Diversity and Potential Plant Growth-Promoting Traits of Cultivable Bacteria Associated with Three Halophytes

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Abstract

Salt-tolerant bacteria associated with halophytes enhance plant resistance to environmental stress and promote plant growth through their metabolic activities. The current study explored the diversity and potential of bacteria associated with three halophytes from the salt-affected land of Wujiaqu, Xinjiang, China. The cultivable bactria were isolated from *Salicornia europaea* L., *Kalidium foliatum* (Pall.) Moq., and *Suaeda aralocaspica*, by using the culture-dependent method. The isolated bacteria were classified based on the differences between their 16S rRNA gene sequences and screened for plant growth-promoting traits. Our findings showed that the bacteria isolated from selected plants' parts (roots and shoots) and soil (rhizosphere and bulk) belonged to 567 strains, four phyla, six classes, 25 orders, 36 families, and 66 genera. This study revealed that the tested strains could possess one or more plant beneficial traits. Among them, 20 strains representing *Bacillu*s, *Streptomyces*, *Isoptericola*, and *Nocardiopsis* species exhibited several plant growth-promoting activities *in vitro*, including phosphate-solubilization, nitrogen fixation, siderophore production, cell wall degrading enzymes such as protease, and cellulase. Our findings demonstrated that halophytes are a source of plant-beneficial bacteria, which may adapt to various conditions and enhance plant development and fitness in challenging environmental situations.

1. Introduction

Soil salinization is a global issue, seriously affecting our soil resources and destroying the self-regulatory capacity of existing ecosystems (Singh, 2022; Zhao et al., 2022). More than 20% of the world's irrigation regions are thought to be affected by soil salinization due to climate change, and this statistic is expected to increase further by 2050 (Butcher et al., 2016; Singh, 2021). Arid and semi-arid regions' agricultural production is impacted by soil salinization (Peng et al., 2019). About 31.10% of all the cultivated lands in Xinjiang are salinized (Yu et al., 2022). Consequently, the improvement and reclamation of salinizing regions have become a major source of public concern. Effective methods for rehabilitating salt-affected fields may facilitate effective approaches to increase plant productivity and overcome food insecurity issues in the region.

Halophytes, or extremely salt-resistant plants, can flourish in habitats with 200 mM NaCl or more salinity, such as saline semi-deserts, salinized soil, salt marshes, and seashores. (Camacho-Sanchez et al., 2022; Flowers and Colmer, 2008). Microbe-facilitated remediation strategies utilizing halophytes-associated bacteria to remediate saline soils are one of the essential methods to combat salinity (Ebadi et al., 2018; Khoshkholgh Sima et al., 2019). Halophyte-associated microbes developed special biochemical, physiological, and adoption strategies that enable halophytes to thrive in salinity (Christakis et al., 2021; Dif et al., 2021; Razzaghi Komaresofla et al., 2019;). *Priestia megaterium* BP-R2 is an endophytic bacterium that enhances plant growth and stress tolerance under salt and drought conditions (Hwang et al., 2022). *Bacillus* sp. MA17 showed plant growth-promoting (PGP) traits under salt stress and efficiently reduced disease incidence by 65% in wheat (Hadj Brahim et al., 2022). The endophytic bacteria *Acinetobacter johnsonii* PC3 from *Prosopis cineraria* showed the highest biocontrol efficacy with an 82%

reduction in the disease against cucumber damping-off incited by *Pythium aphanidermatum* under saline conditions (50 mM NaCl) (Al-Rashdi et al., 2022). These bacteria showed some PGP traits and antagonistic activity (Bibi et al., 2018; Gao et al., 2021; Hassan, 2019). Therefore, increasing interest in exploiting and utilizing microbes associated with halophyte remediation exists.

The objectives of the present study were as follows: (1) to isolate and identify cultivable bacteria associated with three halophytes (*Salicornia europaea* L., *Kalidium foliatum* (Pall.) Moq., and *Suaeda aralocaspica*) growing in saline soil by using the culture-dependent method and 16S rRNA gene sequencing; (2) to analyze the diversity and distribution patterns of isolated bacteria from three halophytes (3) to evaluate the PGP traits and salt stress tolerance of bacteria. The results will offer a scientific understanding of the range of bacteria linked to three halophytes and their capacity to enhance agricultural productivity in salt-affected areas.

2. Materials and Methods

2.1. Sample Collection

Three halophyte species, i.e., *Salicornia europaea* L., *Kalidium foliatum* (Pall.) Moq. and *Suaeda aralocaspica*, were collected in 2020 from the salinized land of Wujiaqu, Xinjiang, China (N44°13'38.0244", E87°40'16.7736"). Healthy and disease free three plant species in three replications were selected and collected randomly from sampling sites. The roots and shoots were packed in aseptic bags. The rhizosphere and bulk soil were placed in aseptic centrifuge tubes (50 mL) and transported to the laboratory. All samples were stored at 4°C until further processing. Different sample numbers were labeled, as shown in Table 1.

Table 1
Samples sites information

Plant	***Salicornia europaea* L. (P1)**	***Kalidium foliatum* (Pall.) Moq. (P2)**	***Suaeda aralocaspica* (P3)**
Root and Shoot	P1PE	P2PE	P3PE
Rhizosphere Soil	P1RR	P2RR	P3RR
Bulk Soil	P1RS	P2RS	P3RS

2.2. Sterilization of plant materials

Running tap water was used to wash away dirt, mud, and silt from each plant sample's root system and surfaces. Subsequently, plant samples were washed several times by sonification for 15 min at 45 kHz until the water became clear. The surface sterilization of the plant samples was done by immersing them

in 75% alcohol for 1 minute, followed by 5% NaClO for 2 minutes, then rinsing three times with sterile distilled water in a laminar airflow chamber (Liu et al., 2017). assess the efficiency of surface sterilization, 100 µL of the final rinsing water of each plant sample was spread on the two selective isolation media (Gao et al., 2021) (M1 (Yeast: 0.25 g/L; K_2HPO_4: 0.5 g/L; NaCl: 30.0 g/L; Agar: 20.0 g/L), M2 (Tryptone: 15.0 g/L; Soya peptone: 5.0 g/L; NaCl: 30.0 g/L; Agar: 20.0 g/L) and incubated at 30°C for a week. The aseptic plant samples were dissected into 1–2 cm fragments using a sterile surgical blade and desiccated within a laminar flow chamber. All specimens were subjected to the sterilized commercial blender for crushing (Joyoung, JYL-C012) (Li et al., 2018; Musa et al., 2020) and subsequently preserved at a temperature of -20°C.

2.3. Isolation, Purification, and Preservation of Bacteria

Each sample of 2 g was completely ground using sterile mortar and pestle. The samples were transferred into a conical flask of 50 ml with 18 mL sterile double-distilled water and shaked at 100 rpm/min for 30 min at 30°C with a Shaken Incubator (Shanghai Tensuc Lab Instruments Manufacturing Co., Ltd., Shanghai, China). The serial dilutions to a final concentration of 10^{-2} and 10^{-5} were made, and 100 µL of each dilution was plated on the two selective isolation media (three replicates) and incubated for 15 days at 30°C. Colonies with distinct morphology were picked and purified by successive streaking four times on marine agar 2216 (MA; Difco). The isolates were routinely cultured on MA at 30°C, maintained as a 20% (w/v) glycerol suspension, and stored at -80°C.

