2004).

PGPR are a heterogeneous group of bacteria that can be found in the rhizosphere, both at the root surface and in endophytic associations, and that can improve the extent or quality of plant growth (Rothballer et al. 2009). PGPR can facilitate plant growth and development in two different ways: directly or indirectly. The direct promotion of plant growth by PGPR generally entails providing the plant with a compound that is synthesised by the bacterium or facilitating the uptake of nutrients from the environment. The indirect promotion of plant growth occurs when PGPR reduce or prevent the deleterious effects of pathogens on plants by producing inhibitory substances or by increasing the natural resistance of the host (Cartieaux et al. 2003; Liu et al. 1995; Schuhegger et al. 2006). The direct growth-promoting mechanisms are as follows: (1) N_2 fixation, (2) phosphate solubilisation, (3) complexation of insoluble ferric iron by siderophore production, (4) production of phytohormones such as auxins, cytokinins, and gibberellins, and (5) lowering of the ethylene concentration (Mayak et al. 2004). The field of salt-tolerant rhizobacteria was recently reviewed by Egamberdiyeva and Islam (2008). The information on halotolerant diazotrophic PGPR is still limited; some examples of salt-tolerant PGPR include *Azospirillum halopraeferens* (Reinhold et al. 1987), *Swaminathania salitolerans* (Loganathan and Nair 2004), and the *Azospirillum brasilense* strain NH (Nabti et al. 2007). Because soil salinity is increasing in many parts of the world, there is a need for general improvement of plant performance and biological nitrogen fixation (BNF).

Salicornia brachiata Roxb. (Dicotyledons, Caryophyllales, Amaranthaceae) is an extreme halophyte growing in coastal areas. In the coastal areas of Gujarat, where cultivation is negligible, *S. brachiata* is grown to produce biomass for vegetable salt (US patent no. 6929809), tender shoots as green salad and greening the vast barren coastal saline area. It is of commercial value and of ecological importance. Therefore, a better understanding of the presence of the diazotrophic PGPR in *Salicornia* is important. There are few reports of PGPR in *Salicornia* spp., such as *Klebsiella pneumoniae* in *S. bigelovii* (Rueda-Puente et al. 2003), *Halomonas maura* in *Salicornia* spp. (Argandonña et al. 2005) and *Pseudomonas pseudoalcaligenes* in *S. europea* (Ozawa et al. 2007). To date, there have been no reports of PGPR in *S. brachiata*, the most salt-tolerant species among the *Salicornia* spp. Thus, this plant was selected for isolation, enrichment and characterisation of any halotolerant diazotrophic PGPR that were associated with it. 16S rRNA gene analysis was used for identification and phylogenetic characterisation of the isolates. Acetylene reduction assay and *nifH* gene amplification were carried out to confirm their N_2-fixing potential. Additionally, isolates were also evaluated in relation to their potential to promote plant growth by screening for IAA production, siderophore production, phosphate solubilisation, ACC deaminase activity, and the presence of the *acdS* gene. Finally, preliminary evidence for growth-promoting effects of two isolates could be demonstrated in axenically grown *S. brachiata*.

Materials and methods

Sample collection

Salicornia brachiata plants were collected from coastal marshy swamps of the Bhavnagar district (Navabandar coast) in Gujarat (21°45′N, 72°14′E), India, and transferred aseptically to the laboratory in sterile boxes. Bacteria were isolated from roots as described below.

Isolation of PGPR from *Salicornia* roots

The roots collected from young *S. brachiata* plants were washed thoroughly with a 0.5× PBS solution. After washing, the roots (0.5 g fresh weight) were homogenised with a sterile mortar in 9.5 ml of 0.5× PBS. Aliquots (50 μl) of serial dilutions were inoculated into vials containing 5 ml of the nitrogen-free semisolid NFb medium with malate as the carbon

source (Doebereiner 1995) and up to 4% NaCl concentration. Under these conditions, after 6–7 days at 30ºC, a diffuse subsurface growth pellicle appeared in vials with an inoculum dilution of 10^{-5} or less. Bacteria from the vial with the highest dilution and showing pellicle formation were transferred to the fresh sterile semisolid medium with the appropriate NaCl concentrations for second and third incubations. After the pellicle formation, a loop full of culture from these vials was streaked onto nitrogen-free solid NFb medium with a trace amount of yeast extract (20 mg l^{-1}). Single, separated colonies growing on these plates were re-inoculated into fresh semisolid NFb medium. Finally, cultures with subsurface growth pellicles were streaked onto non-selective ½ DYGS agar plates (Kirchhof et al. 2001).

The salt tolerance of the isolates in the presence of a nitrogen source was examined in nutrient broth medium supplemented with 1–20% NaCl. Fresh bacterial cultures (50 µl) were inoculated in 5 ml medium and incubated in a shaking incubator at 200*g* for 24 h at 30ºC. The absorbance of the cultures was recorded at 600 nm using uninoculated medium as a blank.

Biochemical characterisation

Biochemical tests for citrate utilisation, lysine decarboxylase, ornithine decarboxylase, urease, phenylalanine deaminase, nitrate reduction and H_2S production were performed using biochemical assay kits (Himedia, Mumbai, India). Ammonia production and activities for some of the important enzymes, such as oxidase, catalase, gelatinase, cellulase, amylase, protease, pectinase, and lipase, were tested as per standard protocols.

