BACTERIAL COMMUNITY OF RHIZOSPHERE ASSOCIATED TO THE ANNUAL HALOPHYTE *Salicornia bigelovii* (Torr.)

Comunidad Bacteriana de la Rizosfera Asociada a la Halófita Anual *Salicornia bigelovii* (Torr.)

Edgar O. Rueda-Puente[1‡], Thelma Castellanos-Cervantes[2], José Luis Díaz de León-Álvarez[3], Pablo Preciado-Rangel[4] and Gustavo Almaguer-Vargas[5]

SUMMARY

The specie *Salicornia bigelovii* (Chenopodiaceae) is a halophyte with promising biological and commercial value as a natural resource from arid zones. However, its productivity depends on the nitrogen supply. Nitrogen fixation by bacteria associated with the roots of *S. bigelovii* is a determinant source of available nitrogen. However, the diversity in *Salicornia*'s rhizosphere is unknown. In order to increase the knowledge of the bacterial diversity associated with the *S. bigelovii* rhizosphere, polymerase chain reaction (PCR) and single-strand conformation polymorphism (SSCP) analysis was performed. This study was conducted in La Paz, BCS, Mexico. A random sample of 25 *S. bigelovii* plants at the flowering stage was collected from 6 areas along the coastal line of La Paz Bay in the southern portion of the Baja California Peninsula. DNA extractions of bacterial DNA through amplification (PCR) of the 16S rDNA, SSCP-profiles analysis, isolation of selected bands of SSCP-profiles, cloning, and sequences were carried out. The SSCP technique reflected the bacterial composition evidencing the available diversity and also the dominant populations. The results obtained reflect the presence of *Rhizobium* spp. and *Bacillus* spp. This is the first report of these microorganisms associated with *S. bigelovii*, a novel halophyte crop. Further studies on diversity of the microbial community in the *Salicornia bigelovii* rhizosphere are required to clarify and describe this ecological association under saline conditions.

Index words: *plant growth-promoting bacteria (PGPB), biofertilizer, soil ecology.*

RESUMEN

La especie *Salicornia bigelovii* (Chenopodiaceae) es una halófita con un promisorio valor biológico y comercial en zonas áridas. Sin embargo, su productividad depende de la aportación de nitrógeno. La fijación de nitrógeno por bacterias asociadas a la raíz de *S. bigelovii*, es una fuente determinante para disponer de nitrógeno. Sin embargo, la diversidad de estos microorganismos en *Salicornia* se desconoce. Por lo anterior, el objetivo del presente trabajo consistió en conocer la comunidad bacteriana, asociada a la rizósfera de *S. bigelovii*, mediante la técnica de reacción en cadena de la polimerasa (PCR) y la técnica de conformación polimórfica de cadena simple (SSCP). El estudio fue desarrollado en Bahía de La Paz, BCS México, mediante un muestreo al azar de 25 plantas de *S. bigelovii* en etapa fenológica de floración, las cuales fueron colectadas de 6 áreas del sistema costero. Se realizaron extracciones de ADN de la población bacteriana a través de amplificación por PCR del 16 S rDNA, análisis mediante SSCP, aislamiento de bandas seleccionadas de SSCP, clonación y, secuenciación. Los resultados mostraron que la técnica SSCP evidencia una diversa composición bacteriana con poblaciones dominantes de las cuales los resultados obtenidos indican la presencia de *Rhizobium* spp. y *Bacillus* spp. Este es el primer informe de este tipo de microorganismos asociados a *S. bigelovii*. Estudios relacionados con la diversidad microbiana en rizósfera de *Salicornia bigelovii* deben ser desarrollados para clarificar y describir las asociaciones ecológicas presentes bajo condiciones de salinidad.

[1] Universidad de Sonora, Campus Santa Ana. Carretera Internacional y Avenida 16 de Septiembre s/n, Col. La Loma. 84600 Ciudad Santa Ana, Sonora, México.
‡ Autor responsable (erueda04@santana.uson.mx)
[2] Centro de Investigaciones Biológicas del Noroeste. Mar Bermejo No. 195, Col. Playa Palo Santa Rita. Apartado Postal 128. 23090. La Paz, Baja California Sur, México.
[3] Universidad Autónoma de Baja California Sur. Apartado Postal 128. 23080 La Paz, Baja California Sur, México.
[4] Instituto Tecnológico de Torreón. km 7.5 carretera Torreón-San Pedro. 27170 ejido Ana, Torreón, Coahuila, México.
[5] Universidad Autónoma Chapingo. 56230 Chapingo, estado de México.

Recibido: abril del 2010. Aceptado: noviembre de 2010.
Publicado como nota de investigación en
Terra Latinoamericana 28: 345-353.

Palabras clave: *bacterias promotoras del crecimiento vegetal (BPCV), biofertilizantes, ecología del suelo.*

INTRODUCTION

Halophytes, particularly *Salicornia bigelovii* (Chenopodiaceae), are promising plant resources in arid coastal zones because they are able to tolerate high saline conditions. Such salt-adapted plant resources are commonly found along coastal plains (Glenn *et al*., 1991). These important plants hold the potential to be incorporated into traditional agriculture to help support the agricultural economy in coastal areas (Glenn *et al*., 1995). In the states of Baja California Sur and Sonora, Mexico, *S. bigelovii* is widely distributed along the coasts. It has been suggested elsewhere that this halophyte has real prospects for commercial exploitation (Glenn *et al*., 1994; Troyo *et al*., 1994). However, in the southern part of these states, halophyte productivity is limited by a lack of available nitrogen. This condition affects growth, reproduction, and nutrient nitrogen levels within the plant biomass (Rueda *et al.,* 2004). Traditionally, to solve soil nitrogen deficiency, farmers apply synthetic fertilizers, such as urea, ammonium sulfate, and others. However, their indiscriminate use severely damages soil microbiological flora (Banwari and Rao, 1990; Al-Nahidh and Gomah, 1991; Akhavan *et al*., 1991), and also gradually increases the level of soil salinity.