2.4. DNA Extraction, PCR Amplification, and 16S rRNA Gene Sequencing

DNA extraction was performed using the Chelex-100 method (Walsh et al., 1991). The colonies were treated in 50 µL 5% Chelex-100 and boiled for 15 min. The supernatant was used as template DNA to amplify the 16S rRNA gene sequence via PCR using universal primers 27F (5'-GAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GAAAGGAGGTGATCCAGCC-3') (Biomed, Beijing, China) (Bredow et al., 2015). The PCR mixture (50 µL) contained 4 µL (20–30 ng) template DNA, 25 µL 2×*Taq* PCR Master Mix, 2 µL of each primer (10 µmol/L), and supplemented DNase/RNase-Free Deionized Water to 50 µL. PCR amplification on a C1000 Touch™ Thermal Cycler (Bio-Rad Laboratories, USA) was under the following conditions: pre-denaturation at 94°C for 6 min, followed by 35 cycles of denaturation for 30 s at 94°C, annealing for 30 s at 55°C, and extension for 90 s at 72°C, with a final extension step at 72°C for 7 min. The amplified products were purified and sequenced by Sangon Biotech (Shanghai). The 16S rRNA gene sequences aligned with the EzBioCloud database (https://www.ezbiocloud.net/, accessed on 15 May 2022) via an online alignment search tool (16S-based ID). Sequence similarity of less than 98.65% was considered a putative novel species (Kim et al., 2014). The 16S rRNA gene sequences of bacteria determined in this study were deposited in GenBank under accession numbers OP662619-OP663185.

2.5. in vitro screening for plant-beneficial traits of bacterial isolates

2.5.1. Phosphate solubilization

Phosphate solubilization was screened by inoculating isolates onto solid Pikovskaya's medium supplemented with $Ca_3(PO_4)_2$ (5.0 g/L) and Bromophenol Blue (0.025 g/L) as described with some modifications (Abdelshafy Mohamad et al., 2020; Paul and Sinha, 2017). The Petri dishes were examined after incubation for 7 days at 30°C. The formation of clear zones or the change of color from blue to yellow around the colonies indicated the utilization of tricalcium phosphate.

2.5.2. Siderophores production

Siderophore production was screened by inoculating isolates onto the CAS medium (Li et al., 2018). After incubation for seven days at 30°C, the change of color from an orange/purple or purple/red halo zone indicated the production of siderophores (Alexander and Zuberer, 1991).

2.5.3. Assays for proteolytic and cellulolytic activity

The protease activity of bacterial isolates was determined by inoculating isolates onto skim milk agar 5% (v/v) medium using the spot inoculation technique (Abdelshafy Mohamad et al., 2020; Tiru et al., 2013). After incubation for three days at 30°C, the formation of clear zones surrounding the colonies indicated positive results. Cellulose activity of bacterial isolates was tested by inoculating isolates onto CMC-Na medium (KH_2PO_4: 1.5 g/L; NaH_2PO_4: 2.5 g/L; Peptone: 2.5 g/L; Carboxymethylcellulose sodium: 20.0 g/L; Agar: 20.0 g/L, pH 7.0–7.5). After incubation for seven days at 30°C, plates were stained with 5 mL 0.1% Congo red solution for 15 min and destained with 5 ml 1 M NaCl (Teather and Wood, 1982). The formation of a transparent or lightly colored halo surrounding the colonies indicated a positive reaction. The ability to solubilize phosphate and produce siderophores, protease, and cellulase was calculated using the following formula (Gao et al., 2021):

$$E = \frac{d1}{d2} \times 100\%$$

Where (d1) refers to the diameter of the clear zone and (d2) refers to the diameter of the bacterial isolate. All experiments for plant-beneficial traits were performed twice, with three replicates for each isolate.

2.5.4. Nitrogen fixation activity

Nitrogen fixation was assayed by inoculating isolates onto Ashby and NFC medium (Li et al., 2018; Sen and Sen, 1965). After incubation for seven days at 30°C, colonies grew on both media, indicating nitrogen fixation activity.

2.5.5. Salt-tolerance Assay

Tolerance to different NaCl concentrations (5, 10, 15, and 20%, w/v) was tested using an R2A medium. After incubation for seven days at 30°C, salt-tolerant bacteria were detected based on colony growth on the agar plates.

2.6. Statistical Analysis

All obtained data were statistically analyzed and visualized using Microsoft Excel 2019 and R (version 4.0.3).

3. Results

3.1. Isolation and identification of cultivable bacteria

A total of 567 strains belonging to four phyla, six classes, 25 orders, 36 families, and 66 genera were isolated and purified from the plant and soil samples (root and shoot, and rhizosphere and bulk soil) of three halophytes (Supplementary Table S1). At the phylum level (Fig. 1A), *Actinomycetota* (50.62%) was the dominant bacterial community in three halophytes, followed by *Bacillota* (24.87%), *Pseudomonadota* (23.99%), and *Bacteroidota* (0.53%). At the class level (Fig. 1B), *Actinomycetia* (50.62%) was the dominant group. The remaining isolates were *Bacilli*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Flavobacteriia*, and *Betaproteobacteria*, with 24.87%, 19.75%, 3.88%, 0.53%, and 0.35%, respectively. The predominant genera were *Streptomyces*, *Halomonas*, and *Bacillus*, accounting for 15.70%, 12.35%, and 12.17%, respectively (Fig. 1C).

Three halophytes' associated cultivable microorganisms showed various levels of diversity. (Table 2). About 23 strains were isolated and purified from P1PE, belonging to three phyla, four classes, six orders, six families, and seven genera. P2PE harbored three phyla, five classes, 11 orders, 16 families, 24 genera, and 67 strains; P3PE harbored three phyla, four classes, 16 orders, 18 families, 25 genera, and 101 strains; P1RR harbored three phyla, four classes, 11 orders, 13 families, 16 genera, and 53 strains; P2RR harbored three phyla, four classes, 15 orders, 17 families, 21 genera, and 77 strains; P3RR harbored four phyla, five classes, 13 orders, 15 families, 20 genera, and 80 strains; P1RS harbored three phyla, four classes, nine orders, 11 families, 18 genera, and 48 strains; P2RS harbored four phyla, five classes, 12 orders, 14 families, 22 genera, and 60 strains; and P3RS harbored three phyla, three classes, 11 orders, 12 families, 16 genera, and 60 strains.

Table 2
Diversity of cultivable bacteria isolated from different sites

Index	P1PE	P2PE	P3PE	P1RR	P2RR	P3RR	P1RS	P2RS	P3RS
Phylum	3	3	3	3	3	4	3	4	3
Class	4	5	4	4	4	5	4	5	3
Order	6	11	16	11	15	13	9	12	11
Family	6	16	18	13	17	15	11	14	12
Genus	7	24	25	16	21	20	18	22	16
Isolates	23	67	101	53	77	80	48	60	58
Richness	13.00	36.00	47.00	28.00	42.00	37.00	27.00	35.00	27.00
Shannon	2.36	3.39	3.57	3.02	3.47	3.40	3.04	3.29	2.98
Simpson	0.88	0.96	0.96	0.93	0.96	0.96	0.94	0.95	0.93
Pielou	0.92	0.93	0.93	0.91	0.93	0.94	0.92	0.92	0.90

The biodiversity profiles of the cultivable bacterial communities with different parts of three halophytes were compared based on the diversity indices computed by the R package vegan, including species richness and α-diversity indices (Shannon and Simpson) and Pielou evenness. Our findings showed that the species richness and Shannon diversity index of P3PE was the highest, and P1PE was the lowest. The Simpson diversity index of P2PE, P3PE, P2RR, and P3RR were the highest, and P1PE was the lowest. The Pielou evenness of P3RR was the highest, and P3RS was the lowest. There was no significant difference in the diversity of the PE (endophytic bacteria), RR (rhizosphere bacteria), and RS (bulk soil bacteria) cultivable bacteria.

