Systematic and Applied Microbiology xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Systematic and Applied Microbiology

journal homepage: www.elsevier.de/syapm

Moderate halophilic bacteria colonizing the phylloplane of halophytes of the subfamily *Salicornioideae* (*Amaranthaceae*)

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ARTICLE INFO

Article history:
Received 16 April 2015
Received in revised form 25 May 2015
Accepted 27 May 2015

Keywords:
Halophyte
454-pyrosequencing
Endophyte
Epiphyte
Halophiles

ABSTRACT

Halophytes accumulate large amounts of salt in their tissues, and thus are susceptible to colonization by halotolerant and halophilic microorganisms that might be relevant for the growth and development of the plant. Here, the study of 814 cultured strains and 14,189 sequences obtained by 454 pyrosequencing were combined in order to evaluate the presence, abundance and diversity of halophilic, endophytic and epiphytic microorganisms in the phytosphere of leaves of members of the subfamily *Salicornioideae* from five locations in Spain and Chile. Cultures were screened by the tandem approach of MALDI-TOF/MS and 16S rRNA gene sequencing. In addition, differential centrifugation was used to enrich endophytes for further DNA isolation, 16S rRNA gene amplification and 454 pyrosequencing. Culturable and non-culturable data showed strong agreement with a predominance of *Proteobacteria*, *Firmicutes* and *Actinobacteria*. The most abundant isolates corresponded to close relatives of the species *Chromohalobacter canadensis* and *Salinicola halophilus* that comprised nearly 60% of all isolates and were present in all plants. Up to 66% of the diversity retrieved by pyrosequencing could be brought into pure cultures and the community structures were highly dependent on the compartment where the microorganisms thrived (plant surface or internal tissues).

Introduction

Plants are naturally associated with microorganisms, and these relationships range from beneficial to pathogenic interactions [48]. In many cases, they can play an important role in the growth and development of plants [25,32]. The beneficial functions reported are as diverse as: promotion of plant growth [39,64], N_2-fixation [11,22,61], protection against plant pathogens [12] and synthesis of secondary metabolites [42]. Such microorganisms can colonize both external surfaces and internal tissues. Those associated with the phylloplane and rhizoplane are termed epiphytes [4], and those invading tissues for all or part of their life cycle are called endophytes [67,68], and these seem to be ubiquitous in the plant [37]. The microbial community residing in the phylloplane (leaf epiphytes) faces a variable environment characterized by fluctuating temperatures, humidity, UV radiation, wind, plant topography, and the quality of the photosynthate [1,4,36], whereas endophytes may colonize a more stable environment.

According to their salinity tolerance, plants can be divided into glycophytes and halophytes. The former include sensitive and relatively salt-tolerant species [49], whereas halophytes are plants that can tolerate high salt concentrations and can complete their whole life cycle in soil with NaCl concentrations higher than 200 mM [20,30]. In general, the exploration of the microbial diversity in plants has been focused on the phylloplane of glycophytes, and especially on the major commercial crop species [18,29]. *Bacteria* generally constitute the microbial fraction. On the other hand, *Archaea* have been reported associated only with the rhizosphere [8,13,44,51] and phyllosphere [29], and have never been convincingly detected in the internal tissues [54]. The microbial community association with halophytes has rarely been investigated, and the few existing reports have focused only on the rhizosphere

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http://dx.doi.org/10.1016/j.syapm.2015.05.004
0723-2020/

[56]. Nevertheless, the phyllosphere is generally predicted to be more diverse from evidence using cultivation-dependent [17] and -independent [7] approaches. Furthermore, there is a study describing the bacterial communities found on the leaves of *Atriplex halimus*, a salt-excreting plant [60], but there are no reports of endophytic microorganisms isolated from the endophyllosphere of halophytes.

In this study, the presence and community structures of halophilic microorganisms colonizing halophytes were investigated by means of culture-dependent and -independent (high-throughput pyrotagging) approaches. This pilot work was centered on the endophytic and epiphytic microbiota associated with the leaves of *Salicornioideae* growing under natural conditions in five different locations. In addition, a protocol for the enrichment of the endophytic microbial fraction was optimized in order to address the drawbacks of chloroplastidial and mitochondrial DNA interferences.

Materials and methods

Collection and identification of plant material

The aerial parts of plants, comprising the stems and green leaves of five halophytes, were collected during the months of March and April 2013 in Pichidangui (PI: 32°08′21.56″ S, 71°31′16.26″W, Chile), Lo Valdivia (LV: 34°41′50.16″S, 72°00′42.86″W, Chile), Alicante (AL: 38°21′03.3″N, 3°00′44.3″W, Spain), Campos (CA: 39°21′03.3″ N, 3°00′44.3″ E, Spain) and Ses Fontanelles (SF: 39°32′4.64″N, 2°43′56.41″E, Spain). Individual stems with green leaves of the plants were excised at approximately 5 cm above the soil level and stored in zip-lock plastic bags using sterile gloves. The five plants were identified in the Biology Department of the University of the Balearic Islands (UIB). Additionally, genetic identification was performed in order to verify the identity of plant specimens. Plant DNA isolation was performed using the DNeasy Plant Mini Kit (Qiagen). The maturase K gene (*matK)* was amplified using MasterMix (5 PRIME GmbH, Germany) following the manufacturer's instructions. The reaction was carried out in a final volume of 25 μL with the specific primers F2cariophyllales and R2cariophyllales using previously published conditions [15]. A fragment of approximately 800 bp was visualized on 1.5% agarose gel stained with ethidium bromide (1 μg mL^{-1}) and amplicons were purified using the MSB® Spin PCRapace kit (INVITEK GmbH, Berlin). Purified PCR products were sent to Secugen (Madrid, Spain) for DNA sequencing. Sequences were trimmed using the software Sequencher *v* 4.8 (Gene Codes Corporation, Michigan). The new sequences were aligned and compared with reference sequences in the GenBank database using the ClustalW aligner implemented in the ARB software package [38]. The identity values between the sequences were also calculated with the ARB package.

Culture-dependent analyses

Surface sterilization and isolation of epiphytic and endophytic microorganisms

Approximately 150 g of 7–10 cm shoots from each plant were randomly selected, with damaged plant material being previously excised and removed. Shoots were carefully manipulated to avoid tissue damage [10], and the excision areas were first hot cauterized using an incandescent metal loop to avoid the loss of sap. Isolation and cultures of the plant-associated microbiota were performed on agar plates with five different salt concentrations (5%, 15%, 20%, 25% and 30%) using sea water (SW) culture media [55] supplemented with 0.05% yeast extract. Three different fractions or conditions: epiphytic (P), endophytic (N) and sterile test (S) were taken after each manipulation step. Briefly, 25 g of plant material were placed into 50 mL tubes with 35 mL SW of each of the five different salt concentrations, gently vortexed for 3 × 45 s, and then the plant material was removed and placed in a sterile tube. This initial suspension was considered as the P inoculum. The plant material surface was sterilized immediately with sequential washes: 10 min in sterile distilled water, 5 min in 0.2% Triton X-100, 10 min in sterile distilled water, 5 min in 2% bleach (NaClO), 10 min in sterile distilled water, 2 min in 70% ethanol and, finally, two rinses of 5 min in sterile distilled water. Between steps, plant material was dried with sterile paper towels. The sterilization test was performed by submerging and mixing the sterilized plant material in SW media, following the same mixing process used for isolation of epiphytes. This suspension was considered as the sterilized inoculum S. Finally, the plant material was mixed with 10 mL of PBS 1× and the mixture was gently macerated using a sterile pestle and mortar. The tissue extract was considered as the N inoculum.

The P, S and N inoculates were serially diluted (to 10^{-8}) in each of the five different salt concentration SW media and spread-plated onto the respective SW agar media supplemented with 10 μg mL^{-1} of the fungicide itraconazol (Bexal Farmacéutica). Samples were plated in triplicates and incubated at room temperature. The colonies were randomly picked and selecting from at least 10% CFUs (colony forming units) growing on plates with abundances between 10 and 100 colonies from each location. Colonies were replicated onto plates with the same culture conditions and, in order to obtain a random subsample of isolates, no attention was paid to the colony morphology. For storage purposes, isolates were grown in liquid SW media with the same salt concentration, and the resultant suspensions were supplemented with glycerol (5%) for storage at −80 °C.

A piece of a shoot of approximately 3 g was selected to verify the efficacy of the surface sterilization by scanning electron microscopy (SEM). For this purpose, an S-3400N variable pressure microscope (Hitachi, Japan) was used. Previously, samples were fixed with 2% glutaraldehyde (4 °C) for 48 h. Shoots were then washed with phosphate buffer (0.1 M, pH 7.2–7.4) for 24 h. Finally, samples were submerged in consecutive concentration steps of acetone of 30–50–60–70–90 and 100% for 30 min each. Micrographs of fifteen areas from each sample were taken using a 10 kV accelerating voltage and 40 Pa of pressure with a low-pressure ESED secondary detector (Hitachi Trademark, Barcelona, Spain). Additionally, with the object of visualizing endophytes, previously sterilized shoots were opened carefully using a sterile scalpel. The opened shoots were fixed and micrographs were taken using the same protocol mentioned above.