Another option to improve the availability of nitrogen is the use of plant-growth-promoting-bacteria (PGPB), which are gaining acceptance worldwide; a large number of bacterial strains have been isolated and evaluated primarily for improving plant growth (Kloeper *et al*., 1991). Strains that fail to demonstrate a positive effect or that induce a negative plant response are routinely discarded and their negative effects are seldom reported (Nehl *et al*., 1997).

These strains can be beneficial or harmful (Nehl *et al*., 1997), saprophytic becoming pathogenic by changing the host, or any of these under different microbe-host relationships (Pimentel *et al*., 1991). Some strains may exhibit beneficial effects on one plant species but may exert harmful or variable effects on others (O'neil *et al*., 1992). Because of the variability in strain's features, some of the groups or strains do not hold properties that enhance growth in some plant species. In this sense, it becomes relevant to know what kind of bacterial population is present in the plant rhizosphere to isolate them for inoculation in other individuals or populations of the same species. For multiple environmental and economic purposes, it is important to increase the populations of salt-tolerant, nitrogen-fixing bacteria (Zahran, 1999), so that potential novel crops, such as *Salicornia bigelovii*, which has already been considered a new plant resource in arid region agriculture (Bashan *et al*., 1992), can thrive.

Few studies on soil ecology and bacterial diversity within the rhizosphere of salt marsh plants are known and available. In previous studies, one nitrogen fixing bacterium was isolated from *Salicornia bigelovii* rhizosphere through conventional techniques, using an N-free media: OAB (Reinhold *et al.,* 1987) and Rennie's medium (Rennie, 1981), evidencing *Klebsiella pneumoniae* as a potential plant-growth-promoting-rhizobacterium (Rueda *et al*., 2003; 2004). Other reported studies were limited to the study of mycoflora, mainly *S. europaea* (Ito *et al*., 1999).

This work focused on the composition of the natural bacterial diversity inhabiting the rhizosphere of the halophyte *Salicornia bigelovii,* by means of the single-strand conformation polymorphism (SSCP) technique.

MATERIALS AND METHODS

Microbial DNA Isolation

Plant and soil samples of 26 plants were collected at flowering stage from six areas along the southern shore of La Paz Bay, in Baja California Sur, northwest Mexico, 17 km west of La Paz City (sample 1: Latitude 24° 8' 16.36" N, Longitude 110° 25' 33.92" O; sample 2: Latitude 24° 8' 14.05" N, Longitude 110° 25' 32.19" O; sample 3: Latitude 24° 7' 55.56" N, Longitude 110° 25' 8.10" O; sample 4: Latitude 24° 7' 50.44" N, Longitude 110° 25' 12.44" O; sample 5: Latitude 24° 7' 48.88" N, Longitude 110° 25' 6.31" O; sample 6: Latitude 24° 7' 30.17" N, Longitude 110° 25' 15.36" O).

Conventional soil and plant physical-chemical analysis were conducted to determine texture class (using Bouyoucos densimeter), organic matter concentration (by titration with ferrous sulfate) salinity of saturated paste (using the Weatston electrical conductivity bridge), sodium balance expressed as SAR (numerical calculation of the sodium adsorption relationship parameter, quantifying Ca^{2+} and Mg^{2+} by EDTA method), and the concentration of major nutrients through bromatological analysis. The general characteristics of the soil present in the six areas sampled are cited in

Table 1. La Paz Bay has different geographical and climatic conditions. The Bay area and the coast are desert, warm (19 ± 4 °C) in the summer and cool in winter (10-17 °C). During the year, precipitation is low (3 mm in February, 5 mm in August and 28 mm in September).

Samples were placed in black plastic bags to prevent an increase in saprophytic microorganisms and labeled with date and location of collection. During the collection process, plants were kept on on ice in a recipient for five hours and then processed at Microbiol and Ecology Lab in the "Centro de Investigaciones Biológicas del Noroeste, S. C." in order to obtain microbial DNA associated with the salicornia rhizosphere . Roots were washed carefully with saline solution at 0.85% of NaCl to detach the adhered soil, avoiding possible damage to the lateral roots. Roots were cut into fragments of 2, 3 and 4 cm. The root pieces were placed in tubes containing 20 mL of NaCl (0.85%) and centrifuged at 5000 g for 3 min at 4 °C, to separate roots from the cellular microbial package according to Ausubel *et al*. (2002). The bacterial cell pellets were lysed with five subsequent cycles of freeze-thawing in liquid nitrogen followed by proteinase K (16 mg mL^{-1}) and incubated at 65 °C during 7 min. From the tubes, 8 mL were taken and settled in 20 mL Eppendorf tubes, to which 800 µL of alcohol phenol - chloroform isoamylic (25:24:1) was added.

