Article

Molecular characterization of rhizospheric bacteria associated with *Echinocactus platyacanthus* in greenhouse and wild

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Abstract

The sweet biznaga (*Echinocactus platyacanthus*) is an endemic cactus of Mexico characterized by its slow growth and low rate of reproduction, which together with a high collection pressure, places wild populations at risk. Currently, the study of bacterial communities associated with cactus is scarce and it is unknown what cultivable bacteria are present in the rhizosphere of sweet biznagas that grow in nature and those that are grown in nurseries. For this study rhizospheric material was collected from wild and cultivated biznagas, in addition to non-rhizospheric soil. A total of 268 morphotypes were isolated and grouped into 41 different ribotypes by RFLP. Representatives of each ribotype were identified by sequencing the 16S rRNA gene. The cultivable fraction of the bacterial community associated with *E. platyacanthus* is mainly composed of members of the genera *Bacillus* (21 strains), *Pseudomonas* (6 strains), *Stenotrophomonas* (4 strains), *Paenibacillus* (2 strains), *Brevibacterium*, *Staphylococcus* and *Cutibacterium* (1 strain, each one), with *Bacillus* genus being the predominant. The genera *Bacillus* and *Pseudomonas* have been previously reported for carrying out beneficial activities for the plants with which they are associated.

Keywords: *Echinocactus platyacanthus*, phylogeny, rhizosphere.

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Introduction

The arid and semi-arid zones constitute around 60% of the Mexican territory (CONAZA, 2019). They inhabit a large number of plant species belonging to the Cactaceae family, whose members make up several of the most representative groups of Mexican biological diversity, placing the country as the main center of cactological diversity in the world (Hernández-Oria *et al*., 2007).

The Toliman quadrant, located at the southern end of the Chihuahua desert, is one of the regions where the diversity of cactus is widely represented. There it is possible to find 55 species of cactus, of which 13 are endemic; however, 17 of them are in some category of risk for their survival (Hernández *et al*., 2007).

Cactaceae are widely used by human communities as construction material, human and livestock food, in addition to giving them medicinal and ornamental uses (Meza-Nivon, 2011). Additionally, excessive consumption, the direct extraction of specimens from their habitat, high endemism, the environmental specificity of their populations, slow growth, low resilience and long life cycles, cause many cactus to be at risk of extinction (Alvárez *et al*., 2004; Arias *et al*., 20055).

Echinocactus platyacanthus, also known as 'sweet biznaga', is a globular-bodied cactus, endemic to Mexico that is distributed in the states of Coahuila, Guanajuato, Hidalgo, Nuevo León, Oaxaca, Puebla, Querétaro, San Luis Potosí, Tamaulipas and Zacatecas (González-Medrano, 2012). It is currently in a risky condition due to the high demand for specimens for human consumption (with it the traditional acitron sweet is made).

It is considered in Appendix II of the Convention on Trade in Endangered Species of Wild Flora and Fauna (CITES), by its NOM-059-SEMARNAT-2010 in the category 'subject to special protection' (Denisse, 2010) and by the red list of the international union for the conservation of nature (IUCN), in the category 'almost threatened' (Castañeda-Romero *et al*., 2016).

The study of the cultivable fraction of bacteria associated with cactus is a relatively little explored field. In the Caatinga region, Brazil, bacteria that could be tolerant to drought were isolated, to be used in the recovery of desert soils (Kavamura *et al*., 2013), meanwhile Chávez-Ambríz *et al*. (2016), used bacteria isolated from the rhizosphere of *Coryphantha radians* and *Mammillaria magnimamma* to promote the growth of *Mammilaria zeilmanniana*.

Other studies used metagenomic approaches for the analysis of the bacterial communities associated with the endemic cactus rhizosphere of the Tehuacan-Cuicatlan Biosphere Reserve (Aguirre-Garrido *et al*., 2012; Torres-Cortes *et al*., 2012) and recently, the effect of soil contamination by Zn, on the diversity of the microbiota associated with *Echinocactus platyacanthus*, was analyzed to explain its high tolerance to environmental stress (Sarria-Carabali *et al*., 2019).