MALDI-TOF/MS analyses, and cluster identification by 16S rRNA gene sequencing

Randomly selected colonies were analyzed by MALDI-TOF/MS (matrix-assisted laser desorption/ionization time-of-flight mass spectrometry), as previously reported [65]. Groups of spectra clustering in the dendrograms were considered as operational taxonomic units (OTUs; see below). OTUs selected from the P and N dendrograms were defined with a cut-off at the 720 and 750 distance levels, respectively. Several representatives of each OTU were selected for their DNA extraction, 16S rRNA gene amplification and sequencing, and phylogenetic reconstruction, as previously reported [65]. The sequences have been deposited at the EMBL repository under the accession numbers (LN651124–LN651155). OPUs (operational phylogenetic units; see below) were circumscribed by manually inspecting the resulting final tree [65].

Clonality estimated by RAPD fingerprinting

RAPD fingerprints were generated for 193 isolates representing the different OPUs. These were randomly chosen in order to cover at

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least 12% of each group. RAPD screening was performed using MasterMix (5 PRIME GmbH, Germany), following the manufacturer's instructions, with the primer RAPD1 and the same conditions as previously reported [47]. The reaction was carried out in a 25 μL volume containing 3 μL of DNA template. The amplified PCR products were visualized by electrophoresis in 2% agarose gel running in 1× TAE buffer, at 25 V for 50 min. The MassRuler™ Express Forward DNA Ladder mix (Thermo Scientific, Massachusetts, USA) was used as a molecular weight standard. Ethidium bromide-stained DNA gels were photographed and analyzed with Bionumerics *v* 7.1 software (Applied Maths, Belgium).

Culture-independent analyses

Separation of plant and microbial fractions

Approximately 60 g of each plant were sterilized and macerated as described above. A total of 20 mL of macerated biomass was centrifuged in five successive steps at 200, 500, 800, 1000 and 3000 × *g* for 20 min at 4 °C, and the pellets were collected after each step. Pellets and the final supernatant (3000 × *g*) were stored at −20 °C. Finally, a sucrose density gradient centrifugation was applied to the pellet at 3000 × *g*. The density gradient was prepared by overlaying six solutions of 4 mL sucrose with increasing concentrations (15%, 20%, 30%, 40%, 50% and 60% w/v) avoiding perturbations and mixing. The 3000 × *g* pellets were mixed with 2 mL sucrose solution at 2.5% w/v and 20 μL blue toluidine (1 mg L^{-1}). They were then placed on the top of the gradient and centrifuged (79,880.3 × *g*, 2:00, 4 °C—Optima TLX Ultracentrifuge Rotor SW30.1 Centrifuge, Beckman Coulter, California, USA). After centrifugation, four layers were observed. Each layer was recovered using a syringe and needle. All layers were stored at −20 °C.

Microbial DNA extraction, PCR amplification and pyrosequencing

Samples of each layer of higher density were used in the microbial DNA extraction as it was expected that mitochondria and chloroplast would be retained in the upper layers (between 10% and 30% w/v of sucrose solution) [68]. A total of 1200 μL of extraction buffer (EB) [34] was added to 2 mL of sample, mixed by vortex (60 s) and centrifuged (7000 × *g*, 15 min, room temperature). The pellet obtained was used to extract DNA, as previously published [34], but with the following modifications: no RNAse was added to the samples, and an initial step with pre-warmed (40 °C) phenol:chloroform:isoamyl alcohol mixture (25:24:1 v/v/v) in a proportion of 1:1 (v/v) was also incorporated before subsequent treatments with chloroform:isoamyl alcohol (24:1). DNA precipitation was carried out using 11% (v/v) 3 M sodium acetate and 60% (v/v) cold (4 °C) isopropyl alcohol, and the air-dried DNA pellet was resuspended in 80 μL of Milli-Q grade water. DNA was quantified using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA).

PCR amplification was performed using bacterial primers GM3 and S (Supplementary Table S1), as previously reported [65]. A second short PCR (five cycles) was performed in a final volume of 25 μL in triplicate in order to incorporate tags and a linker into the amplicon using a 1:25 dilution of the original products as templates, using the same conditions as the first PCR. In this case, the primers GM3-PS and a variant of 907-PS (Supplementary Table S1) were used. The PCR products were visualized by electrophoresis in 1% agarose gel running in 1× TAE buffer at 25 V for 50 min. Two bands of ~1500 bp and ~960 bp were observed. The second band was excised and the PCR product was eluted using the Zymoclean™ Gel DNA Recovery Kit (Zymo Research, California, USA) following the manufacturer's instructions. The concentration of the barcoded-amplicons was measured with a NanoDrop™ and the MassRuler™ Express Forward DNA Ladder mix and, finally, an equimolar mixture of the amplicons was sent to the sequencing company Macrogen Inc., Seoul, Korea. The samples were sequenced using 454 GS-FLX+ Titanium technology. The set of sequences has been deposited at the ENA sequence repository under the study accession number PRJEB7624.

Sequence trimming, OTU clustering and OPU design

Data was processed using Mothur [57] software. Low-quality sequences were trimmed (<300 nucleotides with a window size of 25 and average quality score of 25). No ambiguities and mismatches in reads with primer pairs and barcodes were allowed. Chimeras were removed with the application Chimera Uchime implemented in Mothur. Sequences were clustered into OTUs at 99% using the UCLUST tool included in QIIME [9]. The longest read of each OTU was selected as representative for the OPU design by phylogenetic inference, as previously described [65].

Ecological indices and statistical analysis

A non-parametric Kruskal–Wallis test was performed for comparing the abundances between epiphytes and endophytes in each plant as they did not satisfy assumptions for normality and homogeneity of variances. The relationship between the composition of the microbial communities, compartment (P or N) and locations was examined by non-metric multidimensional scaling (NMDS) [31]. The goodness of the NMDS was evaluated according to the stress value, which for ten samples is considered acceptable if smaller than 0.133 [62]. The abundances of endophytes obtained with both methods (cultured and non-cultured) were further explored using a principal component analysis (PCA). All multivariate statistical analyses were performed using the package *vegan* [45] and FactorMineR [33] in R *v* 3.1.1. (www.r-project.org). OPUs were used to calculate rarefaction curves and the Shannon-Wiener (H'), Chao 1, Evenness (E), and Dominance (D) indexes per sample with PAST *v* 3.01 software [24].

OTU and OPU definitions in this study

In previous studies [21,65], OTUs were defined by clustering the units according to an identity threshold. Here, the OTUs based on MALDI-TOF/MS measures corresponded to each independent cluster formed by profile identity limits, which were dependent on each dataset. On the other hand, the trimmed sequences obtained by 454 pyrotagging were stringently clustered in OTUs sharing ≥99% identity. In both cases, the representative sequences of each OTU were used for phylogenetic inference (by reconstruction for almost full sequences, or parsimony addition for partial sequences) and OPU assignment. An OPU is considered as the smallest clade containing one or more amplified sequences affiliating together with reference sequences available in the public repositories [65]. When possible, the OPUs should include a type strain sequence, and for identity values >98.7% with type strain sequences the amplicons would be considered to belong to the same species using this conservative threshold, as previously recommended [61]. On the other hand, for identity values <98.7% and >94.5% with the closest relative type strains, the OPU would be considered to be the same genus [69] but from a different unclassified species.

Results

Species identification of the host plants

Molecular identification results confirmed the morphological identification, and the five exemplars could be identified as members of the family *Amaranthaceae*, subfamily *Salicornioideae*.