The floating particles were removed at 269 g during 10 min at 4 °C. The top phase (600 µL) was transferred to 1.6 mL Eppendorf tubes, and 600 µL of chloroform:isoamylic alcohol (24:1) were added and centrifuged at 10 700 g during 5 min at 4 °C (Ausubel *et al*., 2002). Again, from the top phase of the tubes, 600 mL were transferred to other Eppendorf tubes, and 600 mL of iso-propanol or cold methanol were added. Tubes were kept at -20 °C during 24 h; then they were centrifuged at 19,810 g during 7 min. The tubes were placed on a table during 30 min to evaporate the alcohol. Finally, the DNA obtained was homogenized in 10 µL of buffer solution (TE) at pH 8 (Ausubel *et al*., 2002).

Once we obtained the microbial DNA, the SSCP technique was carried out according to Tebbe *et al*. (2001), which consisted in the PCR amplification of a specific region of the 16S rRNA gene using initiators (primers) non-phosphorilated and phosphorilated (Primer name Com1: CAG CAG CCG CGG TAA TAC; targeted microorganisms: bacteria; targeted regions of SSU rRNA gene: Variable region 4 and Variable region 5 (~407 bp size of amplification product). A digestion of the phosphorilated strand was performed by means of the enzyme exonuclease (LAMBDA) in order to obtain simple strands. Electrophoresis was then developed in an acrylamide gel (MDE 2X). Electrophoresis was carried out at 20 °C with 400 V during 16 h. Positive bands from known microorganisms, such as *Bacillus liquenifornis, Rhizobium trifolli, Flavobacterium jashmoniae* and *Agrobacterium tumefasciens*, were considered. Bands were revealed by the method of silver staining (Bassam *et al*., 1991). For the staining procedure we used cleaned stainless steel trays (18 / 8). The gel, attached to the cover glass, was transferred into a tray filled with 10% aqueous acetic acid solution and incubated for 30 min at room temperature with slow shaking (0.05 g) for fixation. The solution was then removed and the gel was washed for 5 min in distilled water. The washing procedure was repeated, and then the staining solution (1 g silver nitrate, 1.5 mL 37% formaldehyde [v/v] in 1 L of twice distilled water) was added. Gels were protected from light by a lid and incubated under slow shaking for 30 min at room temperature. The staining solution was removed and the gel was rinsed with double distilled water for 10 s. Then the gel was transferred to another tray and, in order to remove remnants of the staining solution, was washed with a small amount of developing solution [56.3 g sodium bicarbonate (decahydrate), 2 mL formaldehyde (37% v/v), and 1 mL sodium thiosulfate (0.2% v/v stock solution) in 1 L of water, stored at 8 °C] for 20 s.

Afterward, the gel was incubated in fresh developing solution with slow shaking at room temperature. The staining of DNA was observed and, when the patterns

Table 1. Recorded values for edaphic variables in a *Salicornia* sampled areas near La Paz, BCS, Mexico.

Variable	Unit	Minimum	Maximum	Mean
pH		8.08	8.59	8.33
Organic matter	%	0.11	1.38	0.74
Electrical conductivity	dS m^{-1}	12.20	22.00	17.10
Sodium adsorption		14.22	29.40	21.81
Sodium (Na^+)	meq L^{-1}	110.00	190.70	150.35
Potassium (K_2O)	kg ha^{-1}	222.00	774.00	498.00
Calcium (Ca^{2+})	meq L^{-1}	50	50	50
Magnesium (Mg^{2+})	meq L^{-1}	55	155	105
Phosphorous (P_2O_5)	kg ha^{-1}	50.00	54.00	52.00
Total nitrogen	%	0.03	0.06	0.03

become clearly visible, the developing procedure was stopped by transferring the gel to another tray filled with acetic acid solution (10% v/v).

Cloning-Sequence and Analysis of DNA Sequence

For sequence analysis, selected bands of the SSCP-profiles were cut from dried gel matrices and DNA was recovered. In this study we considered only seven bands that appeared on all samples but with different intensities, in order to have the most representative of the *Salicornia* rhizosphere. In this stage, the thick, narrow bands are evidence of criteria for selecting bands. For this study we considered thin and faint bands, since these appeared to be the ones that could provide us information about new microorganisms and how a bacterial population can be constituted (Castellanos, 2002).

The isolated bands were re-amplified by PCR (Tebbe *et al.*, 2001). Selected bands of SSCP-profiles were cut with a razor blade and transferred into a microfuge tube (total volume 1.5 mL). The gel slices were crushed with a disposable pipette tip against the wall of the tube and subsequently incubated with 50 µL of elution buffer (0.5 M ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0 and 0.1% dodecil sodium sulfate) at 37 °C for 3 h. For this purpose, we used a thermo-mixer for microfuge tubes at 34 g. Samples were centrifuged for sedimentation of the gel particles. A total of 40 µL were carefully transferred with a pipette to a fresh microfuge tube while avoiding possible co-transfer of gel particles. DNA was precipitated with 2 volumes of ethanol (96%), incubated for at least 2 h at -20 °C, and centrifuged at 24 000 × g for 15 min at 4 °C. The pellet containing DNA was dried for 15 min at 37 °C and re-dissolved in 10 µL of 0 mM Tris HCI (pH 8.0). The amplification of the extracted DNA molecules was performed with PCR using the same primers and conditions as those applied for the generation of community profiles. The PCR-products were purified as described above in the recovery of SSCP bands. To control the quality of the re-amplified products, a single strand was digested and the re-amplified products on SSCP-gels were compared with the original community profile.

