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Exploring the Potential of Four Novel Halotolerant Bacterial Strains as Plant-Growth-Promoting Rhizobacteria (PGPR) under Saline Conditions

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Abstract: Soil salinity, due to natural phenomena or human activities, alters the water potential, which in turn affects plant growth, negatively influencing their nutrient and water uptake. Plant-growth-promoting rhizobacteria (PGPR) can be used to counteract these negative effects, especially in glycophytes. The aim of our study was to characterize physiologically, genetically, and biochemically the novel halotolerant/halophilic bacteria isolated in our previous work. We evaluated the plant-growth-promoting (PGP) features and NaCl regulation's roles in them. In this regard, analysis based on 16S rDNA sequences confirmed that our isolates are distinct bacterial strains, probably belonging to new species, which we named *Bacillus* sp. M21 and M23 and *Halomonas* sp. QH23 and QH24. In literature, it is known that many species of *Halomonas* and *Bacillus* genera produce factors regulating plant development, such as indole-3-acetic acid (IAA), ammonium, and siderophores; and their efficiency in promoting plant growth and productivity was also demonstrated in vivo. We demonstrated that the newly isolated strains exhibit different PGP activities, highlighting how the latter are regulated by NaCl and in a strain-dependent manner. In particular, the main results showed that NaCl negatively affects the production of IAA in QH23, M21 and M23, whereas it promotes it in QH24, where it is strictly salt-dependent. Both *Halomonas* strains produce ammonium only in the presence of NaCl, directly correlated to its concentration. The opposite occurs in *Bacillus* strains, where salt reduces its production up to ten times. Overall, the results underline how halotolerance is a preferable, but not sufficient, condition for considering a PGPR potentially useful in applications aimed at improving the tolerance and productivity of crops in saline soils.

Keywords: salinity; NaCl tolerance; bacterial consortium; *Halomonas*; *Bacillus*

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1. Introduction

The last century has been characterized by climate change, a global reduction in water resources, environmental pollution, and soil depletion. Soil salinization is among the most significant phenomena contributing to the loss of cultivable soils; in fact, every year, more and more land is degraded by salinization, and it is estimated that more than 50% of the cultivated land could deal with high salt concentration by 2050 [1]. In particular, salinization is a process causing a progressive accumulation of salts such as as sulphates, sodium, or chlorides in the soils [2]. It is frequent in arid and semi-arid areas, in which soluble salts precipitate and accumulate on the surface layers of the soil, causing desertification phenomena. Salt accumulation has different origins: through natural processes (primary salinization) or anthropic activity (secondary salinization) [3]. The primary salinization depends on the lithology of the substrate, the chemical and physical properties of the soil, and its hydrological characteristics; on the contrary, the secondary salinization is related to agricultural land use, and specifically inadequate agricultural management practices, such as the use of poor-quality water (e.g., rich in salts) and the excessive usage of inorganic fertilizers [4]. Based on the values of salinity and sodium quantity, estimated by electrical

conductivity (EC), sodium adsorption ratio (SAR), or percentage of exchangeable sodium, saline soils can be classified into: (i) saline, (ii) saline-sodic, and (iii) sodic [5]. Generally, soil is classified as saline when the EC of the saturated paste extract is greater than 4 $dS \cdot m^{-1}$ [6]. In this regard, a saline soil is characterized by low water content and high ion concentrations (e.g., Na^+ and Cl^-), causing significant imbalances in plants' biochemistry and physiology, which in turn negatively affect morphology, growth, and seed germination [7]. Specifically, salinity negatively affects plant reproduction (flowering and fruiting pattern), influencing crop yields and biomass by reducing its productivity, especially in glycophyte plants (e.g., rice, maize, and wheat) [8]. Moreover, salt excess decreases the amount of chlorophyll and the efficiency of the photosystems and also electron transport mechanism in salt-sensitive plants [9]. Furthermore, salt stress induces the generation of reactive oxygen species (ROS), affecting enzyme activities, membrane and cell wall functions, root and shoot development, and growth [10].

One potential approach to counteracting plant stresses, and in particular, the negative effects of soil salinization on glycophytes crops, is the application of plant-growth-promoting rhizobacteria (PGPR). In fact, PGPR promote plant development and the sequestration of metal excess, and consequently reduce biotic and abiotic stresses.

The rhizosphere is considered the region of soil surrounding plant roots up to a distance of 1–3 mm [11], and where the interaction between the plant and microorganisms is beneficial for both of them [12]. In fact, plants host a complex microbial community (bacteria and fungi) that closely adhere to the roots, and among them, the PGPR can improve the fitness of the plant through their metabolic activity.

The PGPR community depends on plant species and soil's chemical and physical conditions [13]. They promote plants' growth directly, synthesizing hormones, facilitating the up-take of nutrients, or indirectly inhibiting plant pathogens [14]. In particular, they are able to: (i) produce indol-3-acetic acid (IAA) implementing root development and its architecture to increase the surface area available for nutrient uptake [15]; (ii) produce 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase, which is able to reduce the level of plant ethylene and enhancing plant tolerance to abiotic stresses [16]; (iii) produce siderophores—secondary metabolites able to scavenge iron from environmental stocks forming soluble Fe^{3+} complexes that are actively taken up via specific receptors [17]; (iv) mineralize or solubilize some nutrients, such as phosphate-compounds. Moreover, PGPR can suppress the growth and activity of plant pathogens by producing antibiotics and degrading enzymes [18].