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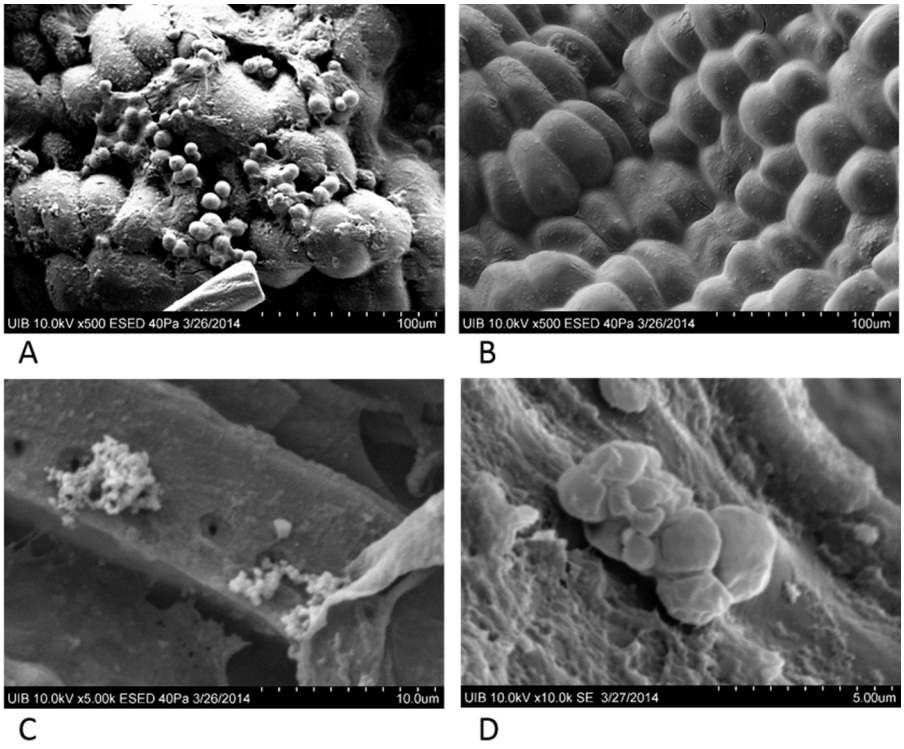

Fig. 1. Scanning electron microscopy micrographs of the untreated phyllosphere plant surface (A) and after the sterilization process (B). Endophytic microorganisms (C and D) from the phyllospheric area after sterilization of the surface. Sample corresponding to *Arthrocnemum macrostachyum* obtained from Ses Fontanelles, Spain.

The affiliation observed with the *matK* gene confirmed the identification of two very close species: *Allenrolfea vaginata*, taken from LV, and *Arthrocnemum macrostachyum* taken from PI, AL, CA and SF (Supplementary Table S2).

Culturable abundances

The number of heterotrophic colony forming units (CFU) was enumerated 3, 7, 15, 25 days after plating. After day 25, CFU were checked every 15 days for the following seven months. None of the S plates (sterile tests) exhibited growth. Accordingly, all areas observed under SEM showed the presence of colonizing epiphytic microorganisms in the untreated tissues, whereas after the sterilization of plant surfaces no traces of attached microorganisms could be detected (Fig. 1A and B), which confirmed that attached epiphytic microbiota were successfully removed from the plant material. Moreover, the micrographs of sectioned sterile plant material exhibited the presence of endophytic microcolonies (Fig. 1C and D).

Plate counts showed abundance values of aerobic heterotrophic epiphytes with a maximum of 2.7×10^6 CFU g^{-1} (PI), whereas the major value for endophytes was 2.7×10^4 CFU g^{-1} (CA), both in 5% SW. No growth was detected in 30% SW after seven months incubation with the unique exception of SF endophytes, which reached values of 1.4×10^4 CFU g^{-1} (Supplementary Table S3). There was a decreasing pattern of microbial abundances with the increase of salt concentration in the culture media for both epiphyte (P) and endophyte (N) samples (Supplementary Table S3). The abundances of CFU's were considerably different between endophytes and epiphytes (Fig. 2). In general, endophytes were more abundant than epiphytes and the differences were significant for all samples (Kruskal–Wallis X^2, d.f. = 1, $p < 0.05$; Supplementary Table S3), except for LV that did not show significant differences (X^2, d.f. = 1, $p > 0.05$; Supplementary Table S3).

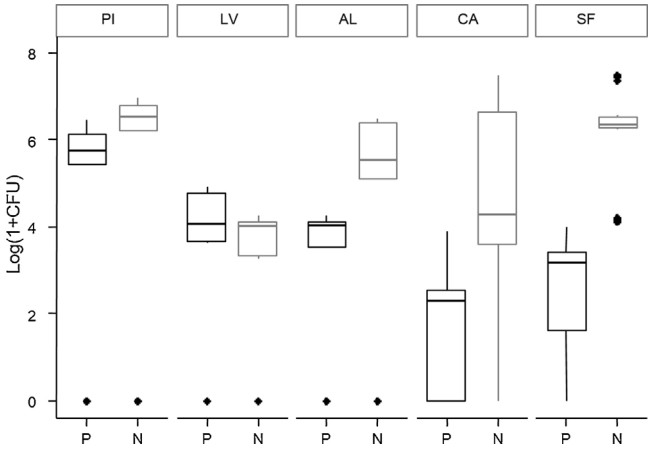

Fig. 2. Total abundance box plot of endophytes and epiphytes in each location. PI = Pichidangui, LV = Lo Valdivia, AL = Alicante, CA = Campos, SF = Ses Fontanelles, P = epiphytes and N = endophytes.

MALDI-TOF/MS analysis of isolates and identification of OTUs by sequencing

A total of 813 colonies corresponding to 318 epiphytes and 495 endophytes from different plants: PI ($n = 247$), LV ($n = 173$), AL ($n = 170$), CA ($n = 79$) and SF ($n = 144$) were analyzed by MALDI-TOF/MS. The dendrograms clustered the MALDI-TOF/MS profiles into 32 distinct OTUs (i.e. clusters based on profile similarities; Supplementary Figs. S1 and S2). Representative isolates of each OTU were identified by 16S rRNA gene sequence affiliation with their closest relative type strains (Table 1, Fig. 3 and Supplementary Table S4). In all cases, the new isolates and the closest type strains were at least 98.7%. The 32 OTUs could be grouped into a total of 17 OPUs (i.e. unique phylogenetic clades affiliating the new sequences with reference sequences; note that the numeration

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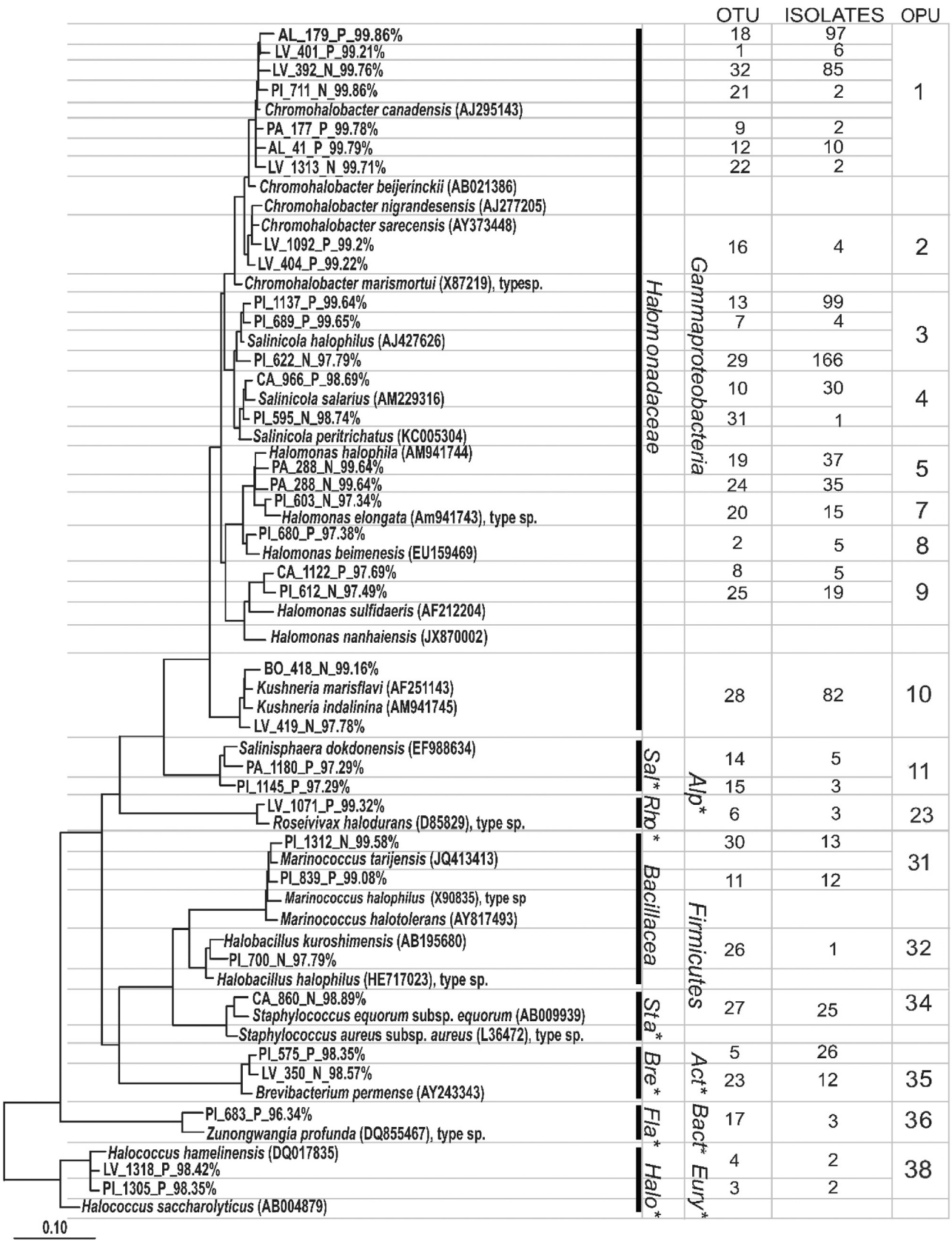

Fig. 3. 16S rRNA phylogenetic reconstruction of representative isolates and their close relative type strains, including additional reference sequences. Sequence names are formed by: location (PI = Pichidangui, LV = Lo Valdivia, AL = Alicante, CA = Campos and SF = Ses Fontanelles), number of isolate, origin (N = endophyte and P = epiphyte) and identity percentage with the closest type strain. Columns indicate (from left to right): family (*Sal = Salinisphaeraceae, *Rho = Rhodobacteraceae, *Sta = Staphylococcaceae, *Bre = Brevibacteriaceae, *Fla = Flavobacteriaceae and *Halo = Halobacteriaceae); class or phylum; (*Alp = Alphaproteobacteria),*Act = Actinobacteria, *Bac = Bacteroidetes and *Eury= Euryarchaeota); OTU number in the dendrograms; number of isolates; number of OPU. .