Cloning was carried out according to Tebbe *et al.* (2001), using the pGEM-T vector system (Promega). Fragments of 500 bp were ligated into the vector and transformed into *Escherichia coli* JM109 competent cells. Clones with inserted sequences were detected by blue-white selection (β-galactosidase activity) on LB agar plates amended with ampicillin, IPTG and X-Gal, as described by the manufacturer´s protocol. White colonies were cultured on the same growth agar; from there, colonies were transferred with a toothpick to microfuge tubes (one colony per tube). The bacterial cells were treated with 50 µL lysis solution (0.05 M NaOH), 0.25% SDS) at 95 °C for 15 min with rotary agitation at 34 g. A total of 450 µL sterile double distilled water was added and vortexed. The suspension was then centrifuged at 10 000 × g for 4 min at room temperature. The PCR-insert amplification was performed with flanking vector primers matching positions 2952 to 2970 (forward) and complementary to positions 174 to 193 (reverse) in the pGEM-T sequences (see www.Promega.com). PCR conditions were identical to those described for re-amplification (total volume 50 µL), except that an annealing temperature of 53 °C was used. The size and amount of the amplified PCR product was analyzed by running 5-8 µL of the PCR solution by electrophoresis in agarose gels (1.2% in TBE). Amplification reactions, which yield products of the expected size, were purified (Qiaquick PCR Purification Kit, Qiagen) and used as a template in cycle-sequencing reactions.

For DNA sequencing, we used the SequiTherm EXCEL II DNA sequencing Kit-LC (Epicentre Technologies, Madison, WI). Primers labeled with an infrared sensitive dye were used since the subsequent sequence analysis was conducted with a Li-Cor system (Li-Cor, Lincoln, NE). Sequencing primers hybridize to vector sequences and are therefore widely applicable, regardless of the sequence of the inserted DNA (in contrast to direct sequencing). Positions of the sequencing primers were used, corresponding to positions 2960 to 2977 of the coding strand and positions 161 to 177 of the non-coding strand in the Laboratory of Institute of Agroecology of Johann Heinrich von Thunen Institut (vTI) in Braunschweig, Germany. The optimum annealing temperatures for the primers were 54 °C (2960-2977) and 50 °C (161-177), respectively. Primer concentrations of 80 nM and a DNA template concentration of 50 - 100 mg were added to the sequencing reactions. All other conditions correspond to the manufacturer's recommendations.

The analysis and interpretation of SSCP profiles were carried out at two levels: i) the pattern composition of profiles, and ii) identification of single components of a profile by DNA-sequencing and comparison to known sequences in databases (BLAST, Gen Bank,

Japan data base, GelCompar and WinCam). The direct result of community-SSCP is a banding pattern on a polyacrylamide gel. These bands become detectable after a silver staining procedure.

RESULTS AND DISCUSSION

With the methodology used in this study, results showed a diversity of microflora in the rhizosphere of *Salicornia bigelovii* (Figure 1), although sample 2 was not amplified.

The sequences of products amplified from a simple strand were compared to public data bases using Megablast al NCBI (http://www.ncbi.nim,nih.gov/BLAST) (Table 2). According to a dendrogram created (Figure 2), and with the purpose of seeing relationships resulting from grouping the data and even among the groups, the results indicated that the microbial community of sites 3 and 5 are similar; the same similarity is observed between sites 1 and 6. However, site 4 is more similar to sites 3 and 5. Thus, the successive subdivisions can give us an idea about how they can be grouped and the distance between the data according to established relationships.

Linking the evidences with previous experiments, the results obtained are a good example of what can be expected from the applied method: classical morphological characteristics as well as biochemical traits can be obtained from *Klebsiella pneumoniae* (Rueda *et al*., 2003). But based on SSCP, strains of *Rhizobium* spp., *Bacillus* spp., Cyanobacteria and Proteobacteria groups (Table 2) may be considered diazotrophic bacteria in association within the environments where the halophyte *Salicornia bigelovii* grows naturally, performing a role (although in low populations) related to the decomposition of organic matter, availability of nutrients, reduction of toxicity due to toxic metals, modification of pH (Table 1), and production or stimulation of plant growth substances or plant growth-promoting-substances (PGPS) (El-Shatanawi *et al*., 2001), serving as an ecological source of biologically available nitrogen in these systems (Hanson, 1983).

In this sense, diazotrophic bacteria are ubiquitous mashes, and according to evidences, nitrogen fixation is carried out exclusively by certain species of Bacteria and Archea (Postgate, 1998), with the highest rates of activity occurring on and around plant root surfaces (the rhizoplane and rhizosphere, respectively (Campbell and Greaves, 1990), which was also found in this study. On the other hand, we found that culture methods may not accurately reproduce microorganisms as they occur in natural habitats (Ward *et al*., 1998). The culture approach, however, is essential for the thorough