In the literature are reported many mechanisms to counteract salt stress in plants by PGPR. In particular, they reduce the detrimental effects of high ethylene levels on plants, such as chlorosis, abscission and senescence [19]. Additionally, under salinity stress, NaCl leads a rapid K^+ loss from the cellular cytosol of the plant, whereas PGPR maintains a greater amount of K^+ in order to preserve a higher K^+-Na^+ ratio and avoid osmotic imbalance [1]. Furthermore, PGPR contributes to the reduction of reactive oxygen species (ROS) induced by NaCl in the plant cells, increasing the levels of ROS-scavenging enzyme [20].

Therefore, the use of PGPR in agriculture is a sustainable and biological approach that is able to ameliorate the soil's fertility and quality [21].

The effects of salt on PGP features remain little known, and few studies focusing on NaCl's effect on IAA production have been reported for fungi and bacteria. Specifically, negative effects were detected on IAA by increasing the NaCl concentration in *Bacillus* and *Pseudomonas* species, and in some enterobacteria [22,23].

The aim of our study was to characterize halophilic/halotolerant PGPR strains with biotechnological potential in the recovery of salinized agricultural soil and to expand the knowledge on salt's role in the regulation of bacterial PGP activities. In this regard, we characterized physiologically, genetically, and biochemically, novel PGPR isolated in an our previous work [24] from the rhizospheres of *Zea mays* L. and *Chenopodium quinoa* Willd., exposed to high concentrations of NaCl.

2. Materials and Methods

2.1. Characterization of Strains and Culture Conditions

The bacterial strains were isolated from *Z. maize* (cv. DDK 7430-Dekalb) and *C. quinoa* (cv. Regalona) rhizospheres at the Plant Biology Laboratory of the University of Salerno (Campania, Italy) in our previous work [24]. Afterward, bacteria were cultured on Lauria-Bertani (LB) agar medium (in $g \cdot L^{-1}$: tryptone 10.0, yeast extract 5.0, NaCl 10.0, agar 15.0) and incubated at 28 ± 2 °C. Morphological characteristics were investigated after 48 h of incubation. The API20NE kit (bioMérieux Italia S.p.A, Florence, Italy) was used for biochemical characterization of the strains. For the catalase assay, one drop of ID color Catalase solution (bioMérieux Italia S.p.A, Florence, Italy) was applied to one bacterial colony; the appearance of bubbles was considered as a positive reaction. The determination of cytochrome oxidase was evaluated by dispensing one drop of TestOxidase™ (Pro-Lab Diagnostics, Richmond Hill, CA, USA) on one bacterial colony crawling on absorbent paper. The development of purple coloration was considered as positive.

2.2. Amplification and Sequencing of 16S rDNA

The bacterial DNA was extracted by means of REDExtract-N-Amp ™ Tissue PCR kit (Merck Life Science Srl, Milan-Italy) following the supplier's instructions. The universal primers 8-27F (5′ AGAGTTTGATCCTGGCTCAG 3′) and 1507-1492R (5′ TACCTTGTTACGACTT 3′) were used for 16S rDNA amplification (amplicon size range 1189–1398 pb—Table S2), and PCR conditions are reported in Table S1. Finally, the 16S rDNA sequencing was performed by the BMR Genomics Service (Padova, Italy).

2.3. Identification of Bacteria by Analysis of 16S rDNA Sequences

The sequences of our isolated were identified by a similarity search using the Basic Local Alignment Search Tool platform (BLAST) function of GenBank at the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov, accessed on 20 February 2023). The sequence lengths of our identified strains are reported in Table S2. The phylogenetic tree was built using the software MEGA11 [25]. The 16S rDNA sequences (1300–1500 nucleotides) of several type strains were collected from GenBank datasets and aligned by Clustal W software. Phylogenetic analysis and tree construction were performed using the Kimura two-parameter algorithm and the neighbor-joining method. The robustness of the inferred phylogenies was determined by bootstrap analysis based on 1000 data resamplings. Bootstrap values less than 50% were not considered for the tree construction because they were not considered statistically significant [26].

2.4. Salt and Temperature Tolerance

Salt tolerance of our bacterial strains was evaluated by spotting 3.0 µL of an LB overnight culture diluted to 0.01 OD_{600} on a LB agar medium without NaCl (control), or with some concentration of NaCl (1.5, 2.0, 2.5, 3.0, or 3.5 M), and incubating the plate at 28 °C for 48 h. Additionally, to better characterize these bacteria, we evaluated their growth temperature ranges by inoculating on an LB agar medium the same amount of suspension (3.0 µL) as above. Finally, the plates were incubated at 28, 37, 45, 50, or 55 °C for 48 h.

2.5. Plant-Growth-Promoting Features

PGP features were evaluated in three different conditions: without addition of NaCl, at 75 mM and 600 mM NaCl. The concentration 75 mM NaCl was chosen, since it is a strongly limiting concentration for glycophyte crops. In fact, it was determined by a preliminary dose/toxicity evaluation of the salt on *Z. maize* (cv. DDK 7430) (Figures S1 and S2) and *Solanum lycopersicum* L. (cv. Microtom) seedlings. Among the different tested concentrations (15–100 mM NaCl), the seedlings showed the greatest growth reduction at 75 mM—remaining viable, however. The used concentration of 600 mM NaCl corresponds to the mean value of the sea salinity.