The analysis of the dominant species at the genus level (top 20 dominant genera) of cultivable bacteria in different sites showed the high relative abundance of *Streptomyces* in P2RR, P3RR, and P2RS, *Halomonas* in P2RS and P3RS, *Bacillus* in P1PE, P3PE, and P1RS, *Nocardiopsis* in P1RR, and *Brevibacterium* in P2PE (Fig. 2A).

Similarities and differences in the appearance of cultivable bacterial communities were reflected in the number of shared and exclusive species displayed throughout nine sections of the Flower plot. (Fig. 2B). Only one species, *Bacillus swezeyi*, was present in all nine samples, representing that the cultivable bacterial community composition was not very similar. In PE, the unique species of P3 was 23, P2 was 20, and P1 was 7. In RR, the unique species of P1, P2, and P3 were 5, 9, and 7, respectively. RS's amazing P1, P2, and P3 species were 10, 11, and 8 (Supplementary Table S2). When comparing PE, RR, and RS, the cultivable bacterial composition of PE indicated more variability.

3.2. Potential Novel Bacterial Strains of Three Halophytes Cultivable Bacteria

Based on 16S rRNA gene identification, the 16S rRNA gene similarity of 147 strains was less than 98.65% belonging to 29 genera and 57 novel species, accounting for 25.93% of the total isolated strains. At the genus level, the predominant genera of the potential novel strains were *Streptomyces* (37.41%), followed by *Alkalihalobacillus* (13.61%), and *Microbacterium* (7.48%) (Fig. 3).

Potential new taxa were isolated from each isolation site of three halophytes, and each isolation site showed different isolation effects (Fig. 4). The three halophytes in RR showed the most potential for isolating novel strains. Only novel *Streptomyces* species were isolated from P1PE, and only a few potentially novel strains were found in P3RS. For potentially novel strains, P2 showed the best isolation effect overall. In saline-alkali environments, microbial resources are rich and untapped for excavation and utilization.

3.3. Comparison of Isolation Effects between Two Media

The dominant phylum grown on both media was *Actinomycetota*. However, the proportion of *Actinomycetota* on M2 (Fig. 5B) was more significant than on M1 (Fig. 5A), indicating that M2 was more selective in isolating *Actinomycetota* species. In addition, *Bacteroidota* were isolated only on M1. On the other hand, compared to the two media at the genus level, the number of bacteria species isolated on M1 was more than on M2. While the quantity of strains obtained from M2 at P2RR was more significant than that obtained from M1, the number of genera isolated by M2 was still lower than that of M1.

Moreover, the number of isolated strains was more than 30 in P2PE, P2RR, P2RS, P3PE, and P3RR, and the number of genera was more than 18. The overall isolation effect of P1 was not as good as P2 and P3, especially since the number and species of strains isolated from the P1PE were the lowest. Only three endophytic bacteria distributed in two genera were isolated on M2. Therefore, the two media were unsuitable for the isolation of P1PE (Fig. 5C).

The 147 potential novel strains were isolated on M1 and M2 media (98 and 49). Different plants showed different effects on the potentially novel strain rate (Fig. 6). In P1, the rate of novel strains isolated on M1 media was 10%, whereas no novel strains were isolated on M2 media. In P3, M2 media showed better results than M1. Interestingly, the number of bacteria species isolated on M2 in P2PE, P3PE, P3RR, and P3RS was less than on M1, but the rate of novel strains was higher than on M1. It's also worth noting that M2 had a higher success rate in isolating new strains than M1.

3.4. in vitro Screening of Bacterial Strains for Plant Beneficial Traits

In the present study, 213 strains, where PE, RR, and RS had 72, 79, and 62, respectively, were screened to investigate multi-beneficial traits *in vitro* to determine the most promising bacterial isolates (Supplementary Table S3). For isolates with phosphate-solubilizing ability, their proportions in PE, RR, and RS bacterial populations were 40.28%, 53.16%, and 41.94% (Fig. 7), respectively. Only one endophytic bacterial isolate, EGI P1K047, showed vigorous phosphate-solubilizing activity and was identified as *Advenella kashmirensis* subsp. *methylica* (similarity: 99.72%) (Supplementary Table S1). The proportions of strains producing siderophores in PE, RR, and RS populations were 29.17%, 64.56%, and 61.29% (Fig. 7), respectively. Most of them were low-active strains. The proportions of strains producing protease in PE, RR, and RS populations were 38.89%, 39.3%, and 29.03% (Fig. 7), respectively. Six strains EGI P1B044, EGI P2B047, EGI P2K012, EGI P3B010, EGI P3B014, and EGI P3K010 demonstrated protease-producing solid activity. They were identified as *Isoptericola halotolerans* (similarity: 98.98%), *Isoptericola halotolerans* (similarity: 99.62%), *Isoptericola halotolerans* (similarity: 98.94%), *Zhihengliuella halotolerans* (similarity: 99.41%), *Alkalihalobacillus berkeleyi* (similarity: 98.40%), and *Halomonas elongata* (similarity: 99.78%), respectively (Supplementary Table S1). The proportions of cellulase-producing strains in PE, RR, and RS were 25.00%, 31.65%, and 25.81%, respectively. Highly active cellulase-producing strains in RR bacterial populations were 16.46%, which was higher than in PE (8.33%) and RS (8.06%) (Fig. 7). A total of 24 strains exhibited vigorous cellulase-producing activity, and these isolates included members of the genera *Streptomyces*, *Bacillus*, *Isoptericola*, *Nocardiopsis*, *Arthrobacter*, *Halomonas*, *Microbulbifer*, and *Salininema* (Supplementary Table S1).

The tested strains with nitrogenase activity in PE (68.06%), RR (46.84%), and RS (46.84%) were grown both on Ashby and NFC medium (Fig. 8). They included:*Streptomyces*, *Bacillus*, *Nocardiopsis*, *Zhihengliuella*, *Paracoccus*, *Brevibacterium*, *Isoptericola*, *Microbacterium*, *Alkalihalobacillus*, *Brachybacterium*, *Cytobacillus*, *Kocuria*, *Nesterenkonia*, *Staphylococcus*, *Corynebacterium*, *Dietzia*, *Halomonas*, *Kushneria*, *Advenella*, *Alcanivorax*, *Amycolatopsis*, *Exiguobacterium*, *Gordonia*, *Mammaliicoccus*, *Marmoricola*, *Mycolicibacterium*, *Prauserella*, *Priestia*, *Pseudarthrobacter*, and *Rhizobium* genera (Supplementary Table S1 and Table S3). The results of the tested strains from three halophytes showed high salt tolerance. All tested strains could resist 5% NaCl concentration, more than 80% could resist 10% NaCl concentration, about 50% could withstand 15% NaCl concentration, and still, some strains in PE (19.44%), RR (8.86%), and RS (12.9%) could resist 20% NaCl concentration (Fig. 8). The strains that could resist 20% NaCl concentration belonged to *Halomonas*, *Kushneria*, *Staphylococcus*, *Cytobacillus*, *Georgenia*, *Gracilibacillus*, *Halobacillus*, *Bacillus*, *Exiguobacterium*, *Mammaliicoccus*, *Marinococcus*, *Nesterenkonia*, and *Oceanobacillus* genera (Supplementary Tables S1 and S3).