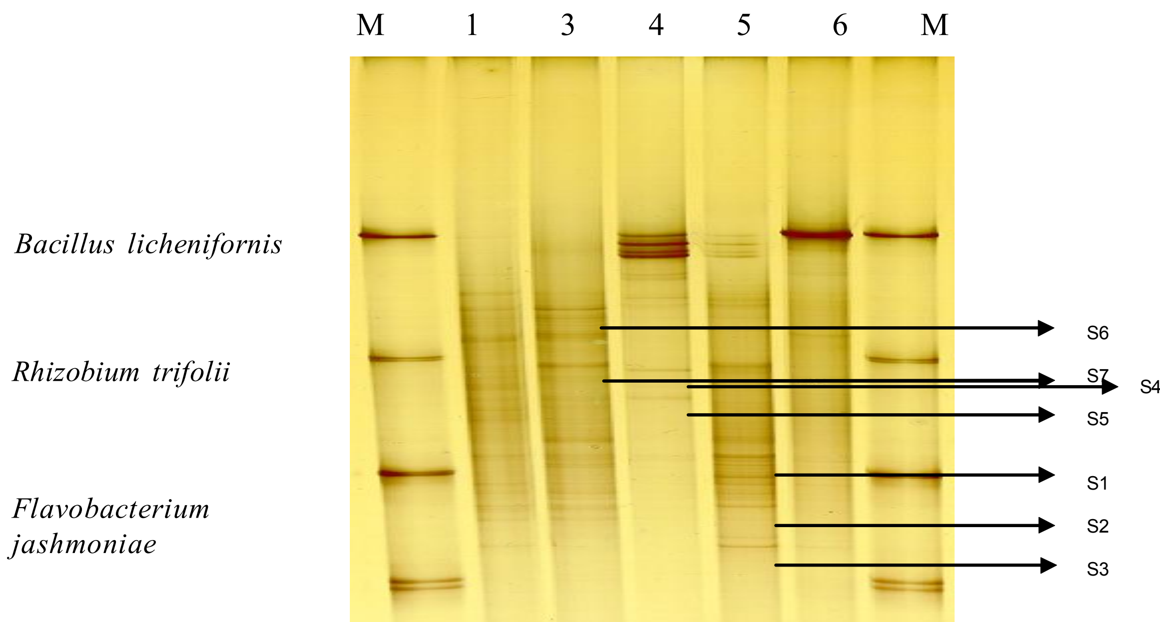

Figure 1. SSCP-profiles of PCR amplified fragments 16S rDNA from rhizosphere DNA of *Salicornia bigelovii.* The numbering of lanes (M) corresponds to the numbering of sites sampled. Arrows indicate the position of bands which were further characterized by DNA sequencing and phylogenetic assignments. For results, see Table 1.

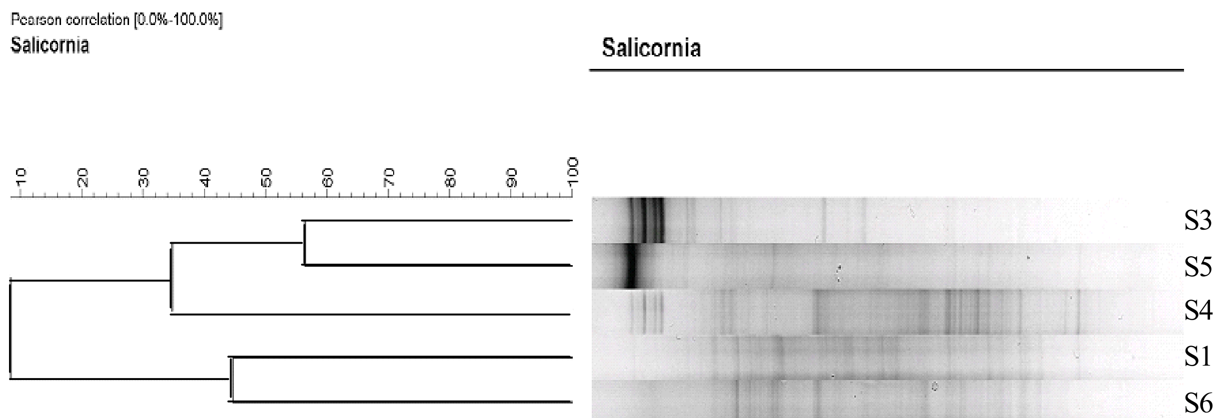

Figure 2. Dendrogram comparing profiles microbial communities of *Salicornia bigelovii* rhizosphere of each sampling.

characterization of microorganisms, as it is considered the basis for understanding microbial physiology and genetics. It is desirable to match isolated strains with their counterparts in nature. Only then, the gained physiological data from culture studies can begin to be confidently extrapolated to natural conditions (Castenholz, 1989). In many cases, however, inadequate culture conditions leading to the loss of various morphological characteristics, the inability to grow certain microorganisms in the laboratory, and the misidentification of strains in culture collections make it difficult to apply taxonomic assignments based on culture to open field populations.

CONCLUSIONS

Molecular studies have led to new approaches that are revolutionizing the applications of microbiology by providing an evolutionary framework and by enabling new ecological approaches. Although we analyzed only some of the most representative bands present at different intensities in all sampled sites, the obtained results evidenced the possibility of finding niche-complexity due to micro-environmental heterogeneity in the rhizoplane and rhizosphere of plants, presumably resulting in the formation of many different niches *in situ* (Bowen 1980, Bagwell & Lovell, 2000), which support an abundant and diverse bacterial community assemblage within these root microenvironments (Bagwell *et al.* 1998, Piceno *et al.* 1999, Lovell *et al.* 2000). Based on SSCP, strains of *Rhizobium* spp., *Bacillus* spp., Cyanobacteria and Proteobacteria groups could be considered diazotrophic bacteria in association, within the environments where the halophyte *Salicornia bigelovii* grows naturally in La Paz Bay, serving as an ecological source of these systems. For further applications, the rhizoplane rhizosphere microflora assemblage of *Salicornia bigelovii* should be studied extensively; in this study we demonstrate a substantial associated bacterial community, which possibly inhabits other ecosystems.

ACKNOWLEDGMENTS

We thank Consejo Nacional de Ciencia y Tecnología (CONACyT Ph. D. scholarship 70548 for E.O. Rueda-Puente). In memory of Mr. Carlos Mota Urbina, the first researcher devoted to halophyte studies; he provided us with *Salicornia bigelovii* references. Thanks to the personnel of Johann Heinrich von Thunen Institut (vTI) (Germany), especially to Dr. C. Tebbe, for helping us with the SSCP technique.