2.5.1. IAA Production

A suspension of each NaCl-resistant strain was inoculated to the point of 0.005 OD_{600} in 5.0 mL of LB liquid medium (LB, 10.0 $g \cdot L^{-1}$ tryptone, 5.0 $g \cdot L^{-1}$ yeast extract) in the presence or absence of tryptophane (0.5 $g \cdot L^{-1}$), with or without NaCl (75 and 600 mM), and then incubated overnight at 28 °C and 200 RPM. Afterward, the indole acetic-3-acid (IAA) was measured according to Castiglione et al. [24].

2.5.2. ACC Deaminase Quantitative Analysis

The ACC deaminase activity was measured according to the method of Penrose and Glick (2002) [27], which quantifies the amount of a-ketobutyrate produced as a result of the enzyme ACC deaminase hydrolyze ACC.

In brief, bacterial cultures were inoculated in LB liquid medium and incubated overnight at 28 °C at 200 RPM. Next, the samples were centrifugated at 8000 RCF and 4 °C for 10 min. The supernatant was removed, and the bacterial pellet was washed with Dworkin and Foster medium (DF $g \cdot L^{-1}$: 4.0 KH_2PO_4, 6.0 KH_2PO_4, 0.2 $MgSO_4 \cdot 7H_2O$, 2.0 glucose, 2.0 citric acid, 2.0 gluconic acid, and trace elements: 1 mg $FeSO_4 \cdot 7H_2O$, 10 µg H_3BO_3, 11.19 µg $MnSO_4$ H_2O, 124.6 µg $ZnSO_4 \cdot 7H2O$, 78.22 µg $CuSO_4 \cdot 5H_2O$, and 10 mg MoO_3) without any nitrogen source. The samples were centrifugated again, supernatant was removed, and the pellet was resuspended in 7.5 mL of DF medium with 45 µL of ACC, for a final concentration of 3.0 mM. Afterward, the samples were incubated at 28 °C for 24 h. At the end of the incubation, bacterial cultures were centrifugated at 8000 RCF and 4 °C, for 10 min. The supernatant was discarded, and 5 mL of TRIS-Hydocloride buffer (0.1 M–pH 7.6) was added to the pellet. Again, the samples were centrifugated at 8000 RCF and 4 °C, for 10 min; then, the pellet was suspended in 1 mL of the above buffer and centrifugated again at 16,000 RCF for 5 min. The supernatant was discarded, the pellet was suspended in TRIS-Hydocloride buffer (0.1 M–pH 8.5), and 30 µL of toluene was added. Finally, the samples were mixed on a vortexer for 30 s. Afterward, 200 µL was used for ACC detection and another 100 µL for protein-extract quantization. To 200 µL of toluenized cells were added 20 µL of ACC 0.5 M; the solution was mixed on a vortexer and incubated for 10 min at 30 °C. At the end of the incubation, the samples were mixed with 1.0 mL of HCl 0.56 M, 300 µL of 2,4 dinitrophenyl hydrazine solution (0.2% in HCl 2M) was added, and then the solution was incubated at 30 °C for 30 min. Finally, 2 mL of NaOH 2M was added to each sample, and the absorbance was read spectrophotometrically at 540 nm. The standard curve was obtained by evaluating different concentrations of α-ketobutyrate ranging from 0.02 to 1.0 µmol.

2.5.3. Protein Extract Quantization

In order to quantify the protein extract, 100 µL of toluenized bacterial cells (see above) were mixed after adding 100 µL of NaOH 0.1 M and incubating them at 100 °C for 10 min. Afterward, the samples were cooled on ice, 10 µL was transferred to a new collection tube, and 1.0 mL of Bradford reagent (0.2 mL Bradford reagent, 0.8 mL distilled water) was added. The absorbance was evaluated at 595 nm.

2.5.4. Production of Siderophores

The bacterial strains were tested for siderophore production using the chrome Azurol S (CAS) agar plate method [28], at three different concentrations of NaCl (0, 75, 600 mM). Hence, 3.0 µL of a LB overnight culture (0.25 OD_{600}) was inoculated on each CAS plate, and they were incubated for 5 days at 28 °C. Siderophore production was determined by the presence of an orange halo around each bacterial colony. The diameters of the growth spots and orange halos were estimated by ImageJ v.1.52t software (NIH-Wayne Rasband) https://imagej.nih.gov/ij/notes.html.

2.5.5. Phosphate Solubilization Capacity

The Pikovskaya agar plate method [29] was employed to estimate the bacterial strains' capacity of solubilizing tricalcium phosphate [$Ca_3(PO_4)_2$]. For this reason, 3.0 µL of a LB overnight culture (0.25 OD_{600}) was spotted on the Pikovskaya plates and then incubated for 5 days at 28 °C. The identification of phosphate solubilizer strains was realized through the observation of a discernible halo around the bacterial colonies.