Among all tested strains, about 20 were positive for five plant-beneficial traits *in vitro*, including phosphate-solubilization, nitrogen fixation, production of siderophores, protease, and cellulase, belonged to various species within *Bacillus*, *Streptomyces*, *Isoptericola*, and *Nocardiopsis* genera (Supplementary Table S1 and Table S3).

4. Discussion

4.1. Cultivable bacterial community compositions associated with three halophytes from Wujiaqu, Xinjiang

Using the culture-dependent method, we isolated 567 strains from the different ecological niches associated with three dominant halophytes growing in Wujiaqu, Xinjiang, China (Supplementary Table S1). The strains were affiliated with four phyla, six classes, 25 orders, 36 families, and 66 genera. The bacterial diversity of RR was higher than that of RS in P2 and P3, but the bacterial diversity of RR in P1 was lower than that of RS. The diversity of rhizosphere bacteria is not always more than that of bulk soil bacteria, which may be related to host plants and environments (Qiu et al., 2022; Tkacz et al., 2015). By comparing the species detected at each classification level in the culture-independent method, we found that there is still a limit to using the culture-dependent method for comprehensive analysis of the diversity of bacteria associated with halophytes. These two methods showed different results about the predominant genera of endophytic and rhizospheric bacteria from three halophytes. The analysis of dominant species of cultivable endophytic bacteria (P1PE, P2PE, P3PE) at the genus level (top 20 dominant genera) showed the high relative abundance of *Bacillus* in P1PE and P3PE, and *Brevibacterium* in P2PE. The rhizospheric bacteria (P1RR, P2RR, P3RR) showed a high proportion of *Nocardiopsis* in P1RR, and *Streptomyces* in P2RR and P3RR (Fig. 2A). Gao et al. (Gao et al., 2022) reported the OTU abundance of the top 100 taxa among different sample groups. The endophytic bacteria (P1EB, P2EB, P3EB) showed the dominant genus *Enterobacter* in P1EB, the unassigned taxa in P2EB, and *Staphylococcus* in P3EB. The rhizospheric bacteria (P1RB, P2RB, P3RB) showed a high proportion of *Enterobacter* in P1RB, *Pantone* in P3RB, and *Rickettsia* with *Thalassospira* in P2RB. From the Flower plot, we know the similarities and differences in the composition of cultivable bacterial communities in nine sites. Only one common species in nine sites was identified as *Bacillus swezeyi* (Fig. 2B). But Gao et al. (Gao et al., 2022) detected 6021 OTUs from 39 soil and plant samples for a bacterial community associated with three halophytes, and they found 432 OTUs common to all the samples. Both approaches demonstrated that the microbial diversity of three halophytes differed to varying degrees across ecological niches, indicating that the community structures of these niches shared some similarities but also displayed some key differences.

4.2. Isolation of Bacteria Associated with Halophytes

We adopted two selective media to isolate bacteria associated with three halophytes according to Gao et al. (Gao et al., 2021). The dominant phyla *Actinomycetota* (50.62%) were obtained from two media, which were different from the high-throughput sequencing result that revealed the top 100 abundant bacteria belonged to 9 phyla, where *Pseudomonadota* accounted for 54%, but *Actinomycetota* only for 7% (Gao et al., 2022). This fact indicates that both media were suitable for isolating *Actinomycetota* from the halophytes. We isolated 147 potential novel strains that accounted for 25.93% of the total isolated strains from different ecological niches using only two isolation media, indicating that three halophytes had abundant potential novel bacterial taxa. Our previous research (Gao et al., 2022) applied high-throughput sequencing to reveal the diversity and community structure of endophytic and rhizospheric bacteria associated with three halophytes. We found the abundant potential novel bacterial

taxa. The predominant genera of those novel strains were *Streptomyces* (38.41%), followed by *Alkalihalobacillus* (13.61%) and *Microbacterium* (7.48%), respectively (Fig. 3). It also showed that we could obtain some bacteria using a culture-dependent method.

Interestingly, *Streptomyces* also had the highest proportion of potential novel taxa, suggesting that the two media, particularly M2, were more suitable for isolating actinomycetes from halophytes. At the genus level, the number of species isolated on M1 media was more significant than those on M2, showing that M1 had a more powerful isolation effect, which has a reference value in future relevant research. The phylum *Euryarchaeota* was detected in the rhizosphere microbiome of halophytes, although in a low abundance, including a taxon of the class *Halobacteria*, which survived extreme salt concentrations (Gao et al., 2022; Gontia-Mishra et al., 2017). Therefore, there is still a large number of microbial resources associated with halophytes that need to be explored and utilized. The isolation of halophilic archaea can also be considered in future isolation processes.

4.3. Screening of the bacterial isolates by the PGP traits and resistance to salinity

Halophytes are potential sources of salt-tolerant bacteria with PGP abilities. These beneficial bacteria can influence and enhance the availability of soil nutrients and partially reduce the requirement of fertilizers for plants without interfering with the natural soil microbial community structure (Chaudhary et al., 2020). In this study, 213 strains from different ecological niches of three halophytes were screened in vitro for multiple beneficial traits to determine the promising bacterial strains using plate screening. More than 40% of the tested strains had the phosphate-solubilizing ability, but only one endophytic bacterial strain *Advenella kashmirensis* subsp. *Methylica* EGI P1K047 showed potent activity. Zhang et al. (Zhang and Yao, 2020) screened the bacteria from plant rhizosphere and found *Advenella kashmirensis* subsp. *Methylica* MD2(2) with strong and stable ability to dissolve inorganic phosphorus up to 283.69 μg/mL. *Advenella kashmirensis* subsp. *Methylica* PK1 - the first known methylotroph of the genus *Advenella*, was isolated from plant rhizosphere and could grow in the presence of NaCl up to 3% (Poroshina et al., 2015). The methylotrophs can utilize C_1 compounds as a sole carbon and energy source and play a key role in the carbon cycle between methane and CO_2 (Iguchi et al., 2015). *Advenella kashmirensis* subsp. *methylica* EGI P1K047 could resist 10% NaCl concentration. Due to the unique ecological niche of isolation, it may have more potential application value to be explored. In the tested strains with nitrogenase activity in PE (68.06%), RR (46.84%), and RS (46.84%), the proportion of nitrogen-fixing bacteria in PE was higher than in RR and RS. Katarzyna et al. (Hrynkiewicz et al., 2019) found that diazotrophic endophytic bacteria were abundant in *Salicornia europaea* L., which is consistent with the higher ratio of nitrogen-fixing endophytic bacteria found in the current research. Gao (2022) also found that nitrogen metabolism in endophytic bacterial sample groups was universally higher than in rhizospheric bacterial sample groups in halophytes, especially in the roots. It means that the halophytes' roots contained more nitrogen-fixing microorganisms. All the tested strains could resist 5% NaCl concentration, less than 20% of the tested strains could tolerate 20% salt concentration, and these bacteria belonged to *Halomonas*, *Kushneria*, *Staphylococcus*, *Cytobacillus*, *Georgenia*, *Gracilibacillus*,

Halobacillus, *Bacillus*, *Exiguobacterium*, *Mammaliicoccus*, *Marinococcus*, *Nesterenkonia*, *Oceanobacillus* genera. These strains had great growth-promoting potential under salt stress, especially *Halomonas* and *Bacillus* (Kearl et al., 2019).