Carbohydrate utilisation test

The basal medium used for the carbohydrate assimilation test contained 0.1% $(NH_4)_2SO_4$, 0.3% KH_2PO_4, 0.7% K_2HPO_4 and 0.01% $MgSO_4.7H_2O$, and the pH was adjusted to 7.0. Different carbon sources were used at a final concentration of 0.2%. A basal medium without any carbon source was used as the negative control. All experiments were carried out in triplicate.

Plant growth-promoting traits

Biological nitrogen fixation abilities of the isolates were tested using the acetylene reduction method in nitrogen-free semisolid NFb (Doebereiner 1995). Indole acetic acid production was examined using the colorimetric method described by Patten and Glick (2002). Phosphate solubilisation by the isolates was determined according to Goldstein (1986). Siderophore production was detected by the formation of orange-yellowish halos surrounding bacterial colonies on CAS agar plates after 48 h incubation at 30ºC (Schwyn and Neilands 1987).

Isolates were tested for utilisation of ACC as a sole nitrogen source. The isolates were grown in NFb medium supplemented with 3 mmol l^{-1} ACC, at 30ºC for 72 h at 175*g*. Bacterial growth was measured by determining the absorbance at 600 nm. Determination of ACC deaminase enzyme activity was carried out according to the method described by Penrose and Glick (2003).

16S rRNA gene sequence analysis

Genomic DNA of the halotolerant cultures was isolated with standard bacterial procedures (Sambrook et al. 1989). The primers fD1 and rP2 were used for PCR amplification of the 16S ribosomal DNA (Weisburg et al. 1991). Phylogenetic analysis of the 16S rRNA gene sequences was performed with MEGA version 4 (Tamura et al. 2007). The phylogenetic trees were inferred using the Neighbour-Joining method (Saitou and Nei 1987), and bootstrap analyses were performed (Felsenstein 1985). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004).

PCR amplification of the *nifH* gene fragment

Genomic DNA was isolated using standard bacterial procedures (Sambrook et al. 1989). Primers PolF and PolR were used to amplify a 360-bp fragment of *nifH* (Poly et al. 2001).

PCR amplification of the ACC deaminase gene (*acdS*)

To identify the gene coding for the ACC deaminase enzyme, DNA was extracted, and primers for PCR amplification were designed on the basis of the consensus regions of known ACC deaminase gene (*acdS*) sequences from *Pseudomonas fluorescens* (EF635249), *Enterobacter cloacae* (AF047710), *Var-*

iovorax paradoxus (AY604531), *Sinorhizobium meliloti* (EU003994) and *Rhizobium leguminosarum* (EF525260). The primers were located at positions 109 and 886 of the *acdS* reference nucleotide sequence of *E. cloacae*, corresponding to an expected amplification product of approximately 800 bp. Amplification of the *acdS* gene was performed in a final volume of 50 μl containing genomic DNA (50 ng), 20 pmol of forward (5′-GCCAARCGBGAVGACTGCAA-3′) and reverse (5′- TGCATSGAYTTGCCYTC-3′) primers, a mixture of dNTPs (Sigma) (each at a concentration of 200 μM), 10× *Taq* polymerase buffer and 2.5 U of *Taq* DNA polymerase (Sigma). The reaction conditions were as follows: an initial denaturation step at 94ºC for 4 min, 35 amplification cycles of denaturation at 94ºC for 1 min, annealing at 54ºC for 1 min and primer extension at 72ºC for 2 min, followed by a final extension at 72ºC for 7 min with a MyCycler™ PCR System (BioRad). The *acdS* gene sequence was determined by PCR direct sequencing (Macrogen, Korea). Sequence analysis was performed with the basic sequence alignment BLAST program against the National Centre for Biotechnology Information database [website http://www.ncbi.nlm.nih.gov/BLAST].

Evaluation of two isolates selected for their effects on germination and seedling growth of *S. brachiata* under salt stress conditions

Seeds were collected from mature, dried plants growing in the coastal area of Gujarat. Seeds were disinfected by washing with 75% ethanol for 1 min, followed by washing twice with sterile distilled water. Seeds were then immersed in 2% sodium hypochlorite for 5 min, followed by three times washing with sterile distilled water. To check both the efficiency of the sterilisation process and the presence of seed germination, the seeds were then placed on plates containing MS medium and incubated for 4 days. For bacterisation of the seeds, overnight cultures of reference strain *A. halopraeferens* Au4, *Brachybacterium* saurashtrense. JG 06 and *Pseudomonas* sp. JG 10 were centrifuged at 11,000*g* for 20 min, and the pellet was washed with 10 mmol l^{-1} PBS, pH 7.2. The pellet was re-suspended in PBS, and the optical density was adjusted to 0.6 (~10^8 cfu). Surface sterilised seeds were placed in bacterial suspension containing 0.1% carboxymethyl cellulose for 1 h for bacterisation. Aseptic conditions were maintained throughout the entire process. As a control, seeds were suspended in PBS containing 0.1% carboxymethyl cellulose without bacterial culture.