LITERATURE CITED

Akhavan K., M., W. F. Campbell, J. J. Jurinak, and L. M. Dudley. 1991. Effects of $CaSO_4$, $CaCl_2$, and NaCl on leaf nitrogen, nodule weight, and acetylene reduction activity in *Phaseolus vulgaris* L. Arid Land Res. Manage. 5: 97-103.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struthl. 2002. Short protocols in molecular biology. Wiley. New York, NY, USA.

Bagwell, C. E. and C. R. Lovell. 2000. Microdiversity of culturable diazotrophs from the rhizoplanes of the salt marsh grasses *Spartina alterniflora* and *Juncus roemerianus*. Microb. Ecol. 39: 128-136.

Table 2. Results of characterization (% identity) of associated bacteria in the *Salicornia bigelovii* rhizosphere in Bay of La Paz, Baja California Sur, Mexico.

Clon No.	Closest relative	Group	Identity (%)	Habitat	Author
S1	*Trichodesmium thiebautii AFO91321*	Cyanobacteria	98.8		Janson *et al.* (1995)
	Cylindrospermum sp. AJ133163	Cyanobacteria	88.5	Soils from eastern Amazonia	Lyra *et al.* (2001)
S2	Marine bacterium SCRIPPS_94 AF359545		98.5		Hold *et al.* (2001)
	Humic substances-degrading bact. HS341		98.3		Elbeltagy *et al.* (2001)
	Alpha proteobacterium ABO24595	a Proteobacteria	98.3	Estuarine environment	Esham *et al.* (2000)
S3	*Bacillus* sp. ABO62678	Bacillus/Clostridium group	99.4		Suzuki *et al.* (1997)
	Bacterial species		99.4		Felske (1999)
	Bacterial species	Bacillus/Clostridium	99	Grassland soils	Nogi (1990)
S4	*Rhizobium* sp. AF331662	a Proteobacteria	98	Campos de minas	Macur *et al.* (2004)
	Mesorhizobium loti AP003001	a Proteobacteria	96.8		Kaneko *et al* . (1996)
	Rhizobium lotii	a Proteobacteria	96.8	Lotus rhizosphere	Sullivan *et al* . (1996)
S5	Uncultured bacterium EKHO-20 AF142902	Verrucomicrobia	88.5	Coastal meromitic marine basin	Bowman and McCuaig (2003)
	Ultramicobacterium str. X99391	Verrucomicrobia	87.3	Anoxic rice paddy soil	Janssen *et al.* (1997)
	Ultramicobacterium str. X99390	Verrucomicrobia	87.3	Anoxic rice paddy soil	Janssen *et al.* (1997)
S6	Uncultured bacterium AF142833		95.3	Coastal meromitic marine basin	Bowman and McCuaig (2003)
	Uncultured bacterium AF277501	CFB group	95.3	Sea ice microbial communities *Phaseolus vulgaris*	Ward (1998)
S7	*Rhizobium* sp. U29387	a Proteobacteria	97.3	*Robinia pseudoacacia* L	Gisèle Laguerre *et al.* (2001)
	Rhizobium sp. AJ271902	a Proteobacteria	97.3	Native Shrubbi Southeastern	Ulrich and Zaspel (2000)
	Rhizobium sp. Z94803	a Proteobacteria	97.3	Australia	Lafay and Burdon (1998)

Bagwell, C. E., Y. M. Piceno, A. Ashburne-Lucas, and C. R. Lovell. 1998. Physiological diversity of the rhizosphere diazotroph assemblages of selected salt marsh grasses. Appl. Environ. Microbiol. 64: 4276-4282.

Banwari, L. and V. Rao. 1990. Effect of *Azospirillum brasilense* on growth and nitrogen content of *Cynodon dactylon* under different moisture regimens. Indian J. Plant Physiol. 33: 210-213.

Bashan, Y., G. Holguin y M. Puente. 1992. Alternativa agrícola regional por fertilizantes bacterianos en uso y manejo de los recursos naturales en la Sierra de La Laguna Baja California Sur. pp. 47-67. *In:* A. Ortega-Rubio (ed.). Uso y manejo de los recursos naturales en la Sierra de la Laguna, BCS. Centro de Investigaciones Biológicas del Noroeste S. C. La Paz, BCS, México.

Bassam, B. J., G. Caetano-Anolles, and P. M. Gresshoff. 1991. Fast and sensitive sil ver staining of DNA in polyacrylamide gels. Anal. Biochem. 198: 217.

Bowen, G. D. 1980. Misconceptions, concepts, and approaches in rhizosphere biology. pp. 283-304. *In:* D. C. Ellwood, M. J. Latham, J. N. Hedger, J. M. Lynch, and J. H. Slater (eds.). Contemporary microbial ecology. Academic Press, New York, NY, USA.

Bowman, J. P. and R. D. McCuaig. 2003. Biodiversity, community structural shifts, and biogeography of prokaryotes within antarctic continental shelf sediment. Appl. Environ. Microbiol. 69: 2463-2483.

Campbell, R. and M. P. Greaves. 1990. Anatomy and community structure of the rhizosphere. pp 11-34. *In*: J. M. Lynch. (ed.). The rhizosphere. John Wiley and Sons. New York, NY, USA.