5. Conclusions

A total of 567 bacteria belonging to the phyla *Actinomycetota*, *Bacillota*, *Pseudomonadota*, and *Bacteroidota* were isolated from different ecological niches of three dominant halophytes collected from the salinized soil from Wujiaqu, Xinjiang, China. The distribution of cultivable bacterial communities differed significantly across ecological niches, with only one common species. Several halophyte-associated bacteria have yet to be discovered and used, as our findings showed that more than 25% of the total isolated strains were prospective new strains. The M1 medium, where yeast was used as a carbon-nitrogen source, was the best for isolating bacteria associated with three halophytes from Wujiaqu, Xinjiang. The tested strains had one or more plant-beneficial traits. About 20 strains with phosphate-solubilizing, nitrogen-fixing and siderophore, protease, and cellulase-producing abilities belonged to various species such as *Bacillus*, *Streptomyces*, *Isoptericola*, and *Nocardiopsis.* The findings provide a scientific insight into the diversity of bacteria associated with three halophytes and their potential to improve vegetation of salt-affected lands.

Declarations

Data availability statement

16S rRNA gene sequences of all actinobacterial endophytes were deposited in the GenBank database in NCBI (https://www.ncbi.nlm.nih.gov/) under accession numbers OP662619-OP663185.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

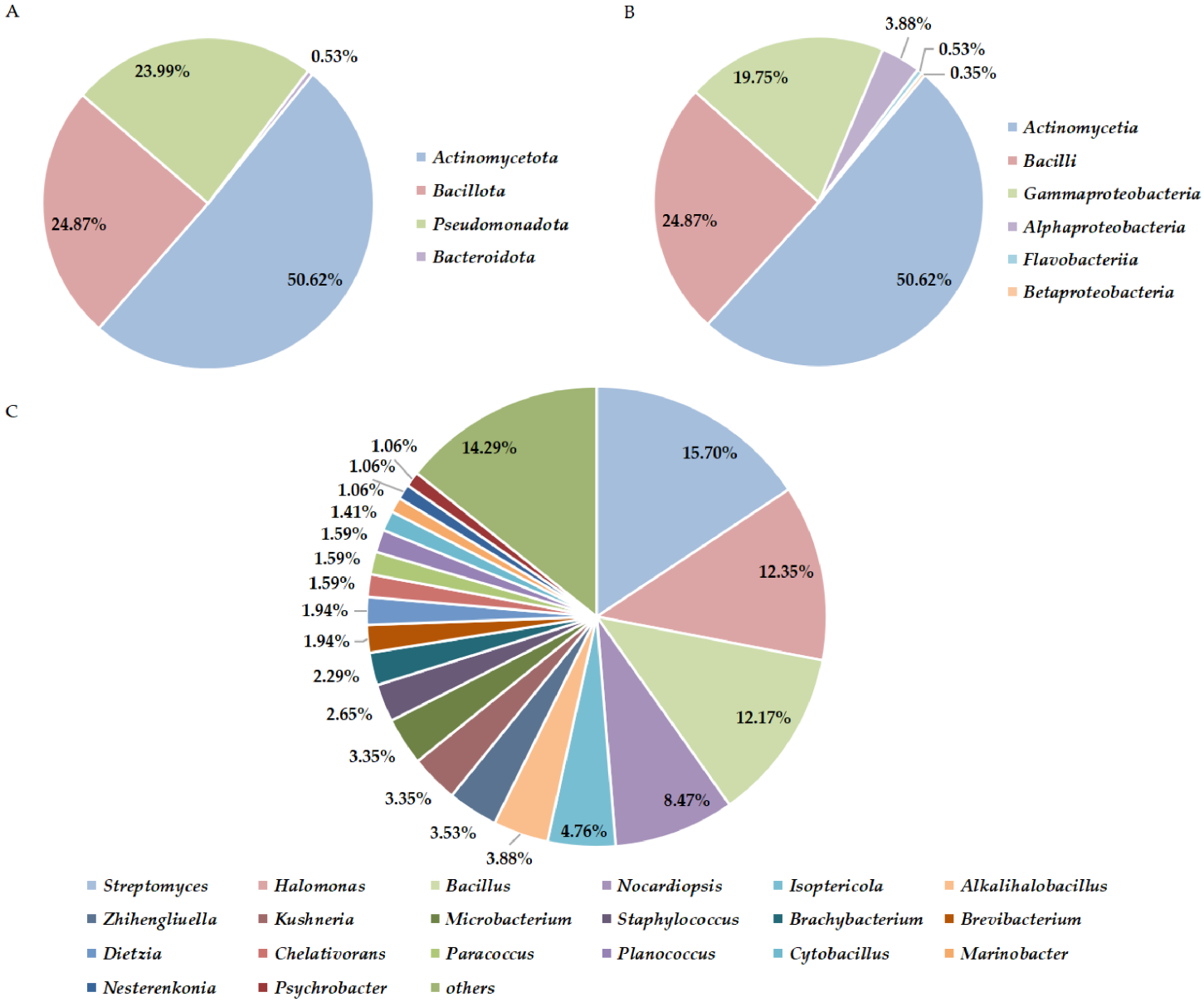

Figure 1

The community composition of culturable bacteria from samples of three halophytes at phylum (A), class (B), and genus (C) (top 20 dominant genera) levels.

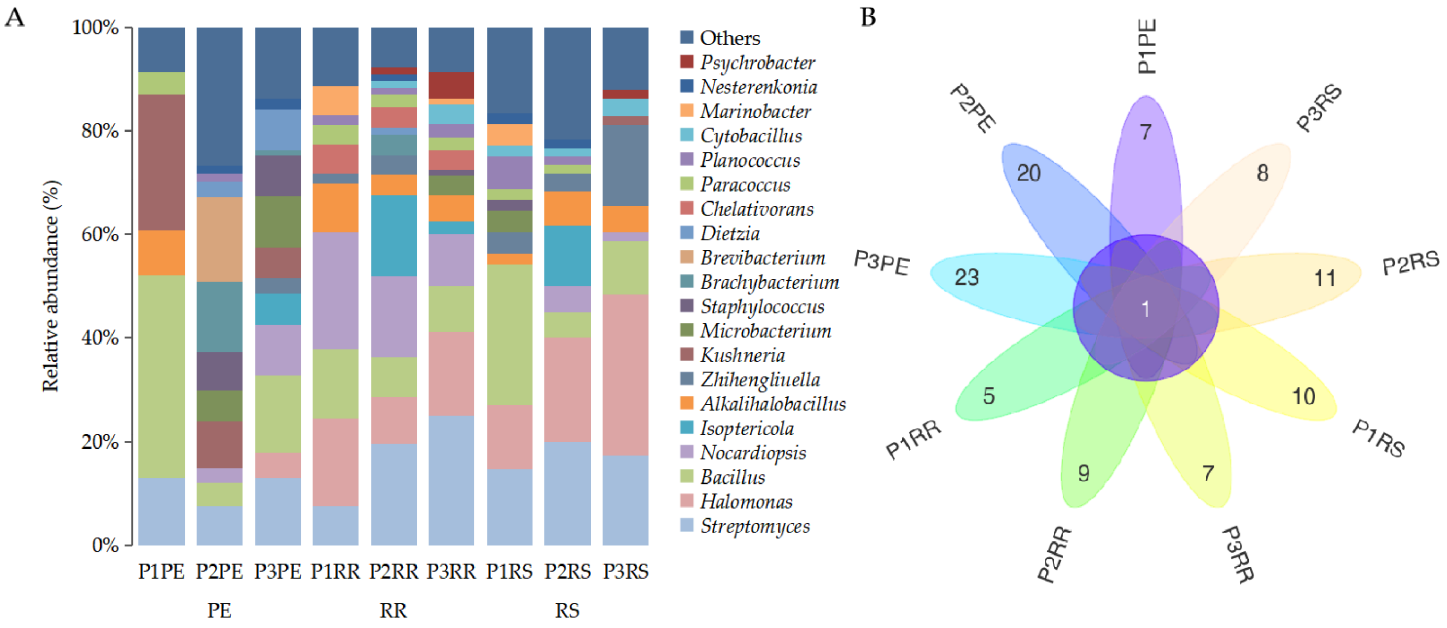

Figure 2

The community composition at the genus level (Top 20 dominant genera) (A) and Flower diagram (B) of cultivable bacteria isolated from different sites.