Germination tests were performed in sterilised petri dishes. The dishes were moistened with NaCl solution (0, 0.25 and 0.5 mol l^{-1}). Germination tests were performed inside a growth chamber at 27±0.5ºC and 35±1% relative humidity (RH), with continuous white light. Every 4 days, 20 ml of the appropriate solution was added to each dish. Seeds were considered germinated when the root was at least 2 mm long. The germination tests were carried out in duplicate sets. The final percentage of germination was measured after 30 days. Thirty-five seedlings from each of the 50 individual units from all treatments were chosen randomly, and seedling growth was measured by recording root length, shoot length and fresh weight on the 30th day. The vigour index was also calculated using the formula described by Abdul Baki and Anderson (1973). Vigour index=(mean root length+ mean shoot length) × germination (%).

Statistical analysis

Data from ten seedlings of *S. brachiata* were collected for each experiment. Each experiment was repeated three times, and the mean values and standard deviations were calculated. For NaCl tolerance (0.25, 0.5 mol l^{-1}) and bacterial treatment, a single factor ANOVA analysis was carried out using Microsoft Excel. The LSDs were calculated at $P=0.05$ to determine any significant difference between the means of each bacterial treatment.

Results

Isolation, biochemical and physiological characterisation

On the basis of cultural, morphological and biochemical characteristics, thirteen different halotolerant N_2-fixing bacteria from the roots of *S. brachiata* were enriched in a nitrogen-free semisolid NFb medium containing up to 4% NaCl and malate as carbon source. According to the highest dilution used for inoculum to get a positive enrichment, the diazotro-

phic bacteria were present up to a density of 4×10^7 per gram root fresh weight. All isolates (Table 1) were tested positive for nitrogen fixation under these conditions using the acetylene reduction assay. The isolates kept their nitrogen fixing ability even after passage through nitrogen-rich conditions and thus met the important criteria of stability of the nitrogen fixation trait according to the ten commandments proposed by Doebereiner (1988). The isolates JG 03, JG 05, JG 06, JG 08 and JG 12 showed the emergence of a growth pellicle in nitrogen-free NFb containing up to 4% NaCl, while the other isolates had a reduced salt tolerance (Table 2). The NaCl tolerance of all the isolates in nutrient broth medium was generally higher when compared to the defined NFb medium (Table 2). In general, the isolates had a rod-like cell morphology, except isolates JG 06 and JG 08, which had coccoid- to ovoid-shaped cell morphologies. Isolates JG 01, JG 02, JG 04, JG 07, JG 09, JG 10, JG 11, JG 12 and JG 13 were motile, whereas isolates JG 03, JG 05, JG 06 and JG 08 were non-motile. The other biochemical and physiological characteristics and the carbohydrate utilisation patterns for the isolates are shown in Table 2. All isolates produced ammonium and showed growth on malic acid.

Plant growth-promoting traits

In the presence of 0.05% L-tryptophan, all the isolates produced IAA in amounts ranging from 30 to 100 μg/ml culture supernatant. Six isolates (JG 03, JG 05, JG 07, JG 08, JG 09 and JG 11) solubilised phosphate from 46 to 69 μg phosphate/mg dry weight of bacteria from media containing poorly soluble tri-calcium phosphate. All the isolates, except JG 04, produced siderophores. ACC utilisation and ACC deaminase enzyme activities ranging from 0.12 to 0.98 μM αKB μg^{-1} h^{-1} were shown by all isolates.

16S rRNA gene sequence analysis

PCR amplification of the 16S rRNA gene using the fD1 and rP2 primer pair yielded amplification products of approximately 1,500 bp. The homologies of 16S rDNA sequences were obtained by blasting the sequences against the NCBI database. The homologies and phylogenetic names of the nearest neighbours are shown in Table 1. The taxonomic positions of the isolates are shown in the phylogenetic tree in Fig. 1. The 16S rRNA gene sequences of all the isolates have been submitted to the NCBI GenBank under the following accession numbers: JG 01, DQ458961; JG 02, DQ458962; JG 03, EU937748; JG 04, EU937747; JG 05 EU937749; JG 06, EU937750; JG 07, EU937751; JG 08, EU937752; JG 09, EU937744; JG 10, EU937753; JG 11, EU937754; JG 12, EU937755; and JG 13, EU937756.

nifH gene sequence analysis

To prove that the isolates were diazotrophs, amplification of the *nifH* gene was carried out using

Table 1 Isolates of diazotrophic halotolerant bacteria and 16S rRNA similarities to reference bacteria

Isolates	Nearest neighbour	16S rDNA similarity (%)
JG 01	*Pseudomonas putida*	99.9
JG 02	*Agrobacterium tumefaciens*	99.4
JG 03	*Zhihengliuella* sp.	97.5
JG 04	*Mesorhizobium* sp.	99.7
JG 05	*Zhihengliuella* sp.	97.5
JG 06	*Brachybacterium* sp.	99.2
JG 07	*Vibrio alginolyticus*	99.4
JG 08	*Brevibacterium casei*	99.7
JG 09	*Cronobacter sakazakii*	99.8
JG 10	*Pseudomonas pseudoalcaligenes*	97.0
JG 11	*Haererehalobacter* sp.	96.0
JG 12	*Halomonas* sp.	97.0
JG 13	*Pseudomonas* sp.	97.0