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Table 1
Most relevant OPUs summing abundances above 91.8% of the total pyrosequencing data. The affiliation of the sequences and isolates is given with the closest relative type strain and the identity value (*id = identity). OPUs marked in bold include those more abundant in all locations.

OPU/affiliation	# accession	% id*	ENDOPHYTES										EPIPHYTES				
			PYROSEQS%					ISOLATES%									
			PI	LV	AL	CA	SF	PI	LV	AL	CA	SF	PI	LV	AL	CA	SF
1 Chromohalobacter canadensis	(AJ295143)	>98%	1.2	0.4	50.6	33.1	37.0	22.4	17.5	15.3	0.0	26.5	50.5	37.7	9.6	15.4	38.1
***2** Chromohalobacter sarecensis*	(AY373448)	>99%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.5	0.0	0.0	0.0
3 Salinicola halophilus	**(AJ427626)**	**>99%**	**0.3**	**2.8**	**0.7**	**1.0**	**0.6**	**32.2**	**43.9**	**63.6**	**0.0**	**16.7**	**30.9**	**21.1**	**80.8**	**0.0**	**16.7**
4 Salinicola salarius	**(AM299316)**	**>97%**	**0.1**	**0.1**	**0.1**	**0.2**	**0.0**	**0.7**	**0.0**	**0.0**	**0.0**	**0.0**	**1.0**	**9.7**	**5.8**	**53.8**	**19.1**
5 Halomonas halophila	**(AM941744)**	**>96%**	**0.6**	**0.8**	**20.0**	**7.8**	**10.0**	**5.9**	**0.0**	**0.0**	**37.9**	**37.3**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
***6** Halomonas meridiana*	(AJ306891)	>96%	0.1	38.1	0.1	2.0	0.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***7** Halomonas elongata*	(FN869568)	>97%	0.0	0.0	0.0	0.0	0.0	9.9	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***8** Halomonas beimenensis*	(EU159169)	>97%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	5.2	0.0	0.0	0.0	0.0
9 Halomonas sulfidaeris	(AF212204)	>98%	0.0	0.2	0.0	0.0	0.0	0.0	0.0	0.8	22.7	0.0	0.0	0.9	0.0	30.8	0.0
10 Kushneria indalinina/K. marisflavi	**(AM941745) (AF251143)**	**>94%**	**0.1**	**38.4**	**0.3**	**1.0**	**0.2**	**24.3**	**17.5**	**19.5**	**1.5**	**10.8**	**0.0**	**0.0**	**0.0**	**0.0**	**0.0**
***11** Salinisphaera dokdonensis*	(EF988634)	>97%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0	0.0	11.9
***12** Pseudomonas seleniipraecipitans*	(FJ22810)	>95%	2.1	12.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***13** Pseudomonas alcaliphila*	(AB030583)	>96%	91.7	0.0	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***14** Pseudomonas pseudoalcaligenes*	(Z76666)	>95%	0.0	0.0	0.0	0.2	1.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***15** Marinobacter daepoensis*	(AY517633)	>94%	0.04	0.03	0.1	0.2	1.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***16** Rudaea cellulosilytica*	(EU741687)	>95%	0.4	0.5	6.5	18.4	13.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***17** Chelonobacter oris*	(EU331064)	>94%	0.4	0.8	5.0	8.4	2.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***18** Nevskia terrae*	(GQ845011)	>98%	0.0	0.0	0.2	0.2	0.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***19** Curvibacter lanceolatus*	(AB021390)	>96%	0.0	0.1	0.1	0.7	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***20** Burkholderia rhynchosiae*	(EU219865)	>97%	0.0	0.3	0.1	2.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***21** Cupriavidus gilardii*	(AF076645)	>98%	0.1	0.4	1.5	3.7	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***22** Nautilia lithotrophica*	(AJ404370)	>80%	0.2	0.0	1.0	1.0	1.4	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***23** Roseivivax halodurans*	(D85829)	>99%	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	2.6	0.0	0.0	0.0
***24** Afifella marina*	(D30790)	>84%	0.0	0.2	0.1	0.1	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***25** Methylobacterium platani*	(EF426729)	>93%	0.0	0.0	0.4	0.2	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***26** Blastochloris viridis*	(AF084495)	>94%	0.2	0.0	0.1	0.1	2.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***27** Gluconacetobacter liquefaciens*	(X75617)	>94%	0.1	0.9	0.0	0.0	0.6	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***28** Rhizobium tarimense*	(HM371420)	>98%	0.0	0.0	0.0	0.0	0.5	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***29** Sphingomonas oligophenolica*	(AB018439)	>98%	0.04	0.6	0.3	4.7	2.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***30** Marinococcus halotolerans*	(AY817493)	>99%	0.1	0.03	0.9	1.2	3.3	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***31** Marinococcus tarijensis*	(JQ413413)	>99%	0.2	0.03	2.8	0.2	0.1	2.0	0.0	0.8	0.0	8.8	4.1	1.8	3.8	0.0	9.5
***32** Halobacillus kuroshimensis*	(AB195680)	>98%	0.0	0.0	0.0	0.0	0.0	0.7	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***33** Alkalibacillus salilacus*	(AY671976)	>85%	0.2	0.8	3.4	1.4	7.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***34** Staphylococcus equorum*	(AB009939)	>96%	0.0	0.1	0.0	1.6	1.0	0.0	0.0	0.0	37.9	0.0	0.0	0.0	0.0	0.0	0.0
***35** Brevibacterium permense*	(AY243343)	>98%	0.2	0.3	1.1	1.4	1.0	0.0	21.1	0.0	0.0	0.0	1.0	21.9	0.0	0.0	0.0
***36** Zunongwangia profunda*	(DQ855467)	>96%	0.2	0.4	0.1	1.2	0.4	0.0	0.0	0.0	0.0	0.0	3.1	0.0	0.0	0.0	0.0
***37** Chitinophaga jiangningensis*	(KF150362)	>89%	0.0	0.0	0.2	0.0	1.2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
***38** Halococcus hamelinensis*	(DQ017833)	>98	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	1.0	0.9	0.0	0.0	4.8
Total			98.8	98.7	95.9	91.8	94.1	98.1	100	100	100	100	100	100	100	100	100

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of the OPUs is unique for both culturing and pyrosequencing approaches; Fig. 3 and Table 1). Six OPUs corresponded only to epiphytic isolates (OPUs 2, 7, 10, 11, 16 and 17), five only to endophytic isolates (OPUs 5, 6, 9, 13 and 14), and six were common to both compartments (OPUs 1, 3, 4, 8, 12 and 15). The bacterial domain was represented by 16 OPUs that harbored 809 isolates (99.5%), while OPU 38 (an epiphyte) with four isolates was the single OPU affiliating with the archaeal domain. Most of the OPUs affiliated with *Gammaproteobacteria* (52.9%) and *Firmicutes* (17.6%), and to a minor extent *Alphaproteobacteria* (0.4%), *Actinobacteria* (4.7%), *Bacteroidetes* (0.4%) and *Euryarchaeota* (0.5%). The most abundant isolates corresponded to OPU 1 that was identified as *Chromohalobacter canadensis* and OPU 3 identified as *Salinicola halophilus*, with 204 and 269 strains, respectively, which represented 58.2% of the total isolates in this study.