2.5.6. Ammonia Production

The ammonia produced by bacterial strains was estimated with Nessler's reagent. Therefore, 6.0 µL of a LB overnight culture (0.5 OD_{600}) was inoculated in 3.0 mL of peptone water (10.0 $g \cdot L^{-1}$ peptone; at three different NaCl concentration: 0, 75, 600 mM). The prepared bacterial suspensions were incubated at 28 °C under constant shaking at 200 RPM for 24 h. Afterward, the suspensions were centrifuged at 6000 RCF for 20 min at room temperature. Then, 1.0 mL of Nessler's reagent (VWR, Leuven, Belgium) was added to 1.0 mL of supernatant. Different concentrations of $(NH_4)_2SO_4$ in Peptone Water liquid medium (from 10 to 1000 $mg \cdot mL^{-1}$) were used to calibrate the standard curve (correlation factor $R^2 = 0.98$).

3. Results

3.1. Genetic Characterization

Two halo-tolerant strains were isolated from the rhizosphere of *C. quinoa* and the other two from *Z. maize*, and then genetically characterized by means of 16S rDNA sequence analysis. The alignment of the obtained sequences indicated an identity of 97.37% for the first two strains and a taxonomically collocation in the *Halomonas* genus (phylum of γ-Proteobacteria, family of Halomonadaceae), and the latter ones showed an identity of 96.81% and were classified as *Bacillus* genus (phylum Firmicutes, family of Bacillaceae). We named the first two isolates *Halomonas* sp. QH23 (QH23) and *Halomonas* sp. QH24 (QH24), and the other ones *Bacillus* sp. M21 (M21) and *Bacillus* sp. M23 (M23).

The phylogenetic analysis of the *Halomonas* and *Bacillus* genus are shown in Figures 1A and 1B, respectively. The *Halomonas* strains branched in the same cluster as *H. alkaliphila*, *H. venusta*, *H. neptunia*, and *H. titanicae*, with QH23 being strongly correlated to *H. alkaliphila* (99.93% identity, 98% bootstrap) and QH24 to *H. titanicae* (98.93% identity, 93% bootstrap). *Bacillus* strains branched with *B. haynesii* and *B. sonorensis*. Specifically, M21 was close to *B. sonorensis* (97.25% identity), and M23 was closer to *B. haynesii* (96.64% identity).

3.2. Morphological and Biochemical Characterization

QH23 and QH24 strains are rod-shaped, Gram-negative bacteria that occur singly or in pairs. Both bacteria on agar plates form circular, smooth, white–cream colonies. They are moderately halophilic, tolerate up to 2.5 M NaCl (15%), and have optimal growth at 28 °C; their maximum growth temperature is <45 °C (Table 1). The two strains can be distinguished by different biochemical characteristics (Table 2), but are similar regarding catalase, oxidase, capacity to reduce NO_3^- to NO_2^- and capacity to assimilate glucose and maltose. M21 and M23 are Gram-positive bacteria. Their cells are rod-shaped and grow singly or in a chain. Both bacteria form leathery, circular, irregularly bordered, white–cream colonies. They tolerate up to 2.0 M NaCl and are able to growth at up to 50 °C (Table 1). M21 and M23 are catalase- and oxidase-positive and reduce NO_3^- to NO_2^- but not reduce NO_2^-. Urease activity, indole production, and glucose fermentation are negative for both strains. They can be distinguished by arginine dihydrolase (ADH)—positive for M23—and hydrolysis of gelatin, which is positive for M21 (Table 2).