Table 2 Biochemical and physiological characteristics of the isolates

Characteristics	JG 01	JG 02	JG 03	JG 04	JG 05	JG 06	JG 07	JG 08	JG 09	JG 10	JG 11	JG 12	JG 13
NaCl tolerance in NFb medium	3%	2%	4%	2%	4%	4%	3%	4%	0%	1%	3%	4%	2%
NaCl tolerance in NB	7%	6%	15%	8%	15%	15%	20%	20%	7%	6%	20%	20%	9%
Methyl red	−	−	−	−	−	+	−	−	−	−	−	−	−
Voges-Proskuer's	+	−	−	−	−	−	+	+	−	−	−	−	−
KOH lysis test	+	+	−	+	−	−	−	−	+	+	+	+	+
Nitrate Reduction	+	−	−	+	+	+	+	−	+	−	−	+	+
H_2S Production	−	−	−	−	−	−	−	−	−	−	−	−	−
Catalase	−	+	+	+	+	+	+	+	−	−	+	+	−
Oxidase	−	+	+	+	+	−	+	+	−	−	+	+	+
Lysine decarboxylase	+	+	−	+	−	−	+	+	−	+	+	+	+
Ornithine decarboxylase	+	+	−	−	−	−	+	+	+	+	+	+	+
Urease	+	−	−	−	−	−	+	−	−	−	−	+	−
Phenylalanine deaminase	−	+	−	−	−	−	−	−	−	−	+	−	−
Amylase	−	−	+	−	+	+	+	+	−	−	−	−	−
Pectinase	−	−	+	−	+	−	+	−	−	−	−	−	−
Gelatinase	+	−	+	−	+	+	+	+	+	−	+	−	−
Protease	+	−	+	−	+	+	+	+	+	−	+	−	−
Lipase	+	+	+	+	+	+	−	+	+	+	+	−	+
Assimilation of carbohydrates													
Maltose	+	+	+	+	+	+	+	+	+	+	+	−	−
Mannose	+	+	−	+	−	+	+	+	+	+	+	−	−
Fructose	+	+	+	−	+	+	+	+	+	+	+	−	+
Ribose	+	+	+	−	−	−	−	+	+	+	+	−	−
Xylose	+	+	+	−	+	+	+	+	+	+	+	−	−
Arabinose	+	+	−	+	−	−	+	+	−	−	−	−	−
Galactose	+	+	−	+	−	+	+	+	+	+	+	−	−
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	−
Glucose	+	+	+	−	+	+	+	+	+	+	+	+	−
Adonitol	−	+	−	−	−	−	−	−	−	−	−	−	−
Lactose	−	−	+	−	−	+	−	−	−	−	+	−	−
Sorbitol	+	+	−	−	−	−	−	−	−	−	−	−	−
Citrate	+	−	−	+	−	−	+	+	+	+	+	+	+

+ Positive, − negative

degenerate *nifH* primers PolF/PolR. The PCR amplification of all the isolates produced the expected 360-bp amplification product. Sequencing of the excised amplification products proved their *nifH* gene sequence relatedness. The *nifH* gene sequence from strain JG 10 was further analysed and showed 96% homology with the *nifH* genes of several uncultured nitrogen-fixing bacteria (AB273227, AB273230 and AB273236). It also showed 87 and 83% homology with the *nifH* genes of bacterium HX148S (HQ204226) and *Celerinatantimonas diazotrophica* (DQ913882), respectively.

ACC deaminase gene analysis

In addition to the demonstration of ACC deaminase activity by some of the isolates, amplification of the *acdS* gene was attempted using PCR with a set of newly designed consensus primers (see above). The isolates JG 04, JG 05, JG 08, JG 11 and JG 12

produced the expected 800-bp amplification product. The *acdS* gene sequences of the isolates showed marked sequence similarities with the *acdS* genes of *Acidovorax*-related bacteria (Fig. 2).

Effect of bacterial inoculation of *Salicornia brachiata* seeds under different NaCl concentrations

Inoculation with *Brachybacterium saurashtrense* JG 06, *Pseudomonas* sp. JG 10 and *Azospirillum halopraeferens* Au4 (as a reference strain) increased percent germination at 0 mol l^{-1} to 0.5 mol l^{-1} NaCl concentrations (Table 3). Inoculations resulted also in significant increases in root length, shoot length and vigour index mostly at elevated NaCl concentrations as compared to the uninoculated control. Inoculation of all strains caused significant increases in the fresh weight at all concentrations of NaCl (Table 3).

Discussion

Gram-positive halotolerant diazotrophs

Halotolerant Gram-positive bacteria representing new N_2-fixing species were isolated from roots of the halophyte *S. brachiata*. These bacteria belong to the genera *Brachybacterium, Brevibacterium*, and *Zhihengliuella* (Fig. 1) and tolerated 3–4% (w/v) NaCl concentrations in NFb semisolid medium and 15–20% NaCl concentrations in complex NB medium. The isolates JG 03 and JG 05 clustered within the genus *Zhihengliuella*. Bacteria of this genus have been isolated from a saline soil sample collected from the Qinghai province in north-west China and can tolerate up to 25% NaCl (Zhang et al. 2007). This is the first report to show an isolate of *Zhihengliuella* sp. with many plant growth-promoting traits, including nitrogen fixation.