Castellanos, C. T. 2002. Detección de microalgas mediante la técnica “conformaciones polimórficas de una sola cadena” (SSCP). *In*: V. Arredondo (ed.). Curso de aplicaciones biotecnológicas del cultivo de microalgas. CIBNOR. La Paz, BCS, México.

Castenholz, R. W. and J. B. Waterbury. 1989. Oxigenic photosynthetic bacteria group I. Cyanobacteria. pp. 1710-1728. *In*: J. Staley, M. Bryant, N. Pfening, J Holt (eds.). Berguey´s manual of systemic bacteriology. New York, NY, USA.

Elbeltagy, A., K. Nishioka, T. Sato, H. Suzuki, B. Ye, T. Hamada, T. Isawa, H. Mitsui, and K. Minamisawa. 2001. Endophytic colonization and in plant nitrogen fixation by a *Herbaspirillum* sp. isolated from wild rice species. Appl. Environ. Microbiol. 67: 5285-5293.

El-Shatnawi, M. K. J. and I. M. Makhadmeh. 2001. Ecophysiology of the plant-rhizosphere system. J. Agron. Crop Sci. 187: 1-9.

Esham, E. C., Y. Wenying, and M. A. Moran. 2000. Identification and characterization of humic substances-degrading bacterial isolates from an estuarine environment. FEMS Microbiol. Ecol. 34: 103-111.

Felske, A. 1999. Reviewing the DA001-files: a 16S rRNA chase on suspect #X99967, a Bacillus and dutch underground activist. J. Microbiol. Methods 36: 77-93.

Glenn, E. P., J. W. O’Leary, M. C. Watson, T. L. Thompson, and R. O. Kuehl. 1991. *Salicornia bigelovii* Torr.: an oilseed halophyte for seawater irrigation. Science 251: 1065-1067.

Glenn, E., T. Lewis, and D. Moore 1994. Synthesis of selected research results on *Salicornia bigelovii*. pp. 1-97. *In*: Proceeding of Halophytes. Gallawa. Halophyte Enterprises: Kino Bay. Sonora, Mexico.

Glenn, E., N. Hicks, and J. Riley. 1995. Seawater irrigation of halophytes for animal feed. pp. 221-236. *In*: R. Choukr-Allah, C. Malcolm, and A. Hamdy (eds.). Halophytes and biosaline agriculture. Marcel Dekker, New York, NY, USA.

Hanson, R. B. 1983. Nitrogen fixation activity (acetylene reduction) in the rhizosphere of salt marsh angiosperm, Georgia, USA. Bot. Mar. 26: 49-60.

Hold, G. L., E. A. Smith, M. S. Rappé, E. W. Maas, E. R. B. Moore, C. Stroempl, J. R. Stephen, J. I. Prosser, T. H. Birkbeck, and S. Gallacher. 2001. Characterization of bacterial communities associated with toxic and non-toxic dinoflagellates: *Alexandrium* spp. and *Scrippsiella trochoidea*. FEMS Microbiol. Ecol. 37:161-173.

Ito, T., I. Okane, and A. Nakagiri. 1999. Mycoflora of the rhizosphere of *Salicornia europaea* L., a halophytic plant. Inst. Ferment. Osaka Res. Commun. 19: 34-40.

Janson, S., P. J. A. Siddiqui, A. E. Walsby, K. M. Romans, E. J. Carpenter, and B. Bergman. 1995. Cytomorphological characterization of the planktonic diazotrophic cyanobacteria *Trichodesmium* spp. from the Indian Ocean and Caribbean and Sargasso seas. J. Phycology 31: 463-477.

Janssen, P. H., A. Schuhmann, E. Mörschel, and F. A. Rainey. 1997. Novel anaerobic ultramicrobacteria belonging to the Verrucomicrobiales lineage of bacterial descent isolated by dilution culture from anoxic rice paddy soil. Appl. Environ. Microbiol. 63: 1382-1388.

Kaneko, T., S. Sato, and H. Kotani 1996. Sequence analysis of the genome of the unicellular cyanobacterium *Synechocystis* sp. strain PCC6803. II. Sequence determination of the entire genome and assignment of potential protein-coding regions (supplement). DNA. Res. 3: 185-209.

Kloepper, J. W., R. M. Zablotowicz, E. M. Tipping, and R. Lifshitz. 1991. Plant growth promotion mediated by bacterial rhizosphere colonizers. pp. 315-326. *In:* D. L. Keister, P. B. Cregan (eds.). The rhizosphere and p1ant growth. Kluwer Acadernic Publishers. Dordrecht, The Netherlands.

Lafay, B. and J. J. Burdon. 1998. Molecular diversity of *Rhizobia* occurring on native shrubby legumes in Southeastern Australia. Appl Environ. Microbiol. 64: 3989-3997.

Laguerre, G., S. M. Nour, V. Macheret, J. Sanjuan, P. Drouin, and N. Amarger. 2001. Classification of *Rhizobia* based on nodC and nifH gene analysis reveals a close phylogenetic relationship among *Phaseolus vulgaris* symbionts. Microbiology 147: 981 993.

López-Cortés, A., F. García-Pichel, U. Nübel, and R. Vázquez-Juárez. 2001. Cyanobacterial diversity in extreme environments in Baja California, México: polyphasic study. Int Microbiol. 4: 227-236.

Lovell, C. R., Y. M. Piceno, J. M. Quattro, and C. E. Bagwell. 2000. Molecular analysis of diazotroph diversity in the rhizosphere of the Smooth Cordgrass, *Spartina alterniflora*. Appl. Environ. Microbiol. 66: 3814-3822.