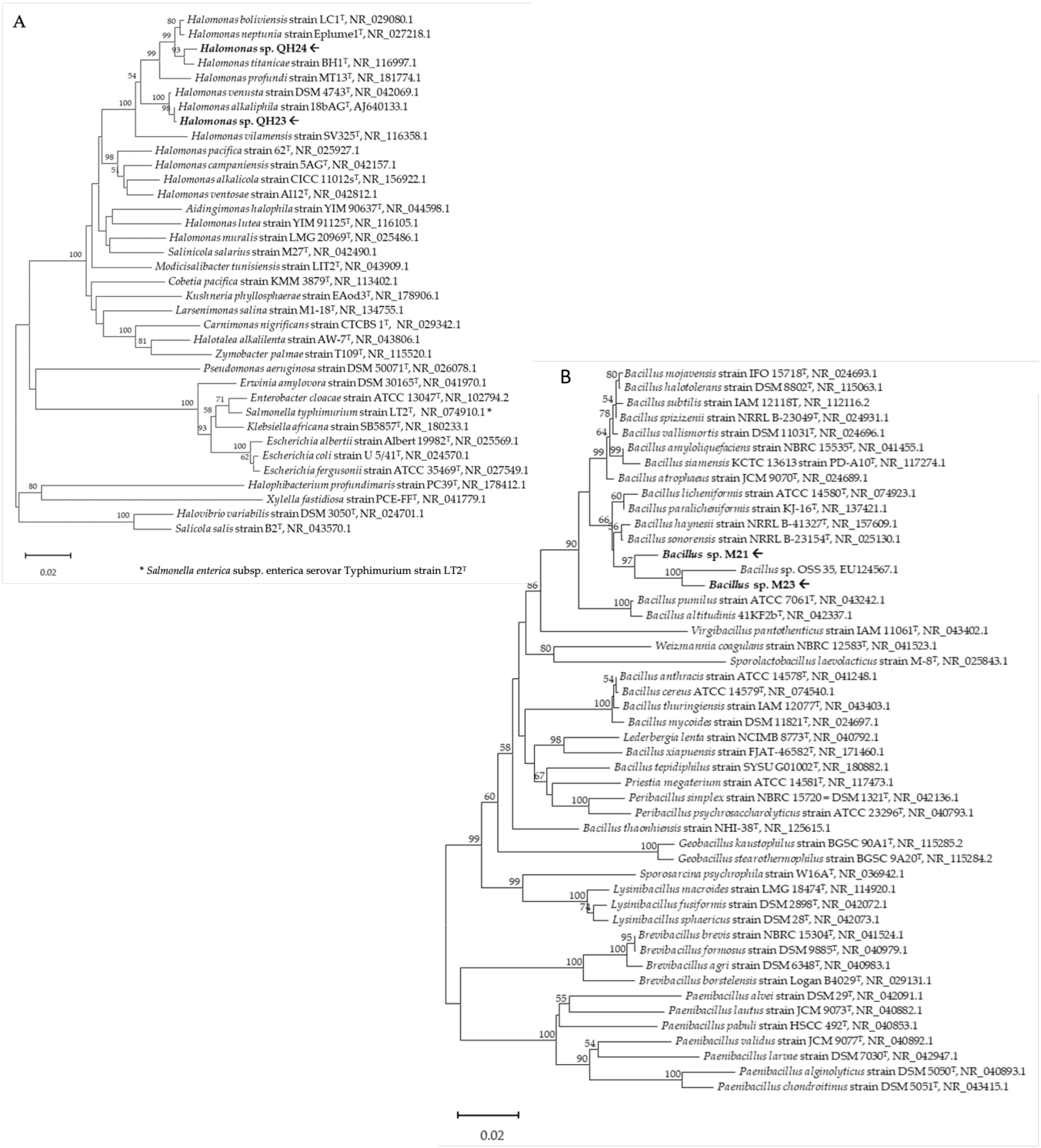

Figure 1. Taxonomical positions of the isolate strains based on the 16S rDNA sequence. Phylogenetic trees, obtained by the neighbor-joining method, show the relationships of *Halomonas* sp. QH23 and *Halomonas* sp. QH24 (**A**), and of *Bacillus* sp. M23 and *Bacillus* sp. M21 (**B**), with the related bacterial species. Bootstrap values ≥ 50 are reported at the branch points. ^{T} indicates the type strains. The access number of the relative strains is indicated.

Table 1. Morphological and physiological characterizations of bacterial strains.

	Colony Characteristics				Microscopy Characteristics				Temperature					NaCl Concentration				
Strain	**Shape**	**Margin**	**Elevation**	**Texture**	**Pigment**	**Shape**	**Arrangements**	**Gram**	**28**	**37**	**45**	**50**	**55**	**1.5**	**2.0**	**2.5**	**3.0**	**3.5**
QH23	Circular	Entire	Flat	Mucoid	White-Cream	Rod	Single or in pairs	-	+	+	-	-	-	+	+	+	-	-
QH24	Circular	Entire	Flat	Mucoid	White-Cream	Rod	Single or in pairs	-	+	+	-	-	-	+	+	+	-	-
M21	Circular	Irregular	Raised	Leathery	White-Cream	Rod	Single or in chain	+	+	+	+	+	-	+	+	-	-	-
M23	Circular	Irregular	Raised	Leathery	White-Cream	Rod	Single or in chain	+	+	+	+	+	-	+	+	-	-	-

Morphological (colony and microscopical analysis) and physiological characterizations (temperature and tolerance to NaCl) are illustrated. Temperature is reported in "°C"; NaCl concentration is shown as molarity (M). "+": growth, "-": no growth. QH23 and QH24: *Halomonas* spp.; M21 and M23: *Bacillus* spp.

Table 2. Biochemical characteristics of bacterial strains.

Strain	**NO_3^-**	**TRP**	**GLU**	**ADH**	**URE**	**ESC**	**GEL**	**PNPG**	**|GLU|**	**|ARA|**	**|MNE|**	**|MAN|**	**|NAG|**	**|MAL|**	**|GNT|**	**|CAP|**	**|ADI|**	**|MLT|**	**|CIT|**	**|PAC|**	**OX**	**CAT**
QH23	+	+	-	-	-	-	-	-	+	-	-	-	+	-	+	-	-	+	-	-	+	+
QH24	+	-	-	-	-	+	-	+	+	+	+	-	-	+	-	-	-	+	-	-	+	+
M21	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+
M23	+	-	-	+	-	+	-	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+

Biochemical differences were evaluated by catalase (CAT) and oxidase (OX) assays and the API20NE standardized system (bioMérieux Italia S.p.A, Florence, Italy). API20NE is based on twenty tests to differentiate non-enteric bacterial strains: NO_3^-: nitrate reduction; TRP: indole production; GLU: glucose (acidification); ADH: arginine dihydrolase; URE: urea hydrolysis; ESC: esculin hydrolysis; GEL: gelatin hydrolysis; PNPG: p-Nitropyhenyl-βD galactopyranoside; |GLU|: glucose assimilation; |ARA|: arabinose assimilation; |MNE|: mannose assimilation; |MAN|: mannitole assimilation; |NAG|: N-acetyl glucosamine; |MAL|: maltose; |GNT|: gluconate;|CAP|: caprate; |ADI|: adipate; |MLT|: malate; |CIT|: citrate; |PAC|: phenyl acetate; OX: cytochrome oxidase. "+": positive reaction; "-": negative reaction. QH23 and QH24: *Halomonas* spp. M21 and M23: *Bacillus* spp.