Based on 16S rRNA gene sequence similarity and DNA-DNA-hybridisation, the isolate JG 06 represents a new species within the genus *Brachybacterium*: *B. saurashtrense* (Gontia et al. 2011). *Brachybacterium* species are known to have high levels of salt tolerance (Schubert et al. 1996), but nitrogen fixation and PGPR activities have not yet been found.

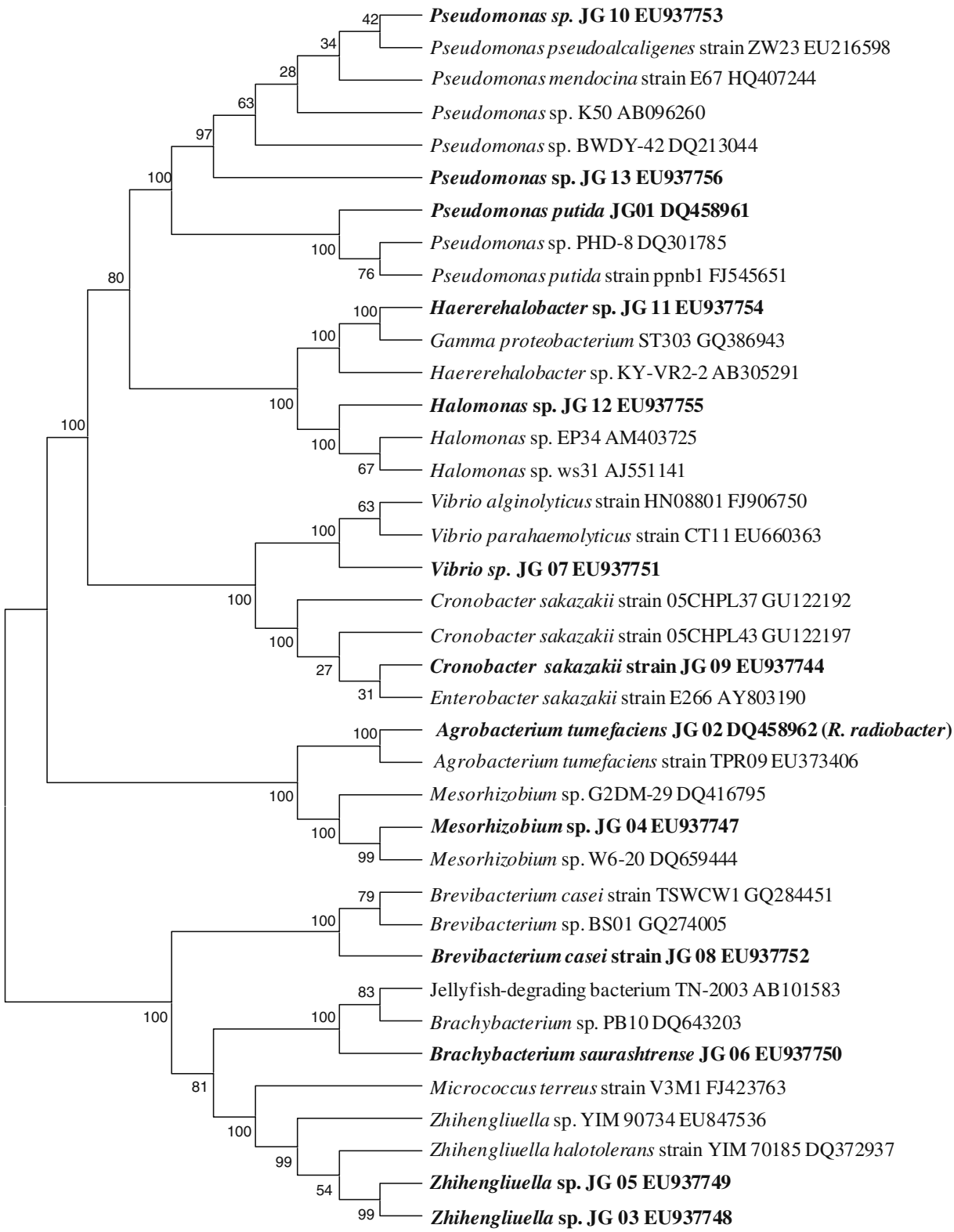
Isolate JG 08 showed close similarity to *Brevibacterium casei*. Generally, *Brevibacterium* spp. are isolated from dairy milk products, humans or poultry as commensals or opportunistic pathogens, and they can be found in both marine and terrestrial environments (Collins 1992). *Brevibacterium* spp. have previously been known for their ability to promote plant growth under chromium stress (Faisal and Hasnain 2006). To the best of our knowledge, this is the first report of the plant growth-promoting properties and the halophilic nature of this bacterium.