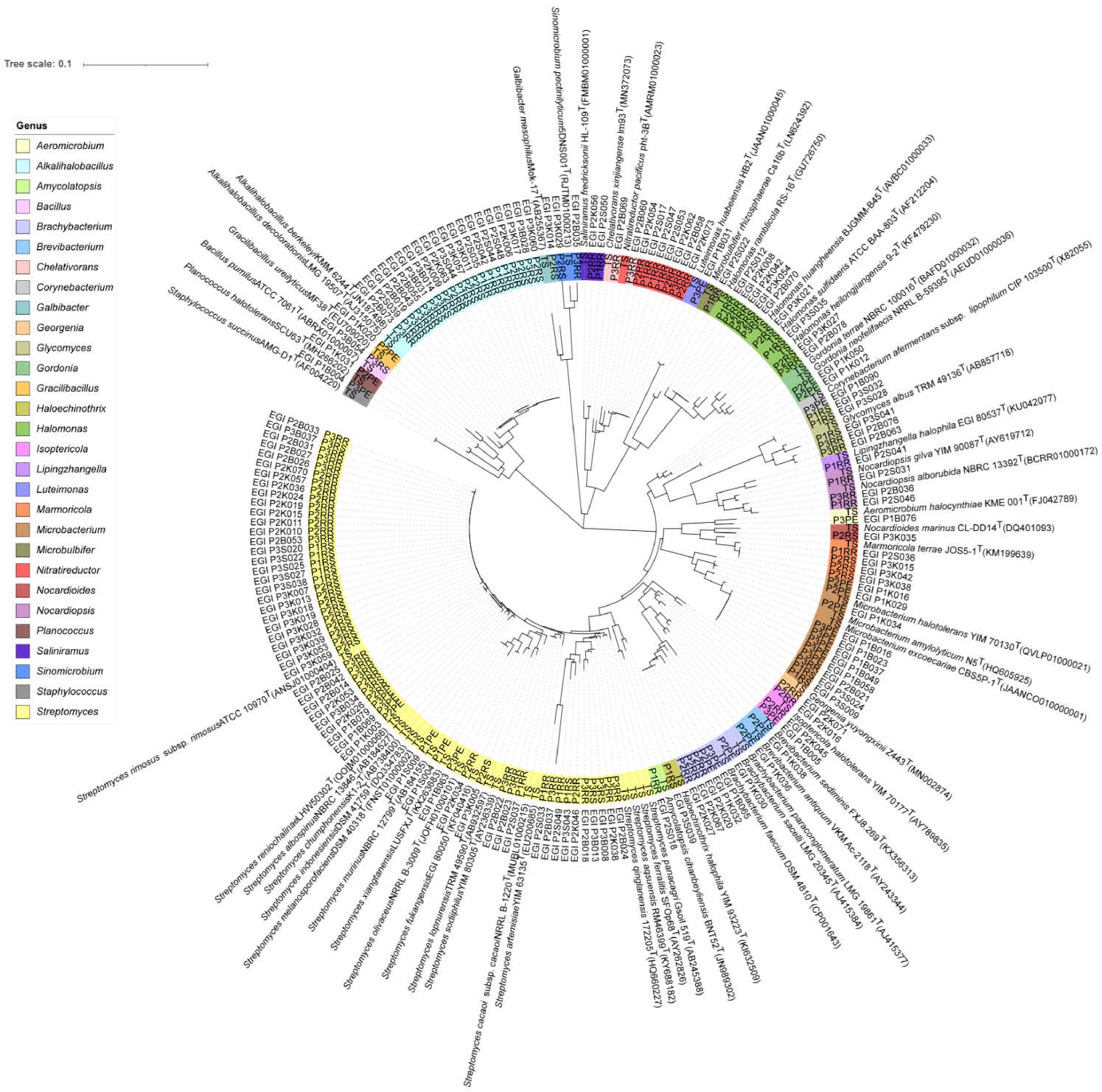

Figure 3

The phylogenetic tree of potential novel bacterial strains. The effective sequence length was 538 bp; bar, the number of substitutions per site.