3.3. PGP Features

In general, bacteria produce IAA for metabolizing the L-tryptophan [30]. There are several methods for studying the production of IAA in bacteria, many of which do not include the addition of purified tryptophan, exploiting that contained in the peptones of the medium for growth. For this reason, we conducted our analysis with or without the addition of the amino acid. We observed a strong increase in IAA correlated to the addition of tryptophan at the culture medium (Tables 3 and 4). This was more evident for QH23 and QH24, where a relevant increase (2.0–3.5 fold) in the IAA production was highlighted. The IAA levels were also dependent on the NaCl concentration. QH23 and M21 showed their greatest IAA production in the concentration range of 0–75 mM NaCl, in either the presence or absence of tryptophan. At 600 mM, compared to the values obtained at 0 mM, in the presence of tryptophan, the production of IAA was reduced in QH23 and M21 by 38% and 62%, respectively. In the absence of tryptophan, at 600 mM NaCl, the reductions in QH23 and M21 were 30% and 50%, respectively. Moreover, in both conditions, M23 and QH24 exhibited the greatest IAA levels at 75 and 600 mM NaCl, respectively. A minor amount of IAA was present at 600 mM NaCl for M23 (reduction of 33%). It was undetectable for QH24 in the absence of added salt (Tables 3 and 4).

Table 3. IAA production without tryptophan.

Strain	**NaCl 0 mM**	**NaCl 75 mM**	**NaCl 600 mM**
QH23	2.23 ± 0.16	2.19 ± 0.11	1.57 ± 0.04
QH24	-	1.46 ± 0.33	2.01 ± 0.16
M21	1.89 ± 0.10	1.75 ± 0.05	0.96 ± 0.06
M23	1.58 ± 0.05	1.95 ± 0.08	0.96 ± 0.04

The concentration of IAA is indicated as $\mu g \cdot mg^{-1}$ of bacterial biomass at three different NaCl concentrations. "-": no production. QH23: *Halomonas* sp.; QH24: *Halomonas* sp.; M21: *Bacillus* sp.; M23: *Bacillus* sp.

Table 4. IAA production with tryptophan 0.5 g·L^{-1}.

Strain	NaCl 0 mM	NaCl 75 mM	NaCl 600 mM
QH23	8.20 ± 0.12	7.22 ± 0.20	5.16 ± 0.06
QH24	-	3.67 ± 0.04	5.48 ± 0.25
M21	3.08 ± 0.11	2.80 ± 0.15	1.17 ± 0.23
M23	2.56 ± 0.18	2.94 ± 0.25	0.97 ± 0.07

The concentration of IAA is indicated as μg·mg^{-1} of bacterial biomass at three different NaCl concentrations. "-": no production. QH23: *Halomonas* sp.; QH24: *Halomonas* sp.; M21: *Bacillus* sp.; M23: *Bacillus* sp.

Siderophore production capability of the selected isolates was evaluated at different concentrations of NaCl after 1 week of incubation. The data were shown as SPI (Siderophores Production Index) measured as the ratio between the diameter of siderophores diffusion (orange halo) and that of the growth spot (Table 5).

Table 5. PGP features.

		Siderophore Production Index			Phosphate Solubilization Index			Ammonium Production		
Strain	**ACC**	**0 mM**	**75 mM**	**600 mM**	**0 mM**	**75 mM**	**600 mM**	**0 mM**	**75 mM**	**600 mM**
QH23	-	n.d.	n.d.	-	-	-	-	-	7.4 ± 0.4	138.9 ± 3.2
QH24	79.6 ± 18.7	n.d.	n.d.	3.1 ± 0.2	-	-	-	-	51.5 ± 0.3	363.0 ± 21.1
M21	-	n.d.	n.d.	n.d.	-	-	-	533.2 ± 11.4	153.9 ± 51.4	62.3 ± 2.3
M23	-	n.d.	n.d.	3.3 ± 0.5	-	-	-	536.8 ± 6.3	290.5 ± 48.4	48.4 ± 3.9

The ACC activity is expressed as nmol of α-ketobutyrate produced per mg protein^{-1}·h^{-1}; SPI was measured as the ratio between the diameter of orange halo zone to that of the growth spot. Ammonium production is showed as concentration μg·mL^{-1}. The value "n.d." indicates "not detectable" because bacteria did not grow. "-": no production. QH23: *Halomonas* sp.; QH24: *Halomonas* sp.; M21: *Bacillus* sp.; M23: *Bacillus* sp.

In the experimental conditions, the bacterial growth was clearly evident only at high NaCl concentrations (600 mM), except for M21, which was unable to grow at any NaCl concentration. QH24 and M23 (Figure 2B,C) exhibited peak siderophore production at 600 mM NaCl (SPI = 3.1 ± 0.2 and 3.3 ± 0.5, respectively), whereas QH23 did not produce any siderophores (Figure 2A).