A collection of bacteria was obtained from the rhizosphere of wild and greenhouse-grown plants of *Echinocactus platyacanthus*, which allowed us to know the composition of the community of cultivable bacteria present in the rhizosphere of the sweet biznaga in the semi-desert area of Queretaro. The isolates were characterized under molecular criteria, using enzymatic restriction assays for amplicons of the 16S rRNA gene and subsequently identified by sequencing the same gene.

Materials and methods

Obtaining the biological material

In 2017, a non-invasive sampling of the rhizospheric material of 20 biznagas was carried out in a wild population of Toliman, Querétaro. Additionally, samples were collected from the rhizosphere of 2 plants grown in nurseries and from non-rhizospheric soil (taken from wild soil, free of the presence of any plant), in order to have represented the entire bacterial community associated or close to the biznaga, which is influenced by the nutritional variations of the different types of soil.

The samples were stored in hermetic bags. They were labeled with the origin of the plant from which they were taken and transferred in a cooler to the Molecular Ecology laboratory of the Autonomous Metropolitan University, Xochimilco Unit, for processing three days after collection.

Viable accounts and isolation

Samples of the same origin (wild, nursery or non-rhizospheric soil) were mixed and suspensions were made with a 1:10 dilution factor with 1 gram of soil and 9 mL of saline (NaCl 0.9%). Serial dilutions were made from the suspensions to count the CFU by the plate extension method (Bonilla *et al*., 2016).

In culture medium for heterotrophs TY (tryptone 5 g L^{-1}, yeast extract 3 g L^{-1}, CaCl2 1 g L^{-1}, pH7, 1.2% agar). Once viable counts were made, bacteria with different colonial morphotypes were isolated from the same boxes. The microscopic morphology of each isolate was determined by Gram staining (Gram, 1884).

Obtaining the genetic material

Genomic DNA from all isolates was obtained with Roche's High Pure PCR Template Preparation Kit (Cat. # 11 796 828 001), following the manufacturer's directions. The ribosomal 16S gene was amplified by PCR with *primers* 8F and 1492R.

The obtained product was purified with the Wizard® SV Gel and PCR Clean-Up Systema kit from Promega (Cat. A9281), following the manufacturer's instructions. Both the integrity of the genomic DNA and the size of the PCR products were evaluated by electrophoresis on 1% agarose gels, stained with ethidium bromide (0.5 µg mL^{-1}), according to a previous report (Aguirre-Garrido *et al*., 2012).

RFLP grouping

For the analysis of the polymorphic length restriction fragments (RFLP), the frequently cut restriction enzymes *Hae*III (GG/CC) and *Msp*I (C/CGG) were used, under the conditions described by Massol- Deya *et al*. (1995). Restriction reactions were analyzed on 2% agarose gels, stained with ethidium bromide (0.5 µg mL^{-1}). From the restriction patterns obtained, the collection of the rhizospheric bacteria collection was grouped.

Phylogenetic analysis

Representatives of each of the restriction patterns (ribotypes) obtained were subjected to phylogenetic analysis. Under the same amplification and purification conditions described above, amplicons were obtained to undergo Sanger-type sequencing from Macrogen (Korea).

Bioinformatic analyzes were carried out from these sequences with the software and platforms: *BioEdit* (Hall, 1999); *ClustalX* (Thompson *et al.*, 1997); *SeaView* (Galtier *et al.*, 1996; Gouy *et al.*, 2010); *JModelTest* (Darriba *et al*., 2012); *Mega6* (Tamura *et al.*, 2013); *Bellerophon* (Huber *et al.*, 2004); *EZTaxon* (Yoon *et al.*, 2017), for the taxonomic identification of bacteria and the establishment of their phylogenetic relationships (Aguirre-Garrido *et al*., 2012).