Comparison between culturable epiphytic and endophytic isolates in different exemplars

Among the 17 OPUs that configured the complete set of phylogenetic clades detected in the culturable fraction, OPUs 1 (*C. canadensis*) and 3 (*S. halophilus*) were common to all samples and also appeared in both the epiphytic and endophytic fractions, with the exception of CA samples where OPU 1 was present only in the epiphytic fraction. In addition, the samples showed other common microbiota, such as OPU 31 (*Marinococcus tarijensis*) that was common to PI, AL and SF; OPU 4 (*Salinicola salarius*) common to all epiphytic communities, but only present as endophytic in PI; OPU 9 (*Halomonas sulfidaeris*) common as epiphytic in CA and LV, and endophytic CA and AL; and the minor OPU 35 (*Brevibacterium permense*) only present in the Chilean PI (only epiphytic) and LV samples (both endophytic and epiphytic). The remaining OPUs detected were either exclusive to the epiphytic fraction, such as OPUs 2 (*Chromohalobacter sarecensis*, exclusive to LV), 8 (*Halomonas beimenensis*, exclusive to PI), 11 (*Salinisphaera dokdonensis*, present in SF and PI), 23 (*Roseivivax halodurans*, exclusive to LV), 36 (*Zunongwangia profunda*, exclusive to PI) and 38 (*Halococcus hamelinensis*, present in PI, LV and SF); or exclusive to the endophytic microbiota, such as OPUs 5 (*Halomonas halophila*, present in PI and SF), 7 (*Halomonas elongata*, exclusive to PI), 32 (*Halobacillus kuroshimensis*, exclusive to PI) and 34 (*Staphylococcus equorum*, exclusive to CA). It was remarkable that OPU 10 (*Kushneria marisflavi–Kushneria indalinina*) was exclusively endophytic and common to all samples (Table 1).

The most relevant OPUs given the high numbers of strains isolated were OPU 1 (*C. canadensis*) with a maximum of 50.5% (PI) of the epiphytic and 26.5% (SF) of the endophytic fractions, and OPU 3 (*S. halophilus*) with 80.8% (AL) and 63.6% (AL) of the respective fractions. Both were the most highly retrieved of the complete dataset comprising 25.1% and 33.1% of the total isolates. From the epiphytes, and after *C. canadensis* and *S. halophilus*, the most highly retrieved groups were OPU 4 (*S. salarius*) with a maximum of 53.8% (CA) and OPU 35 (*B. permense*) with 21.9% (LV) (Table 1). The most important and exclusive endophytic OPU present in all samples was OPU 10 that affiliated with *K. marisflavi–K. indalinina* with 24.3% (PI) of the endophytes (Table 1).

The comparison of abundance and distribution of OPUs between all sites and compartments, visualized by NMDS analysis (Fig. 4), revealed with a stress value of 7.9×10^{-2} that the two distinct compartments (endophytic vs epiphytic) were remarkably different even in the same plant. However, the same compartments in different plants shared a common culturable microbiota with independence of their origin (and plant species for LV). For both compartments, CA samples always exhibited the most different culturable microbiota. With respect to diversity indexes, the richness estimator index (Chao 1 in Table 2) ranged from 3 to 10.5 among all

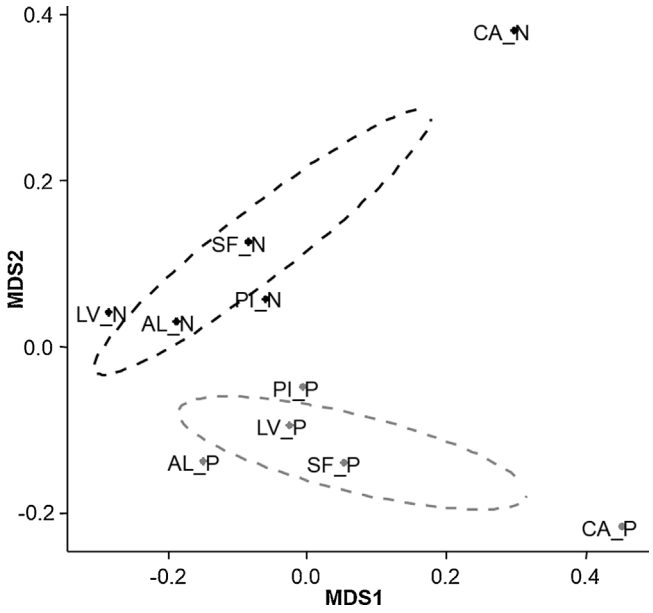

Fig. 4. Two dimension NMDS of epiphytes (P) and endophytes (N) in all locations. Standardization by Wisconsin, Euclidean distance and a stress value of 0.0786.

Table 2
Comparison of the operational phylogenetic units (OPUs) diversity (H'), richness (Chao 1), evenness (E) and dominance (D) indices of the profiles obtained from MALDI-TOF analysis and sequences from pyrosequencing.

	Col/seq*	OTUs	OPUs	H'	Chao 1	E	D
Culturable data							
Epiphytes							
PI	95	12	8	1.30	10.5	0.40	0.37
LV	116	10	9	1.66	10	0.58	0.23
AL	52	5	4	0.69	4	0.50	0.66
CA	13	4	3	0.98	3	0.89	0.40
SF	42	8	6	1.60	6	0.83	0.23
Endophytes							
PI	152	11	10	1.54	10	0.58	0.23
LV	57	5	4	1.30	4	0.92	0.30
AL	118	5	5	0.97	6	0.78	0.47
CA	66	4	4	1.14	4	0.77	0.39
SF	102	6	5	1.47	5	0.87	0.26
Pyrosequencing data							
PI	2334	57	30	0.52	53	0.04	0.84
LV	3145	132	35	1.64	55	0.12	0.29
AL	1747	106	44	1.85	56.4	0.12	0.31
CA	1097	102	55	2.53	63.6	0.21	0.16
SF	1262	94	56	2.53	68.5	0.22	0.17

* Colonies for culturable data and sequences for pyrosequencing data.

samples, and in general was higher in the Chilean samples (PI and LV for epiphytic samples and only PI for endophytic) than those of the Mediterranean area (AL, CA and, SF). On the other hand, rarefaction plots showed similar saturation trends (Supplementary Fig. S3). The diversity measurement (H' index) was shown to be higher in the Chilean (PI and LV) and SF samples, whereas PI, AL and CA showed higher values for endophytes than epiphytes, and SF and LV had the opposite trend. Evenness (E) showed the lowest values for PI, and the highest values were observed for CA (0.9) and SF (0.8) in the epiphytic fraction, and LV (0.9) and SF (0.9) for the endophytes. Dominance (D) gave a maximum of 0.7 in AL (P), and the rest exhibited values under 0.5 (Table 2).

RAPD fingerprinting analysis

In order to understand whether isolates showed clonality, RAPD analyses were performed at the OTU level, since a single OPU could harbor more than one OTU (e.g. OPU 3 was formed by

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Table 3
Sequence distribution obtained by pyrosequencing, including chloroplastidial and mitochondrial sequences detected (PI = Pichidangui, LV = Lo Valdivia, AL = Alicante, CA = Campos and SF = Ses Fontanelles).

	Total	Removed	% Removed	Chloropl.	Mitoch.	Final	OTUs	OPUs
PI	3085	751	24.4	0	85 (5.48%)[a]	2334	56	30
LV	5659	2514	44.4	4 (0.12%)[b]	197 (6.05%)[a]	3145	126	36
AL	2082	335	16.5	5 (0.16%)[b]	180 (5.90%)[a]	1747	97	45
CA	1544	447	23.1	2 (0.07%)[b]	129 (4.60%)[a]	1097	94	56
SF	1472	210	14.2	0	53 (5.13%)[a]	1262	92	56
Total	14,189	4257	–	11	644	9585	465	103
Mean	2837.8	851.4	24.5	2.2	128.8	1917	93	44.6
SD	1683.7	950.8	11.9	2.3	61.11	838.5	24.9	11.7

[a] The percentage represents the number of mithocondrial sequences removed of the trimmed sequences (values not shown).
[b] The percentage represents the number of chloroplastidial sequences removed of the trimmed sequences (values not shown).

OTUs 7, 13 and 29). The profiles exhibited diverse patterns ranging from 2 to 16 bands with product lengths ranging from 250 to 4000 bp (data not shown). In all cases, each OTU showed its own profile composition, and in no case was the same pattern shared by two different OTUs. In addition, all strains of the same OTU belonging to different samples or compartments never shared an identical profile. Clonality was only observed among the strains of the same OTU isolated in the same location. OTUs were found with monoclonal subpopulations (e.g. OTU 6—*R. halodurans* and OTU 24—*H. elongata*) or high percentage clonality (e.g. OTUs 13 and 29–*S. halophilus* for PI; Supplementary Table S5). However, in most cases (60%) OTUs were formed by subpopulations with no clonality (Supplementary Table S5).