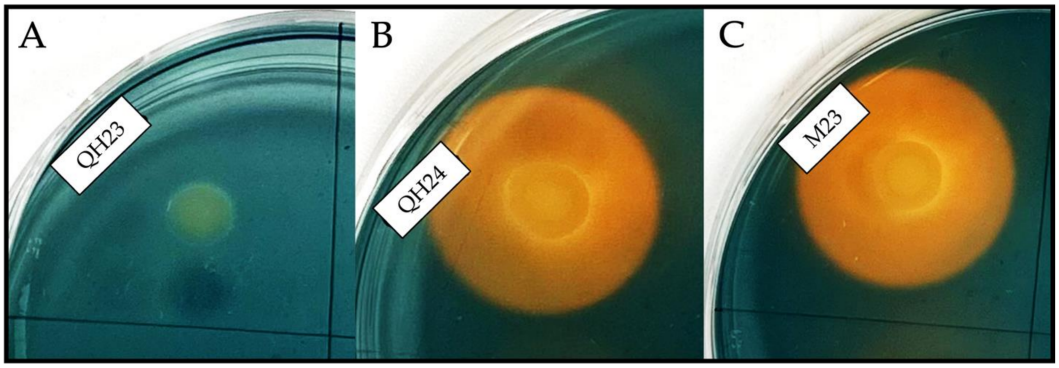

Figure 2. The siderophore production is shown as an orange halo around the growth spot. (**A**) QH23: *Halomonas* sp.; (**B**) QH24: *Halomonas* sp.; (**C**) M23: *Bacillus* sp.

Moreover, we evaluated the production of ammonium, the phosphate solubilization capability, and the ACC deaminase activity. As for IAA and siderophores, the ammonium production was dependent on the concentration of NaCl. In QH23 and QH24 strains, this production was strictly dependent on the presence of salt (non-detectable without NaCl), and they reached their highest levels at 600 mM (138.9 ± 3.2 and 363.0 ± 21.1 μg·mL^{-1} respectively). In M21 and M23, it was downregulated by salt concentration, in fact, the greatest production occurred in the absence of NaCl (533.2 ± 11.4 and 536.8 ± 6.3 μg·mL^{-1}, respectively). Other than the greatest ammonium production in *Bacillus* strains, in contrast to what *Halomonas*

isolates do, M21 and M23's production capabilities were partially maintained at all tested salt concentrations, and moreover, at 75 mM NaCl, their production was comparable to that of *Halomonas* strains grown at 600 mM (Table 5).

Finally, the phosphate solubilization test was negative at all NaCl concentrations and for all the examined strains.

4. Discussion

Soil salinization limits plant growth and agricultural productivity by up to 50%, and it is a very common phenomenon in developing countries and in extremely exploited agricultural lands [31]. Therefore, many researchers are looking for new biotechnologies that are able to improve crop cultivation in these areas. In this regard, the isolation and selection of halotolerant bacteria showing PGP features could be a quick, effective, and sustainable solution at this problem.

In this study, we characterized novel halotolerant/halophilic PGPR and evaluated the effect of increasing the NaCl concentration on their growth-promoting features (PGP).

Comparing the 16S rDNA sequences of QH23 with those of QH24, and those of M21 with M23, we obtained for both strain pairs identity values close to 97% (97.37 and 96.81%, respectively), below the accepted molecular limits for species definition (>98.5%) [32]. Therefore, we can hypothesize that the identified strains may be four different species. The molecular differences were in accordance with the results of the biochemical characterization, which highlighted different profiles for each of the strains.

The phylogenetic analysis showed that QH23 was closest to *H. alkaliphila* species (99.93% identity), a strain isolated for the first time from a salt pool located in Montefredane in Campania Region (South of Italy). QH24 was closest to *H. titanicae* species (98.93% identity), whose type strain, BH1, was isolated from the RMS Titanic wreck site. However, the high identity value, above the threshold of 98.5% for members of this genus, is not enough to hypothesize that both strains belong to their closest respective species. In fact, from our comparative analysis of multiple 16S sequences, it appears that distinct species of *Halomonas* genus can exhibit up to (and more then) 99.93% identity. M21 and M23 are taxonomically close to *B. haynesii*, *B. sonorensis*, and *Bacillus* sp. OSS 35 (an unclassified strain of *B. subtilis*); nevertheless, the 16S rDNA sequence identity among these species, for both strains, was close to 97%, and based on threshold of 98.5%, it is too low to consider possible classification at the species level.

Therefore, further molecular and phenotypic characterization studies are needed to exactly define the species of our four isolates [33].

Several bacterial strains of *Halomonas* and *Bacillus* genera are known for their abilities to tolerate moderate/high concentrations of NaCl and to exhibit many PGP features (IAA production, ACC deaminase activity, etc.) [34,35]. *Halomonas neptunia*, a very similar species to the QH24 strain, and isolated for the first time from a deep-sea hydrothermal-vent [36], exhibits the ability to produce IAA and moderately siderophores, slightly solubilizes phosphate, and shows ACC deaminase activity [37]. Likewise, we showed that QH24 produces significant amounts of siderophores and IAA. According to Penrose et al. [27], who considered moderate ACC deaminase activity, when it ranged from 20 to 300 nmol α-ketobutyrate per mg protein^{-1}·h^{-1}, QH24 exhibits similar ACC deaminase activity (79.6 $\pm$ 18.7 nmol·per mg protein^{-1}·h^{-1}). In this regard, the ACC deaminase activity lowered plant ethylene levels, promoting its growth, and therefore, PGPR could metabolize ACC exuded from seed or plant roots, decreasing its concentration [38]. The IAA is known to regulate the growth and developmental processes of plants, such as cell division, elongation, tissue differentiation, and apical dominance [39]. Moreover, the roots are most sensitive to fluctuations in the IAA level.