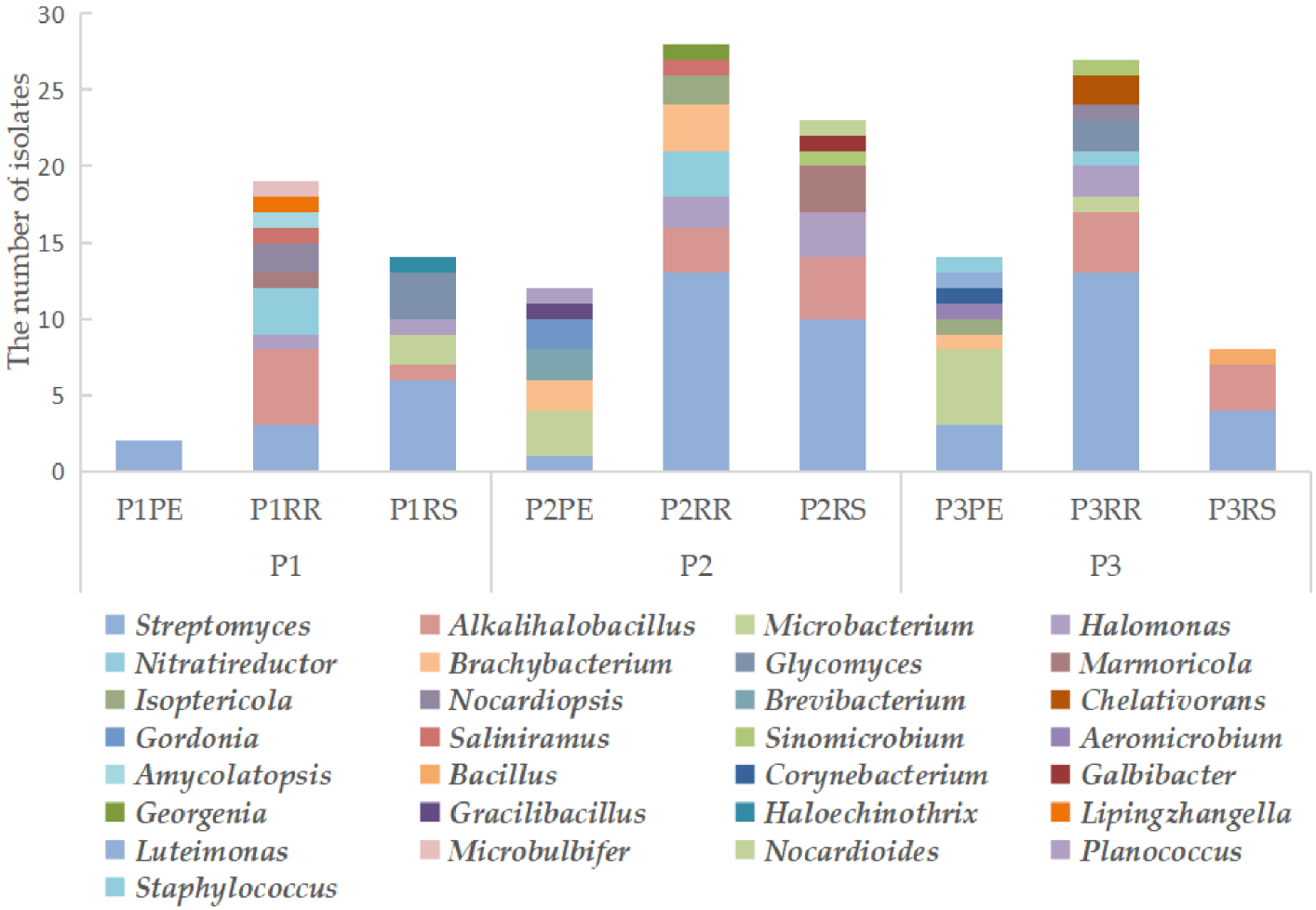

Figure 4

The diversity of potentially novel bacterial strains isolated from different sites at the genus level.

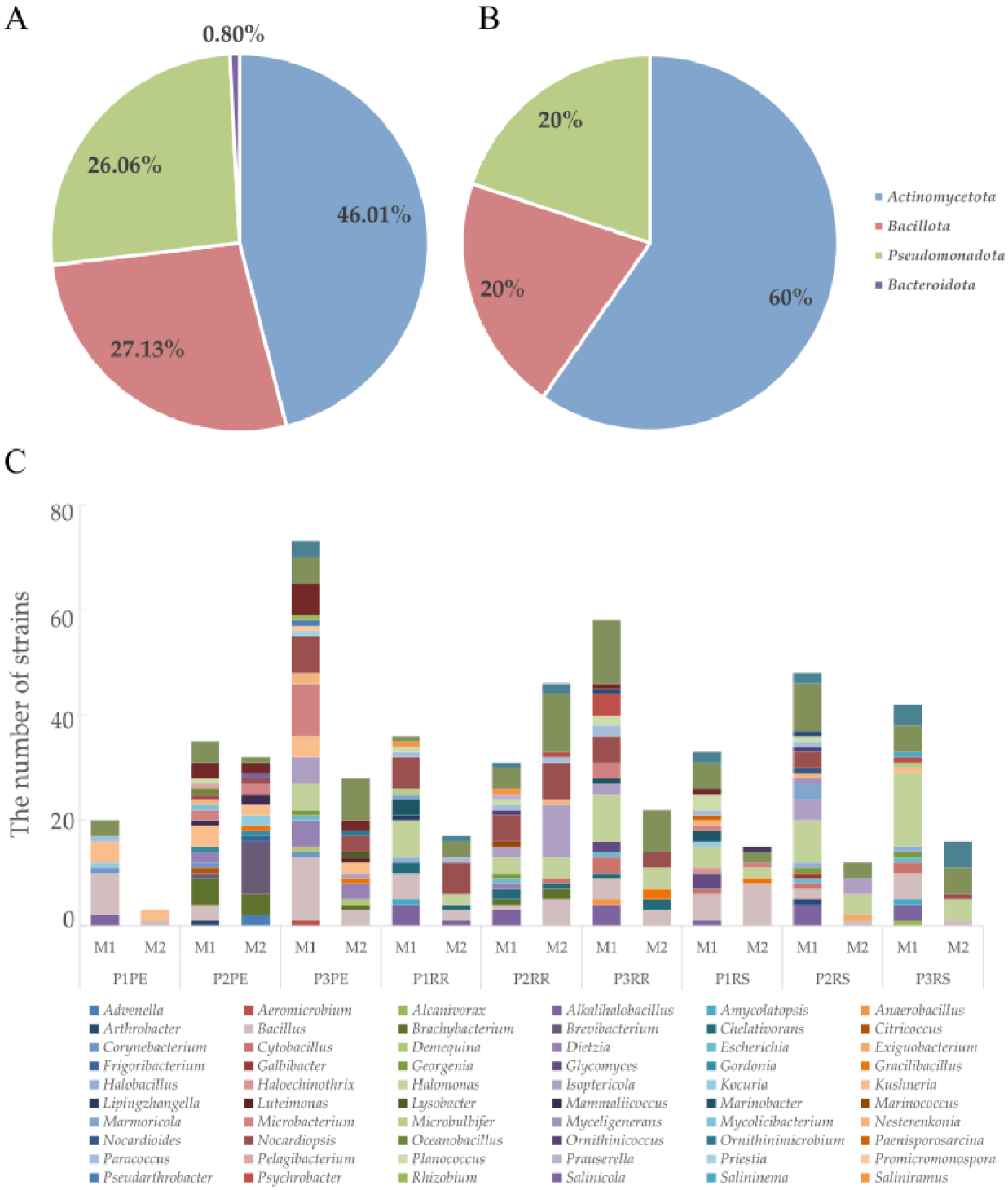

Figure 5

The community of cultivable bacteria on the M1 (A) and M2 (B) media at the phylum level and comparison of isolation effects between the two media among different sites at the genus level (C).