Direct pyrosequencing, OTU affiliation and OPU distribution

A culture-free analysis was performed for the endophytic microbiota fraction so it could be compared with the cultured diversity. In order to enrich the prokaryotic endophyte fraction, a protocol of differential centrifugations was modified and improved using sucrose density gradients (as detailed in the Materials and methods section). Pyrosequencing analysis of the amplified 16S rRNA gene generated a total of 14,189 sequences ranging between 1472 (SF) and 5659 (LV). The mean length of the sequences was 540 bp, as all fragments with size <300 nucleotides were removed. After trimming, the sequence set was reduced to ~76% of the total (Table 3). Sequences affiliating with mitochondria (<6.5%) and chloroplasts (<0.2%) were removed. A total of 465 OTUs (unique clusters of sequences embraced by a minimum threshold of 99% identity, Table 3) were obtained, and for each location the number ranged from a minimum of 56 (PI) to a maximum of 123 (LV). After phylogenetic reconstruction, the diversity of pyrotags was reduced to 103 OPUs (i.e. unique phylogenetic clades affiliating the new sequences with their references), including 28 singletons and 14 doubletons (Supplementary Table S4). Among these, 31 OPUs represented >90% of the sequence diversity, whereas the remaining 72 only generated values <0.8% in their corresponding sample (Table 1 and Supplementary Table S4). Most OPUs affiliated with *Proteobacteria* (64 OPUs comprising 91.7% of the total sequences), of which 32 affiliated with *Gammaproteobacteria* (84.3%), 19 with *Alphaproteobacteria* (3.7%), 7 with *Betaproteobacteria* (2.8%), 5 with *Deltaproteobacteria* (0.2%) and 1 with *Epsilonproteobacteria* (0.7%). *Bacteroidetes* were represented by 7 OPUs (1.0%), *Acidobacteria* (0.2%) by 3 OPUs, *Chlamydiae* (0.01%) by 1 OPU, *Deinococcus-Thermus* (0.1%) by 1 OPU, *Firmicutes* (5.7%) by 18 OPUs, *Armatimonadetes* (0.3%) by 2 OPUs and *Actinobacteria* (1.0%) by 7 OPUs (Table 1 and Supplementary Table S4).

The most representative OPUs (Table 1) due to their abundances and common occurrences were *Alkalibacillus salilacus* (OPU 33), *C. canadensis* (OPU 1), *Halomonas* spp. (OPUs 5, 6 and 7) and *Kushneria* spp. (OPU 10). The first OPU was only detected by pyrosequencing, and the latter three were also present in the culture collection. Finally, two OPUs affiliating with *Pseudomonas* (OPUs 12 and 13) were present in high percentages in the Chilean samples, but were nearly absent in the Mediterranean samples. Among 103 OPUs detected, only 19 (OPUs 1, 3, 5, 6, 10, 15, 16, 17, 21, 29, 30, 31, 33, 35, 36, 81, 95, 99 and 103) were detected in all locations, and they occurred with a remarkably high number of sequences representing 70.3% of the total number of pyrosequencing sequences, with a mean of 70.3% ± 37.1% per location (Table 1 and Supplementary Table S4). In fact, no distribution pattern (Supplementary Fig. S4) seemed to be associated with the microbial community in relation to the location. The richness estimator index (Chao 1 in Table 2) ranged from 53 (PI) to 68.5 (SF), and the rarefaction curves were shown to be close to saturation in all but SF, which seemed to harbor higher diversity (Supplementary Fig. S3). Accordingly, the diversity (H') index and evenness (E) were shown to be higher in SF (2.53 and 0.22), and lower in PI (0.52 and 0.04) and LV (1.64 and 0.12). Dominance (D) presented an inverse behavior in comparison to the H' and E indexes (Table 2).

Comparison between culturable and pyrosequencing endophytic data

Endophytic microbiota could be considered as authentically autochthonous and not incidentally occurring. In this regard, among the 11 OPUs that constituted the endophytic cultivable fraction (Fig. 3), 9 were detected by pyrosequencing (Table 1), and only the rare OPUs 32 (singleton, affiliating with *H. kuroshimensis*) and 7 (affiliating with *H. elongata*) were not detected by the pyrotagging approach. Almost all sequences of cultured OPUs matched their closest relatives (at the genus level) with a high identity (above 97%) obtained by pyrosequencing. Both pyrosequencing and culture data coincided with the principal OPUs, which affiliated with *Proteobacteria* and gave similar abundances (91.7–88.2%; pyrosequencing-culture). Also, *Firmicutes* (5.7–6.3%; pyrosequencing-culture) and *Actinobacteria* (1.0–4.7%; pyrosequencing-culture), showed similar occurrences (Table 1 and Fig. 3). Approximately 45.0% of the pyrosequences corresponded to the cultured taxa *C. canadensis* (24.5%), *S. halophilus* (1.1%), *S. salarius* (0.09%), *Halomonas* spp. (7.9%), *Kushneria* spp. (8.0%), *Staphylococcus* spp. (0.5%), *Marinococcus* spp. (0.7%) and *B. permense* (0.8%) (Table 1). The PCA based on OPUs (Supplementary Fig. S4) did not show any association between pattern and origin of the sample in both the culturable and pyrosequencing fractions. However, in both cases, CA seemed to be slightly different in relation to other samples.

Discussion

To our knowledge, this is the first report of halophilic microorganisms inhabiting the endophyllosphere of halophytes that combines culture-dependent and -independent approaches. Contrary to other studies that reported values of 10^2 to 10^5 CFU g^{-1} [2,19,35] for bulk endo- and epiphytes, and specifically 10^4 to

10^5 CFU g^{-1} for epiphytes [18] and 10^5 CFU g^{-1} for endophytes [17], we could retrieve culturable yields up to two orders of magnitude higher (10^2 to 10^6 CFU g^{-1} for epiphytes and 10^3 to 10^7 for endophytes). Conspicuously, our data more closely resembled the abundances obtained in the rhizosphere, where ranges from 10^4 to 10^{10} CFU g^{-1} have been reported for soybean [32] or *Salicornia* [40]. The results suggested that the halophytes studied here were mainly colonized by moderate halophiles (5–20% salt; 95% of total counts), and to a lesser extent halotolerant (2–5% salt; 3.1% of total counts) or extreme halophiles (20–30% salt; 1.9% of total counts; Supplementary Table S6), in accordance with what has been reported by Mapelli et al. for the *Salicornia* rhizosphere [40].

Culturable and uncultured diversity measured by means of sequence identities showed high consistency, and the paradigm considering that culture-based techniques recover only 1% or less of the true prokaryotic diversity [3] failed. By culture, we could recover a maximum of 62.7% (LV sample) of the pyrosequenced diversity. In addition, from the 38 most relevant OPUs (representing >90% of the diversity), 11 of them were recovered as endophytic culturable microbiota. Only two cultured taxa (OPU 7 and OPU 32 affiliating with *H. elongata* and *H. kuroshimensis*, respectively) were not detected by pyrosequencing, and were possibly part of the rare biosphere [46]. The success in mirroring the most abundant taxa detection for the cultivable and culture-independent fractions was also reinforced by the fact that >95% of the pyrotagging sequences affiliated at the genus level (>94.5% similarity between sequences) [69] with known taxa. Our results contrast with other culture-independent studies that showed a significant percentage of sequences remained unclassified [7,63]. The success of the current study may be related to the fact that most of the microbiota thriving in the phylloplane is readily cultivable [26,56], but also because the identification approach used relied on phylogenetic inference (by means of the OPU approach) [21] and not just on sequence identity clustering (classification by means of OTU clustering and blast matches of their representatives), as commonly used [7,17,26].

Proteobacteria, *Firmicutes* and *Actinobacteria* were the most abundant bacterial phyla associated with the plants studied in this work, which seem to be common in foliar and root areas [7,53]. The principal OPUs in this study (both determined by culture and pyrosequencing) had been previously observed associated with other plants (e.g. the *Cupriavidus* genus) [14], including halophyte species [56]. Some of the genera detected here had been observed associated with the rhizosphere of halophytes, such as *Brevibacterium*, *Chromohalobacter*, *Halobacillus*, *Halomonas*, *Kushneria*, *Marinococcus*, *Zunongwangia* [2,23,28,40,58,59]. Only one archaeal species was detected, which always occurred as epiphytic and affiliated with *Hcc. hamelinensis*. Similarly, *Halococcus* spp. was reported to thrive in the rhizosphere of *Halocnemum strobilaceum* [2]. On the other hand, species such as *A. salilacus* [27], *R. cellulosilytica* [66] and *S. dokdonensis* [6] have not been reported previously in association with plants, but have been isolated from hypersaline environments.

Despite the fact that the plants originated from very different geographical locations, a common trend was observed between the same compartments of the different plants as an indication that there might be host specificity for the moderate halophiles detected. Contrarily to what has been observed for the phyllosphere of *Tamarix* [19], we found shared species as major microbiota between plants of different origins. However, identical clonal varieties of the same species shared by two different plants were not found. RADP fingerprinting showed a very low degree of clonality, and when clonality was observed (only in 14 OTUs) it always occurred in the same plant. Different clonal varieties may be assumed to be different ecotypes that, despite colonizing the same environment, may exhibit distinct phenotypes with distinct ecologic advantages [5]. On the other hand, clear differences were found between both compartments in the same plant, which may reflect the different environmental and nutritional conditions between the plant surface and inside the vegetal fraction. While the external part of the plant is subjected to pressures such as pluvial precipitation, solar radiation and wind, internal tissues represent relatively stable environments. Therefore, epibionts need strategies for surviving on plant surfaces, such as the formation of mats [41] or the production of biologically active compounds and generation of microcolonies [52].