The QH23 strain, based on our data, is phylogenetically close to *Halomonas venusta*. They have common PGP properties. In fact, Ahmed et al. [40] observed that this species is able to produce high amounts of IAA (16.2 $\pm$ 0.1 μg·mL^{-1}). Karamat et al. [41] evaluated the effect of *H. venusta* soil addition in sunflower, observing a significant improvement in

plant growth, an increment in chlorophylls, and, moreover, a protein amount correlated to the production of bacterial auxin (IAA).

Thilagar et al. [42] showed that *B. sonorensis*, a very similar species to M21 and M23 strains, produced IAA (4.3 $\mu g \cdot mL^{-1}$), siderophores, ACC deaminase, and solubilized phosphate, but they also observed, with the inoculation of this bacterium, improved plant growth. Moreover, it is reported in the literature [43] that *B. licheniformis*, another species phylogenetically similar to M21 and M23, produces IAA and siderophores, solubilizes phosphate, and inhibits the growth of dangerous pathogenic fungi, such as *Botrytis cinerea*, *Colletotrichum gloeosporioides*, and *Phytophthora nicotianae*. According to Kwon et al. [44], this *Bacillus* species can be used in fruit orchards to control fungal diseases and increase fruit production. Similarly, M21 and M23 produce IAA, siderophores, and large amounts of ammonia.

Despite the above evaluation of NaCl's effects on PGP features, little is known, and only few studies on *Bacillus* spp. have been reported. Moreover, to our knowledge, no information about *Halomonas* spp. is at present available. In fact, these studies are focused on IAA production, and only small amounts of are available on ammonia production. Here, we showed that QH24 produces significant amounts of IAA, even in the presence of high concentrations of NaCl (600 mM), but in the absence of this salt, no production is observed. QH23, M21, and M23 produce IAA with or without the addition of NaCl; however, unlike QH24, in these strains, salt exerts a negative effect on its production. All bacterial strains analyzed in our study produced ammonium at both 75 and 600 mM NaCl, and we also highlighted how salt sees different regulation in *Halomonas* strains compared to those of *Bacillus*. In QH23 and QH24, the ammonium production was directly positively associated with the NaCl concentration, whereas in M21 and M23, NaCl's addition to the growth medium strongly reduced its production. Finally, for M23 and QH24 strains, it is also possible to highlight, only for 600 mM NaCl, relevant production of siderophores.

Therefore, all our isolates exhibit PGP features that could enhance plant growth and development even under salinity stress conditions. However, further analyses are necessary to verify these potentialities, such as in in vivo tests and thorough experiments in the field involving direct bacterial inoculation of plants.

5. Conclusions

Salinization is one of the major challenges for agriculture, as it limits crop productivity. However, the use of PGPR can greatly improve plant fitness and crop productivity, by limiting the impacts of soils.

In this work, we have characterized four new halophilic/halotolerant bacterial strains with potentially beneficial properties for plants in vitro, at different concentrations of NaCl. In fact, all these isolated bacteria showed PGP features, such as production of IAA, ammonia, siderophores, and ACC deaminase.

Currently, the effects of salt on PGP features remain little known, and few studies have described these effects in *Bacillus* species, *Pseudomonas* spp., and some enterobacteria. Even less is known for *Halomonas*. Here, we have shown how different PGP activities in *Bacillus* and *Halomonas* species are differentially regulated by NaCl, and in a strain-dependent manner. In the same bacterium, a specific activity can be strongly inhibited by salt, and another expressed only in its presence, whereas in another strain, the same activity can has a different or opposite reaction.

Our study contributes to expanding the knowledge on the effects of salt on the PGPR activities, highlighting how halotolerance is not a sufficient characteristic for such microorganisms to be useful for plants under salt-stress conditions, emphasizing the importance of considering the use of bacterial consortia in experimental soil recovery plans and selecting them based on their responses to NaCl. In this way, it is expected that the greatest number of growth-promoting functions will be kept active at high levels in a wide range of salt concentrations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/app13074320/s1. Figure S1: Phytotoxicity experiment with *Zea mays* L. cv. DDK 7730. Plants (5 replicates) are exposed to increasing NaCl concentration, cultivated in greenhouse with a photoperiod of 16 h of light and 8 h of dark and at constant temperature of 21 °C. The picture was taken one month before the material harvesting. At the end of the experiment, all seedlings treated with NaCl 100 mM were not anymore alive. The effects of increasing NaCl concentration (control, 15, 35, 55, 75, 100 mM) on maize seedlings are shown from left to right; Figure S2: Biomass dry weight of Zea mays L. at the end of experiment (Figure S1). Values are the means of 5 replicates. Letters indicate statistical significance. NA = "not available" because the seedlings were dead. The colors indicate different NaCl concentrations; Table S1: PCR conditions; Table S2: 16S rDNA sequence length of isolated strains.

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