Lyra, C., S. Suomalainen, M. Gugger, C. Vezie, P. Sundman, L. Paulin, and K. Sivonen, 2001. Molecular characterization of planktic cyanobacteria of *Anabaena*, *Aphanizomenon*, *Microcystis* and *Planktothrix* genera. Int. J. Syst. Evolut. Microbiol. 51: 513-526.

Macur, R. E., H. W. Langner, B. D. Kocar, and W. P. Inskeep. 2004. Linking geochemical processes with microbial community analysis: successional dynamics in an arsenic-rich, acid-sulphate-chloride geothermal spring. Geobiology 2: 163-177.

Al-Nahidh, S. I. and A. K. M. Gomah. 1991. Response of wheat to dual inoculation with Va-mycorrhiza and *Azospirillum*, fertilized with NPK and irrigated with sewage effluent. Arid Land Res. Manage. 5: 83-96.

Nehl, D. B., S. J. Allen, and J. F. Brown. 1997. Deleterious rhizosphere bacteria: an integrating perspective. Appl. Soil Ecol. 5: 1-20.

Nogi, Y. and Koki Horikoshi. 1990. A thermostable alkaline *ß*-1, 3-glucanase produced by alkalophilic *Bacillus* sp. AG-430. Appl. Environ. Microbiol. 32: 704-707.

O'Neill, G. A, R. A. Radley, and C. P. Chanway. 1992. Variable of effects of emergence-promoting rhizobacteria on conifer seedling growth under nursery conditions. Biol. Fertil. Soils 13: 45-49.

Piceno, Y. M., P. A. Noble, and C. R. Lovell. 1999. Spatial and temporal assessment of diazotroph assemblage composition in vegetated salt marsh sediments using desnaturating gradient gel electrophoresis analysis. Microbiol. Ecol. 38: 157-167.

Pimentel, J. P., F. Olivares, R. M. Pitard, S. Drquiaga, F. Akiba, and Dobereiner. 1991. Dinitrogen fixation and infection of grass leaves by *Pseudomonas rubrisubalbicans* and *Herbaspirillum seropedicae*. pp. 225-229. *In:* M. Polsinelli, R Materassi, M. Vincenzini (eds.). Nitrogen fixation- developements in p1ant and soil sciences. Vol. 48. Academic Publishers. Dordrecht, The Netherlands.

Postgate, J. 1998. Nitrogen fixation. Cambridge University Press. Cambridge, UK.

Reinhold, B., T. Hurek, I. Fendrik, B. Pot, M. Gillis, K. Kersters, S. Thielmans, and J. De Ley. 1987. *Azospirillum halopraeferens* sp. nov., a nitrogen-fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth). Int. J. Syst. Bacteriol. 37: 43-51.

Renie, R. J. 1981. A single medium for the isolation of acetylene reducing (dinitrogen-fixing) bacteria from soil. Can J. Microbiol. 27: 8-14.

Rueda-Puente, E. O., C. Castellanos, E. Troyo-Diéguez, J. L. Díaz de León Alvarez, and B. Murillo-Amador. 2003. Effects of a nitrogen-fixing indigenous bacterium (*Klebsiella pneumoniae*) on the growth and development of the halophyte *Salicornia bigelovii* as a new crop for saline environments. J. Agron. Crop Sci. 5: 273-360.

Rueda-Puente, E. O., T. Castellanos, E. Troyo-Diéguez, and J. L. Díaz de León Alvarez. 2004. Effect of *Klebsiella pneumoniae* and *Azospirillum halopraeferens* on the growth and development of two *Salicornia bigelovii* genotypes. Aust. J. Exp. Agric. 44: 65-74.

Sullivan, J. T., B. D. Eardly, P. van Berkum, and C.W. Ronson. 1996. Four unnamed species of nonsymbiotic rhizobia isolated from the rhizosphere of *Lotus corniculatus*. Appl. Environ. Microbiol. 62: 2818-2825.

Suzuki, M. T., M. S. Rappe, Z. W. Haimberger, H. Winfield, N. Adair, J. Stro-Bel, and S. J. Giovannoni. 1997. Bacterial diversity among small-subunit rRNA gene clones and cellular isolates from the same seawater sample. Appl. Environ. Microbiol. 63: 983-989.

Tebbe, C. C., A. Schmalenberger, S. Peters, and F. Schwieger. 2001. Single-Strand conformation polymorphism (SSCP) for microbial community analysis. pp. 161-175. *In*: P. A. Rochelle (ed.). Environmental molecular microbiology: Protocols and applications. Horizon Scientific press. Wymondham, Norfolk, UK.

Troyo D., E., A. Ortega-Rubio, Y. Maya, and J. L. León. 1994. The effect of environmental conditions on the growth and development of the oilseed halohyte *Salicornia bigelovii* Torr in arid Baja California Sur, Mexico. J. Arid Environ. 28: 207-213.

Ulrich, A. and I. Zaspel. 2000. Phylogenetic diversity of rhizobial strains nodulating *Robinia pseudoacacia* L. Microbiology 146: 2997-3005.

Ward, D. M., M. J. Ferris, S. C. Nold, and M. M. Bateson. 1998. A natural view of microbial biodiversity within hot spring cyanobacterial mat communities. Microbiol. Mol. Biol. Rev. 62: 1353-1370.

Zahram, H. H. 1999. Rhizobium-Legume Symbiosis and Nitrogen Fixation under Severe Conditions and in Arid Climate. Microb. Mol. Biol. Rev. 63: 968-989.