Gram-negative halotolerant diazotrophs

In addition, using the NFb semisolid enrichment method, a series of interesting Gram-negative isolates was found. However, no *Azospirillum* spp. were isolated, probably because members of this genus are not prevalent in the *Salicornia* rhizosphere or they did not reach this elevated level of salt tolerance

The 16S rRNA gene sequence of isolate JG 11 showed 96.0% 16S rRNA gene homology with the gamma-proteobacteria *Haererehalobacter ostrendis* and thus may represent a new species within *Haererehalobacter*. *Haererehalobacter ostendris* is a halophilic bacterium that has previously been isolated from Mediterranean seawater (Hogstrom et al. 2000). Isolate JG 11 is halotolerant and possesses many plant growth-promoting factors such as diazotrophy, IAA production, phosphate solubilisation and ACC deaminase activity. This is the first report to show that *Haererehalobacter* are associated with the roots of a halophyte, harbouring PGPR potential and diazotrophy. Isolate JG 12 clustered within the genus *Halomonas* and has 97% 16S rRNA gene homology with the isolate *Halomona*s sp. EP34. In general, bacteria belonging to genus *Halomonas* are known for their halophilic nature and grow at NaCl concentrations of 0.5–2.5 M (Kushner and Kamekura 1988). In addition to our isolate, *Halomonas maura* has been isolated from the rhizosphere of *Salicornia* sp. (Argandonña et al. 2005).

The 16S rRNA gene sequences of the isolates JG 01, JG 10 and JG 13 clustered within the genus *Pseudomonas*, which harbours many well-known PGPR, although most lack halotolerance. Several N_2-fixing endophytic diazotrophic *Pseudomonas* spp. were found in the rice rhizosphere (You and Zhou 1989; Tripathi et al. 2002b; Jha et al. 2009), and *Pseudomonas pseudoalcaligenes* have been isolated from the endorhizosphere of *Salicornia europea* (Ozawa et al. 2007).


Pseudomonas sp. JG 10 EU937753
Pseudomonas pseudoalcaligenes strain ZW23 EU216598
Pseudomonas mendocina strain E67 HQ407244
Pseudomonas sp. K50 AB096260
Pseudomonas sp. BWDY-42 DQ213044
Pseudomonas sp. JG 13 EU937756
Pseudomonas putida JG01 DQ458961
Pseudomonas sp. PHD-8 DQ301785
Pseudomonas putida strain ppnb1 FJ545651
Haererehalobacter sp. JG 11 EU937754
Gamma proteobacterium ST303 GQ386943
Haererehalobacter sp. KY-VR2-2 AB305291
Halomonas sp. JG 12 EU937755
Halomonas sp. EP34 AM403725
Halomonas sp. ws31 AJ551141
Vibrio alginolyticus strain HN08801 FJ906750
Vibrio parahaemolyticus strain CT11 EU660363
Vibrio sp. JG 07 EU937751
Cronobacter sakazakii strain 05CHPL37 GU122192
Cronobacter sakazakii strain 05CHPL43 GU122197
Cronobacter sakazakii strain JG 09 EU937744
Enterobacter sakazakii strain E266 AY803190
Agrobacterium tumefaciens JG 02 DQ458962 (R. radiobacter)
Agrobacterium tumefaciens strain TPR09 EU373406
Mesorhizobium sp. G2DM-29 DQ416795
Mesorhizobium sp. JG 04 EU937747
Mesorhizobium sp. W6-20 DQ659444
Brevibacterium casei strain TSWCW1 GQ284451
Brevibacterium sp. BS01 GQ274005
Brevibacterium casei strain JG 08 EU937752
Jellyfish-degrading bacterium TN-2003 AB101583
Brachybacterium sp. PB10 DQ643203
Brachybacterium saurashtrense JG 06 EU937750
Micrococcus terreus strain V3M1 FJ423763
Zhihengliuella sp. YIM 90734 EU847536
Zhihengliuella halotolerans strain YIM 70185 DQ372937
Zhihengliuella sp. JG 05 EU937749
Zhihengliuella sp. JG 03 EU937748

◀ **Fig. 1** Phylogenetic tree showing the relationships of the isolates to closely related bacteria. The tree was obtained using the neighbour-joining method. The *numbers* at branching points refer to bootstrap values, based on 100 replicates

Isolate JG 09 showed a close similarity to *Cronobacter (Enterobacter) sakazakii*. Recently, *Enterobacter sakazakii* and two subspecies of *Enterobacter sakazakii* were proposed to be part of a new genus: *Cronobacter* gen. nov. (Iversen et al. 2008). *C. sakazakii* with PGPR properties were isolated from the roots of rice (Yang et al. 1999) and soybeans (Kuklinsky-Sobral et al. 2004). The capability of this isolate to endophytically colonise tomato and maize root hairs was demonstrated by fluorescence in situ hybridisation (FISH) and confocal laser scanning microscopy (CLSM) (Schmid et al. 2009).

The isolate JG 07 exhibited a very close similarity to *Vibrio alginolyticus*, which has been previously isolated from the roots of *Spartina alterniflora*, a prevalent salt marsh grass (Bagwell et al. 1998).

Interestingly, the isolate JG 02 showed very close similarity with *Rhizobium radiobacter* (*Agrobacterium tumefaciens)*. These bacteria are usually known to be phytopathogenic, causing crown gall disease in a wide range of dicotyledonous plants, although non-pathogenic *Rhizobium radiobacter* strains have also been characterised (Sharma et al. 2008). Recently, a *Rhizobium radiobacter* strain was shown to nodulate *Sesbania* successfully (Cummings et al. 2009). Rhizobia are able to colonise the roots of non-legumes like other PGPR (Chabot et al. 1996) and to stimulate plant growth (Yanni et al. 2001). The isolate JG 04 showed very close similarity with the *Mesorhizobium* sp., and *Mesorhizobium ciceri* ch-191 has been reported to be one of the most salt-tolerant, root-nodule bacteria that can form stable symbioses under saline conditions (Soussi et al. 2001).