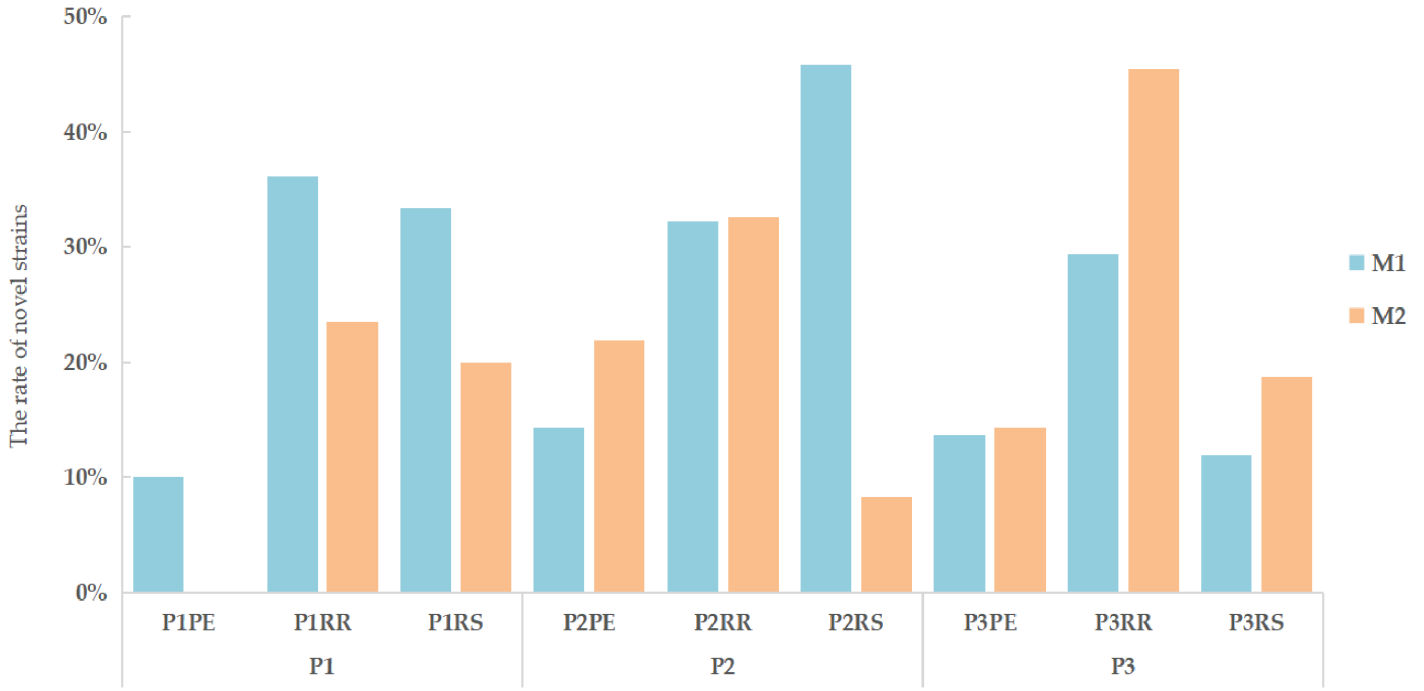

Figure 6

The rate of potential novel strains isolated from different sites on two media.

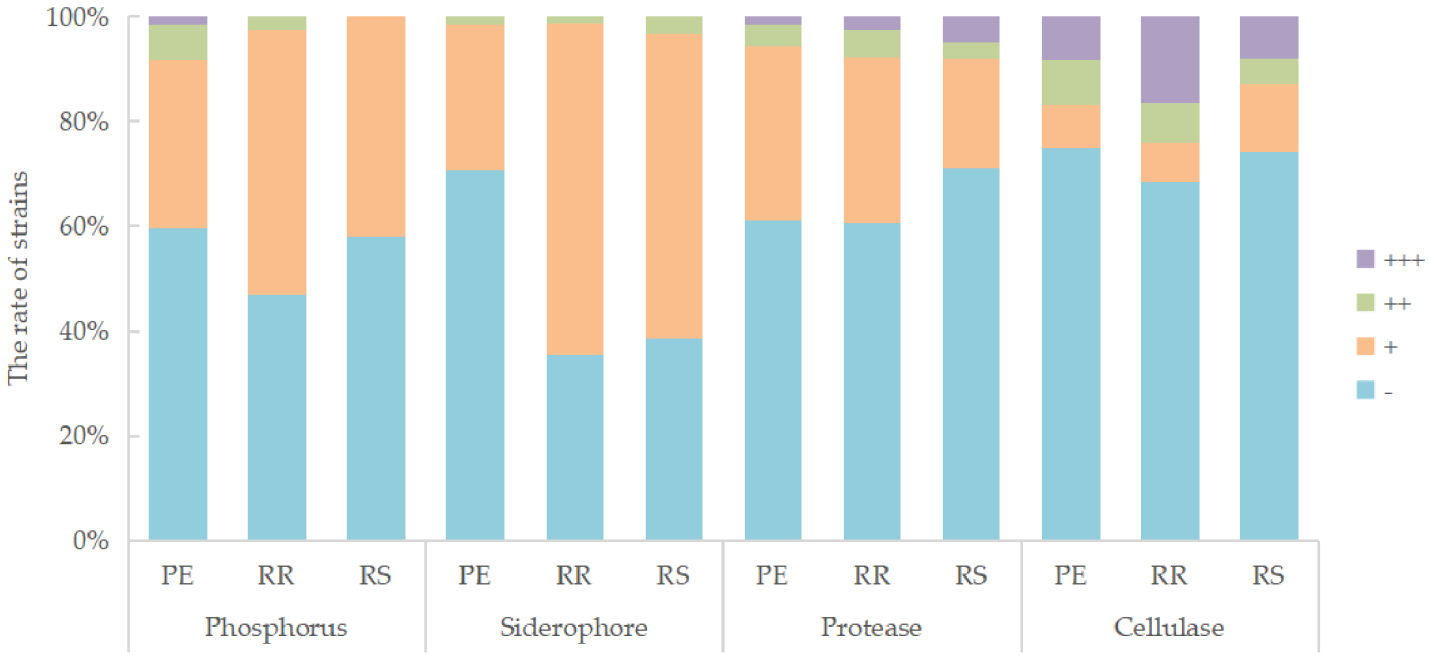

Figure 7

Screening results in bacterial strains solubilizing phosphate, producing siderophores, protease, and cellulase. Notes: “-” - negative (E=1); “+” - weak (1<E≤2); “++” - moderate (2<E≤3); “+++” - strong (E>3).

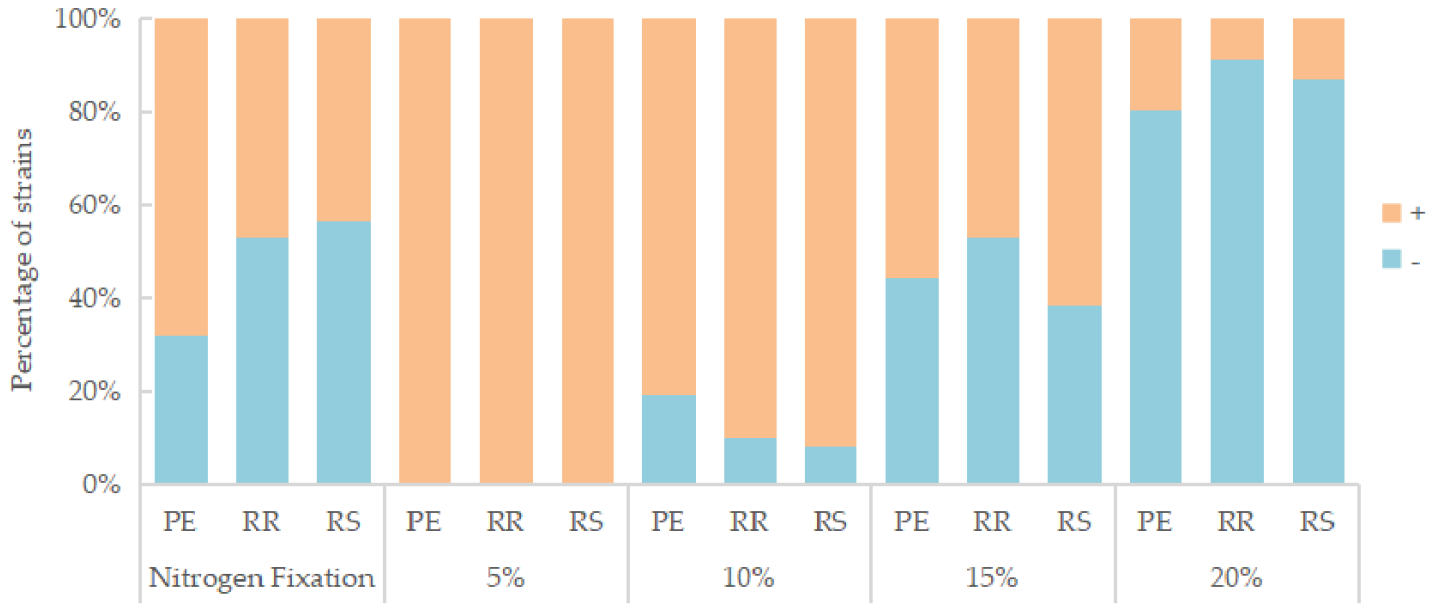

Figure 8

The ability of nitrogen fixation and salt tolerance in bacterial strains. Notes: “-” - negative; “+” - positive.

Supplementary Files

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