Finally, halotolerant and halophylic microorganisms might be an important resource that could help the crop production systems where soils or irrigation waters contain high salt concentrations [56]. In fact, members of *Chromohalobacter*, *Halomonas*, *Halobacillus* and *Kushneria* species have been reported with plant growth promoting activity under salt stress (up to 320 mM NaCl) [16,40,50], and they are expected to be useful in microbial-assisted phytoremediation of saline soils [43]. Consequently, other microorganisms (i.e. *Salinicola*, *Pseudomonas*, *Roseivivax* and *Marinococcus*) isolated in this study could also have positive effects and their relevance in plant development should be addressed in the future.

Acknowledgements

This study was funded by the Spanish Ministry of Economy project CGL2012-39627-C03-03, which was also supported with European Regional Development Fund (FEDER) funds, and the preparatory phase of the Microbial Resource Research Infrastructure (MIRRI) funded by the EU (grant number 312251). MMR's PhD was supported by fellowship CVU 265934 of the National Council of Science and Technology (CONACyT), Mexico. CGD was funded by grant FPI-INIA-2012. FFV was founded by fellowship Javier Benedí 2014 of the IMEDEA UIB-CSIC. The authors thank Joan Rita (Universitat de les Illes Balears) for useful advice and for important suggestions, and Salines de Campos (Oliver Baker) and Salinas de Lo Valdivia (Alejandro Chaparro, Sal de Mar and Turismo Pacífico Central SpA) for access to their installations.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.syapm.2015.05.004

References

[1] Ahmad, P., Sarwat, M., Sharma, S. (2008) Reactive oxygen species, antioxidants and signaling in plants. J. Plant. Biol. 51, 167–173.
[2] Al-Mailem, D., Sorkhoh, N., Marafie, M., Al-Awadhi, H., Eliyas, M., Radwan, S. (2010) Oil phytoremediation potential of hypersaline coasts of the Arabian Gulf using rhizosphere technology. Bioresour. Technol. 101, 5786–5792.
[3] Amann, R., Ludwig, W., Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. 59, 143–169.
[4] Andrews, J.H., Harris, R.F. (2000) The ecology and biogeography of microorganisms on plant surfaces. Annu. Rev. Phytopathol. 38, 145–180.
[5] Antón, J., Lucio, M., Peña, A., Cifuentes, A., Brito-Echeverría, J., Moritz, F., Tziotis, D., López, C., Urdiain, M., Schmitt-Kopplin, P., Rosselló-Móra, R. (2013) High metabolomic microdiversity within co-occurring isolates of the extremely halophilic bacterium *Salinibacter ruber*. PLoS ONE 8 (5), e64701.
[6] Bae, G.D., Hwang, C.Y., Kim, H.M., Cho, B.C. (2010) *Salinisphaera dokdonensis* sp. nov. isolated from surface seawater. Int. J. Syst. Evol. Microbiol. 60, 680–685.
[7] Bodenhausen, N., Horton, M.W., Bergelson, J. (2013) Bacterial communities associated with the leaves and the roots of *Arabidopsis thaliana*. PLoS ONE 8, e56329.
[8] Bomberg, M., Jurgens, G., Saano, A., Sen, R., Timonen, S. (2003) Nested PCR detection of *Archaea* in defined compartments of pine mycorrhizospheres developed in boreal forest humus microcosms. FEMS Microbiol. Ecol. 43, 163–171.
[9] Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Peña, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T.,

Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.
[10] Chase, M.W., Hills, H.H. (1991) Silica gel: an ideal material for field preservation of leaf samples for DNA studies. Taxon 40, 215–220.
[11] Cocking, E.C. (2003) Endophytic colonization of plant roots by nitrogen-fixing bacteria. Plant Soil 252, 169–175.
[12] Compant, S., Duffy, B., Nowak, J., Clement, C., Barka, E.A. (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol. 71, 4951–4959.
[13] Conrad, R., Klose, M., Noll, M., Kemnitz, D., Bodelier, P.L. (2008) Soil type links microbial colonization of rice roots to methane emission. Global Change Biol. 14, 657–669.
[14] Da Silva, K., Florentino, L.A., Da Silva, K.B., De Brandt, E., Vandamme, P., de Souza Moreira, F.M. (2012) Cupriavidus necator isolates are able to fix nitrogen in symbiosis with different legume species. Syst. Appl. Microbiol. 35, 175–182.
[15] De Vere, N., Rich, T.C., Ford, C.R., Trinder, S.A., Long, C., Moore, C.W., Satterthwaite, D., Davies, H., Allainguillaume, J., Ronca, S., Tatarinova, T., Garbett, H., Walker, K., Wilkinson, M.J. (2012) DNA barcoding the native flowering plants and conifers of Wales. PLoS ONE 7, e37945.
[16] Desale, P., Patel, B., Singh, S., Malhotra, A., Nawani, N. (2013) Plant growth promoting properties of *Halobacillus* sp. and *Halomonas* sp. in presence of salinity and heavy metals. J. Basic Microbiol. 54, 781–791.
[17] Emiliani, G., Mengoni, A., Maida, I., Perrin, E., Chiellini, C., Fondi, M., Gallo, E., Gori, L., Maggini, V., Vannacci, A., Biffi, S., Firenzuoli, F., Fani, R. (2014) Linking bacterial endophytic communities to essential oils: clues from *Lavandula angustifolia* Mill. J. Evidence Based Complement. Altern. Med. 2014, 16pages.
[18] Ercolani, G. (1991) Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. Microb. Ecol. 21, 35–48.
[19] Finkel, O.M., Burch, A.Y., Lindow, S.E., Post, A.F., Belkin, S. (2011) Geographical location determines the population structure in phyllosphere microbial communities of a salt-excreting desert tree. Appl. Environ. Microbiol. 77, 7647–7655.
[20] Flowers, T.J., Colmer, T.D. (2008) Salinity tolerance in halophytes. New Phytol. 179, 945–963.
[21] França, L., Lopéz-Lopéz, A., Rosselló-Móra, R., Costa, M.S. (2015) Microbial diversity and dynamics of a groundwater and a still bottled natural mineral water. Environ. Microbiol. 17, 577–593.
[22] Franche, C., Lindström, K., Elmerich, C. (2009) Nitrogen-fixing bacteria associated with leguminous and non-leguminous plants. Plant Soil 321, 35–59.
[23] Gontia, I., Kavita, K., Schmid, M., Hartmann, A., Jha, B. (2011) *Brachybacterium saurashtrense* sp. nov., a halotolerant root-associated bacterium with plant growth-promoting potential. Int. J. Syst. Evol. Microbiol. 61, 2799–2804.
[24] Hammer, Ø., Harper, D.A. 2008 Paleontological Data Analysis, John Wiley and Sons, Malden, USA.
[25] Hardoim, P.R., van Overbeek, L.S., Elsas, J.D. (2008) Properties of bacterial endophytes and their proposed role in plant growth. Trends Microbiol. 16, 463–471.
[26] Jackson, C.R., Randolph, K.C., Osborn, S.L., Tyler, H.L. (2013) Culture dependent and independent analysis of bacterial communities associated with commercial salad leaf vegetables. BMC Microbiol. 13, 274.
[27] Jeon, C.O., Lim, J.M., Lee, J.M., Xu, L.H., Jiang, C.L., Kim, C.J. (2005) Reclassification of *Bacillus haloalkaliphilus* Fritze 1996 as *Alkalibacillus haloalkaliphilus* gen. nov., comb. nov. and the description of *Alkalibacillus salilacus* sp. nov., a novel halophilic bacterium isolated from a salt lake in China. Int. J. Syst. Evol. Microbiol. 55, 1891–1896.
[28] Jha, B., Gontia, I., Hartmann, A. (2012) The roots of the halophyte *Salicornia brachiata* are a source of new halotolerant diazotrophic bacteria with plant growth-promoting potential. Plant Soil 356, 265–277.
[29] Knief, C., Ramette, A., Frances, L., Alonso-Blanco, C., Vorholt, J.A. (2010) Site and plant species are important determinants of the *Methylobacterium* community composition in the plant phyllosphere. ISME J. 4, 719–728.
[30] Kosová, K., Vítámvás, P., Urban, M.O., Prášil, I.T. (2013) Plant proteome responses to salinity stress–comparison of glycophytes and halophytes. Funct. Plant Biol. 40, 775–786.
[31] Kruskal, J., Wish, M. 1978 Multidimensional Scaling. Sage University Paper Series on Quantitative Applications in the Social Sciences No. 11, Sage Publications, Beverly Hills, CA.
[32] Kuklinsky-Sobral, J., Araújo, W.L., Mendes, R., Geraldi, I.O., Pizzirani-Kleiner, A.A., Azevedo, J.L. (2004) Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. Environ. Microbiol. 6, 1244–1251.
[33] Lê, S., Josse, J., Husson, F. (2008) FactoMineR: an R package for multivariate analysis. J. Stat. Softw. 25, 1–18.
[34] Li, H., Luo, J., Hemphill, J.K., Wang, J.-T., Gould, J.H. (2001) A rapid and high yielding DNA Miniprep for cotton (*Gossypium* spp.). Plant Mol. Biol. Rep. 19, 1–5.
[35] Lindow, S.E., Andersen, G.L. (1996) Influence of immigration on epiphytic bacterial populations on navel orange leaves. Appl. Environ. Microbiol. 62, 2978–2987.
[36] Lindow, S.E., Brandl, M.T. (2003) Microbiology of the phyllosphere. Appl. Environ. Microbiol. 69, 1875–1883.
[37] Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E.R., Taghavi, S., Mezgeay, M., der Lelie, D.v (2002) Endophytic bacteria and their potential applications. Crit. Rev. Plant Sci. 21, 583–606.
[38] Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A.W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., Schleifer, K.H. (2004) ARB: a software environment for sequence data. Nucleic Acids Res. 32, 1363–1371.
[39] Lugtenberg, B., Kamilova, F. (2009) Plant-growth-promoting rhizobacteria. Annu. Rev. Microbiol. 63, 541–556.
[40] Mapelli, F., Marasco, R., Rolli, E., Barbato, M., Cherif, H., Guesmi, A., Ouzari, I., Daffonchio, D., Borin, S. (2013) Potential for plant growth promotion of Rhizobacteria associated with *Salicornia* growing in Tunisian hypersaline soils. Biomed. Res. Int. 2013, 13pages.
[41] Morris, P., Soule, D. (2004) The potential role of microbial activity and mineralization in exoseletal development in Microporellidae. In: Cancino, M., Jackson, W. (Eds.), Bryozoan Studies 2004, Taylor & Francis Group, London, pp. 181–186.
[42] Nagarajkumar, M., Bhaskaran, R., Velazhahan, R. (2004) Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluorescens* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. Microbiol. Res. 159, 73–81.
[43] Newman, L.A., Reynolds, C.M. (2005) Bacteria and phytoremediation: new uses for endophytic bacteria in plants. Trends Biotechnol. 23, 6–8.
[44] Ochsenreiter, T., Selezi, D., Quaiser, A., Bonch-Osmolovskaya, L., Schleper, C. (2003) Diversity and abundance of *Crenarchaeota* in terrestrial habitats studied by 16S RNA surveys and real time PCR. Environ. Microbiol. 5, 787–797.
[45] Oksanen, J. 2011 Multivariate analysis of ecological communities in R: vegan tutorial. In: R Package Version 1 [WWW document]. URL http://cc.oulu.fi/~jarioksa/opetus/metodi/vegantutor.pdf [accessed on 20 August 2011].
[46] Pedrós-Alió, C. (2006) Marine microbial diversity: can it be determined? Trends Microbiol. 14, 257–263.
[47] Peña, A., Valens, M., Santos, F., Buczolits, S., Antón, J., Kämpfer, P., Busse, H.J., Amann, R., Rosselló-Mora, R. (2005) Intraspecific comparative analysis of the species *Salinibacter ruber*. Extremophiles 9, 151–161.
[48] Quispel, A. (1992) A search for signals in endophytic microorganisms. In: Verma, D.P.S. (Ed.), Molecular Signals in Plant-Microbe Communications, CRS Press, Boca Raton, FL, pp. 471–490.
[49] Radyukina, N., Kartashov, A., Ivanov, Y.V., Shevyakova, N., Kuznetsov, V.V. (2007) Functioning of defense systems in halophytes and glycophytes under progressing salinity. Russ. J. Plant. Physiol. 54, 806–815.
[50] Ramadoss, D., Lakkineni, V.K., Bose, P., Ali, S., Annapurna, K. (2013) Mitigation of salt stress in wheat seedlings by halotolerant bacteria isolated from saline habitats. SpringerPlus 2, 6.
[51] Ramakrishnan, B., Lueders, T., Dunfield, P.F., Conrad, R., Friedrich, M.W. (2001) Archaeal community structures in rice soils from different geographical regions before and after initiation of methane production. FEMS Microbiol. Ecol. 37, 175–186.
[52] Rao, D., Webb, J., Kjelleberg, S. (2005) Competitive interactions in mixed-species biofilms containing the marine bacterium *Pseudoalteromonas tunicata*. Appl. Environ. Microbiol. 71, 1729–1736.
[53] Rastogi, G., Sbodio, A., Tech, J.J., Suslow, T.V., Coaker, G.L., Leveau, J.H. (2012) Leaf microbiota in an agroecosystem: spatiotemporal variation in bacterial community composition on field-grown lettuce. ISME J. 6, 1812–1822.
[54] Reinhold-Hurek, B., Hurek, T. (2011) Living inside plants: bacterial endophytes. Curr. Opin. Plant Biol. 14, 435–443.
[55] Rodríguez-Valera, F., Ventosa, A., Juez, G., Imhoff, J.F. (1985) Variation of environmental features and microbial populations with salt concentrations in a multi-pond saltern. Microbiol. Ecol. 11, 107–115.
[56] Ruppel, S., Franken, P., Witzel, K. (2013) Properties of the halophyte microbiome and their implications for plant salt tolerance. Funct. Plant Biol. 40, 940–951.
[57] Schloss, P.D., Westcott, S.L., Ryabin, T., Hall, J.R., Hartmann, M., Hollister, E.B., Lesniewski, R.A., Oakley, B.B., Parks, D.H., Robinson, C.J., Sahl, J.W., Stres, B., Thallinger, G.G., Van Horn, D.J., Weber, C.F. (2009) Introducing Mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl. Environ. Microbiol. 75, 7537–7541.
[58] Sgroy, V., Cassán, F., Masciarelli, O., Del Papa, M.F., Lagares, A., Luna, V. (2009) Isolation and characterization of endophytic plant growth-promoting (PGPB) or stress homeostasis-regulating (PSHB) bacteria associated to the halophyte *Prosopis strombulifera*. Appl. Microbiol. Biotechnol. 85, 371–381.
[59] Shiba, T., Shioi, Y., Takamiya, K., Sutton, D.C., Wilkinson, C.R. (1991) Distribution and physiology of aerobic bacteria containing bacteriochlorophyll *a* on the east and west coasts of Australia. Appl. Environ. Microbiol. 57, 295–300.
[60] Simon, R.D., Abeliovich, A., Belkin, S. (1994) A novel terrestrial halophilic environment: the phylloplane of *Atriplex halimus*, a salt-excreting plant. FEMS Microbiol. Ecol. 14, 99–110.
[61] Stackebrandt, E., Ebers, J. (2006) Taxonomic parameters revisited: tarnished gold standards. Microbiol. Today 33, 152–155.
[62] Sturrock, K., Rocha, J. (2000) A multidimensional scaling stress evaluation table. Field Methods 12, 49–60.
[63] Vaz-Moreira, I., Egas, C., Nunes, O.C., Manaia, C.M. (2011) Culture-dependent and culture-independent diversity surveys target different bacteria: a case study in a freshwater sample. Antonie van Leeuwenhoek 100, 245–257.

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[64] Vessey, J.K. (2003) Plant growth promoting rhizobacteria as biofertilizers. Plant Soil 255, 571–586.
[65] Viver, T., Cifuentes, A., Díaz, S., Rodríguez-Valdecantos, G., González, B., Antón, J., Rosselló-Móra, R. (2015) Diversity of extremely halophilic cultivable prokaryotes in Mediterranean, Atlantic and Pacific solar salterns: evidence that unexplored sites constitute sources of cultivable novelty. Syst. Appl. Microbiol. 38, 266–275.
[66] Weon, H.Y., Yoo, S.H., Kim, Y.J., Lee, C.M., Kim, B.Y., Jeon, Y.A., Hong, S.B., Anandham, R., Kwon, S.W. (2009) *Rudaea cellulosilytica* gen. nov., sp. nov., isolated from soil. Int. J. Syst. Evol. Microbiol. 59, 2308–2312.
[67] Wilson, D. (1995) Endophyte: the evolution of a term, and clarification of its use and definition. Oikos 73, 274–276.
[68] Wilson, L., Matsudaira, P.T., Galbraith, D.W., Bourque, D.P., Bohnert, H.J. 1995 Methods in plant cell biology, Academic Press, New York.
[69] Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F.O., Ludwig, W., Schleifer, K., Whitman, W.B., Euzéby, J., Amann, R., Rosselló-Móra, R. (2014) Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. Nat. Rev. Microbiol. 12, 635–645.

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