Halotolerances of the diazotrophic isolates

In the present study, conventional nitrogen-free semisolid medium, modified by the addition of up to 4% (w/v) NaCl, was used to enrich bacteria with nitrogen fixation activity and tolerance to salt-stress conditions. The presence of nitrogen fixation ability was further demonstrated using the acetylene reduction assay and by successful PCR amplification of the *nifH* gene. Amplification of the *nifH* gene has been used to confirm the diazotrophy of PGPR by several researchers, e.g., Chowdhury et al. (2007), Roesch et al. (2007) and Jha et al. (2009).

It is well known that, in halosensitive bacteria, synthesis and activity of the nitrogenase enzyme is inhibited under saline conditions (Hartmann 1988; Tripathi et al. 2002a). In earlier studies, halotolerant

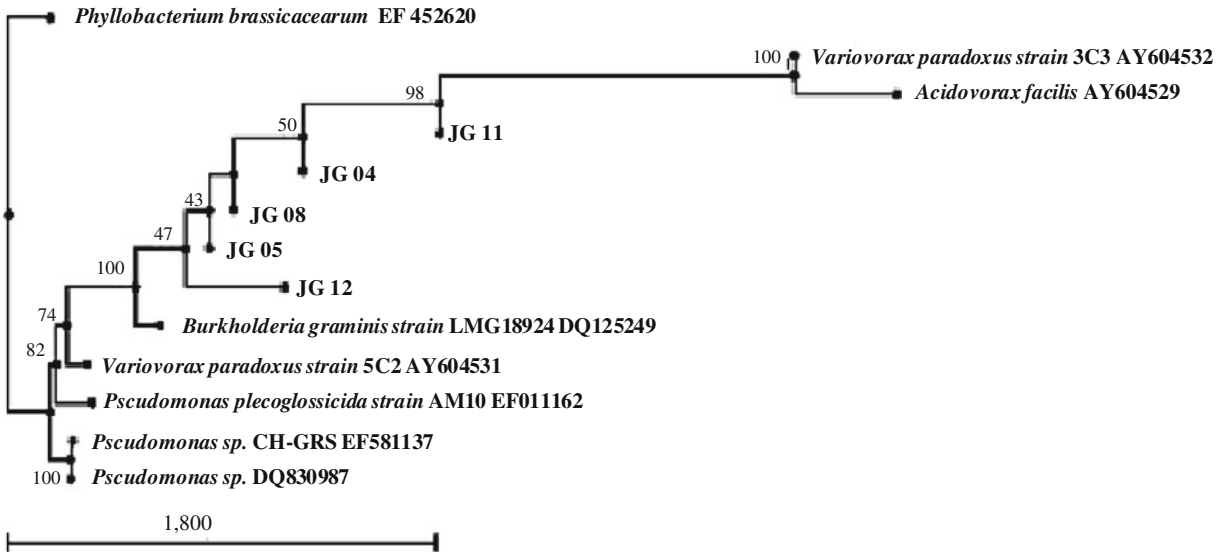


Fig. 2 Phylogenetic tree showing the relationships of the ACC deaminase genes (*acdS*) of the isolates with respect to the *acdS* genes of other bacteria. The tree was obtained using the neighbour-joining method. The *numbers* at branching points refer to bootstrap values, based on 100 replicates

Table 3 Effect of *Brachybacterium saurashtrense* JG 06, *Pseudomonas* sp. JG 10, and *A. halopraeferens* Au4 (reference strain) on root length, shoot length, fresh weight, germination and vigour index of seedlings of *Salicornia brachiata*

Bacterial inoculation	NaCl (mol l^{-1})	Root length (cm)	% Increase	Shoot length (cm)	% Increase	Fresh weight (mg)	% Increase	Germination (%)	Vigour index	% Increase
Control	0	1.04^{f}±0.06	-	0.85^{e}±0.02	-	4.50^{e}±1.27	-	88.3	167.5^{h}	-
Control	0.25	2.32^{d}±0.2	-	1.03^{d}±0.06	-	8.13^{d}±2.12	-	98	328.1^{d}	-
Control	0.5	1.63^{e}±0.07	-	1.04^{d}±0	-	8.16^{d}±0	-	92	243.5^{e}	-
JG 06	0	1.05^{f}±0.02	1	1.04^{d}±0	22	5.00^{e}±1.41	11	98	199.5^{g}	19
JG 06	0.25	2.82^{cd}±0.11	22	1.23^{bc}±0.01	19	10.70^{c}±0.07	32	98	393.5^{b}	20
JG 06	0.5	2.49^{cd}±0.01	53	1.08^{cd}±0.01	4	10.46^{c}±1.7	28	100	356.2^{c}	46
JG 10	0	1.15^{f}±0.01	11	1.16^{cd}±0.03	36	5.90^{e}±0.71	31	100	216.7^{f}	29
JG 10	0.25	3.68^{a}±0.13	59	1.38^{ab}±0.02	34	15.66^{a}±2.4	93	100	480.3^{a}	46
JG 10	0.5	2.80^{bc}±0.25	72	1.20^{c}±0.84	15	11.90^{c}±1.48	47	100	398.0^{b}	63
Au 4 halopraeferens	0	1.12^{f} ±0.12	7.69	1.13^{cd}±0.02	33	5.60^{e}±0.63	24	96	216.7^{f}	29
Au 4	0.25	2.53^{cd}±0.5	9.5	1.45^{a}±0.21	41	14.06^{b}±0.63	73	98	392.7^{b}	20
Au 4	0.5	2.95^{b}±0.3	81	1.21^{c}±0.51	16	11.73^{c}±3.18	44	98	405.6^{b}	67

