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

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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).

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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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[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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