The letters after each number represent the results of statistical analysis. The same letter indicates that no significant difference was observed at $P=0.05$

PGPR, such as *A. halopraeferens*, have been isolated from the halophyte Kallar grass (*Leptochloa fusca*) (Reinhold et al. 1987). Furthermore, salt tolerance was found in *A. brasilense* (Hartmann et al. 1991; Holguin and Bashan 1996; Nabti et al. 2007), *Rhizobium* (Zahran 1999), and *Swaminathania salitolerans* (Loganathan and Nair 2004).

Phenotypic and plant growth-promoting traits

Various biochemical tests were carried out to identify and characterise the isolates, including tests for the activity of hydrolytic enzymes, such as pectinase and cellulase. The enzyme activities for pectinase and cellulase are most relevant in the case of root-associated PGPR because these are the key enzymes for the invasion and colonisation of plant roots (Hallmann et al. 1997; Reinhold-Hurek and Hurek 1998). In the present study, isolates JG 03, JG 05 and JG 07 showed significant pectinase activity (Table 2). In other PGPR that have high to moderate endophytic abilities, such as *Azoarcus* (Reinhold-Hurek et al. 1993) and *Klebsiella* spp. (Kovtunovych et al. 1999), these enzymes are produced in relatively small amounts compared to pathogenic strains and may be completely suppressed once the bacteria are established within the plant. The pectinolytic activity might also be involved in a slight hydrolysis of the middle lamellae of colonised cortical cells without causing cell collapse, which may accelerate water and nutrient uptake by the roots (Sarig et al. 1984).

The isolates exhibited multiple plant growth-promoting traits, including IAA production, phosphate solubilisation, siderophore production, ACC utilisation and ACC deaminase activity. It has recently been shown that the salt-tolerant *A. brasilense* isolate NH produces IAA during salt-stressed conditions, and it was hypothesised that this production may substantially contribute to the increase in salt tolerance of inoculated wheat plants (Nabti et al. 2010). The majority of the isolates had phosphate-solubilising abilities, which is regarded as possible mechanism of PGPR to promote plant growth (Richardson 2001). Almost all isolates showed efficient iron sequestration in the CAS-plate assay for siderophore production. The abundance of microbial siderophores in iron-deficient soils and their ferric (Fe^{3+}) binding capacity significantly contribute to enhancing the mobility of mostly insoluble Fe^{3+}-oxohydroxy complexes in the

rhizosphere, making it more available for microbes and plants.

Many of the PGPR strains possess the enzyme ACC deaminase, which cleaves the ethylene precursor ACC and thereby lowers the level of ethylene in developing seedlings or in stressed plants (Mayak et al. 2004). The ACC deaminase genes for the isolates JG 04, JG 05, JG 08, and JG 11 showed 77, 81, 83 and 83% similarities with ACC deaminase gene from *Phyllobacterium brassicacearum, Pseudomonas* sp. CH-GRS, *Acidovorax facilis* 4P and *Variovorax paradoxus* strain 5C2, respectively (Fig. 2). The ACC deaminase gene for isolate JG 12 did not show homology with any *acdS* gene available in the NCBI database. Similar results were obtained by Saravanakumar and Samiyappan (2007) and Shah et al. (1998) for *Pseudomonas fluorescens* and ACC deaminase genes of other PGPR isolates. To the best of our knowledge, this is the first report of the presence of the ACC deaminase gene (*acdS*) in *Zhihengliuella* sp., *Brevibacterium casei*, *Haererehalobacter* sp. and *Halomonas* sp. PGPR that harbour ACC deaminase activity may facilitate the formation of longer roots and enhance the survival of plant seedlings during various abiotic and biotic stresses (Wang et al. 2000) The reduction of growth inhibitory ethylene levels by ACC deaminase-active root-associated bacteria may also substantially affect the salt tolerance of the inoculated crop plants.

Potential of plant growth promotion in salt-stressed axenic *Salicornia* plants

The growth enhancement of the inoculated *S. brachiata* plants may be attributed to a combination of the PGPR traits present in the inoculants. However, the inoculation experiments have to be extended to include more isolates and repetitive field experiments for realising the full potential of plant growth promotion of these isolates. It has been demonstrated that inoculation of *Salicornia bigelovii* with mangrove rhizosphere bacteria and halotolerant *Azospirillum* spp. promotes growth under saline conditions (Bashan et al. 2000). Similar results were obtained by Rueda-Puente et al. (2003) using *Klebsiella pneumoniae* as a PGPR inoculum for *S. bigelovii*. In addition, inoculation of *S. europea* with *Pseudomonas pseudoalcaligenes* resulted in the promotion of plant growth (Ozawa et al. 2007). These results suggest that halotolerant PGPR could be used to enhance the growth, and possibly the yield, of halotolerant crops and to produce sustainable agriculture in salt-affected areas.

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