Results and discussion

Viable beads and bacterial isolation

Viable counts of the rhizosphere of both wild plants, nurseries, and non-rhizospheric soil are shown in Table 1. Samples of wild *E. platyacanthus* and those of non-rhizospheric soil gave very high CFU values, found by on top of previously reported counts.

Table 1. CFU count of bacteria isolated from the different samples.

Sample	CFU/g of rhizosphere	Standard deviation
Wild	4.27 E+10	6.25 E+10
Nursery	1.06 E +06	5.12 E+05
Non rhizospheric	7.23 E+10	5.13 E+09

For rhizosphere samples from another cactus (Aguirre-Garrido *et al*., 2012). In addition, the values of these two groups of samples are four orders of magnitude higher than the nursery samples. However, the analysis of variance and the corresponding Tukey test ($p \leq 0.05$) indicated that there are no statistically significant differences between the three types of samples.

A total of 268 bacterial strains were isolated: 261 of rhizospheric material and 7 of non-rhizospheric soil. From the total collection, 201 strains were grouped into 41 ribotypes obtained by RFLP, which were named P1 to P41 (Table 2). The remaining 67 strains did not yield reliable restriction patterns that would allow them to be located in any of the ribotypes obtained and were excluded from the analysis.

Table 2. Total number of isolates from the *E. platyacanthus* rhizosphere and their grouping by RFLP.

Restriction pattern	No. of isolates	Origin of isolates			Restriction pattern	No. of isolates	Origin of isolates		
		C	S	nr			C	S	nr
P1	42		42		P22	3	2	1	
P2	47	6	41		P23	1	1		
P3	4	4			P24	4	1	3	
P4	4	4			P25	1		3	
P5	4	2	1		P26	8	1	5	2
P6	1	1			P27	1	1		
P7	5	4	1		P28	2	2		
P8	2	2			P29	1	1		
P9	4	1	3		P30	10		7	3
P10	1	1			P31	1		1	
P11	2	2			P32	1		1	
P12	4	2	2		P33	1		1	
P13	2	1	1		P34	3		3	
P14	4	1	3		P35	1		1	
P15	1	2			P36	1		1	
P16	1		1		P37	4		4	
P17	1		1		P38	5		5	
P18	15		13	2	P39	2		2	
P19	3		3		P40	1		1	
P20	1	1			P41	1		1	
P21	1	1							

C= cultivated; S= wild; nr= non rhizospheric.

The strains grouped in ribotypes P1, P16, P17, P18, P19, P25, P30, P31, P32, P33, P34, P35, P36, P37, P38, P39, P40 and P41 are exclusive to wild samples. While the strains of ribotypes P3, P4, P6, P10, P11, P15, P20, P21, P23, P27, P28 and P29 were isolated from the nursery samples.

The strains grouped in the rest of the ribo types (P2, P5, P7, P8, P9, P12, P13, P14, P22, P24 and P26) were found in both types of samples. In the case of patterns P2 and P26, the largest number of isolates comes from wild samples. The isolates from the non-rhizospheric soil samples were grouped into ribo types P18, P26 and P30, which they share with the wild samples.

Identification and phylogenetic analysis

To establish the identity and phylogenetic relationships of the collection, a representative of each ribo type was selected, except for the case of ribotypes P1, P2, P18 and P26 that have a greater number of isolates. In these cases, two strains were selected in order to corroborate the grouping by RFLP.

The two sequences of the 16SrRNA gene obtained for each isolate were assembled and almost complete sequences of said gene were obtained (> 1350 nr). The assembled sequences were analyzed in *Bellerophone* (Huber *et al*., 2004) to rule out the formation of chimeras. Subsequently, they worked on the EZTaxon platform (Chun *et al*., 2007), which allows for precise taxonomic identification since it has a robust comparison algorithm and only includes sequences from the 16S rRNA gene belonging to type strains.

The P10, P17, P19, P20, P21, P24, P27, P30 and P33 sequences resulted in coverage percentages (exhaustiveness) less than 95% and were excluded from the phylogenetic analysis as they were considered of low quality (Kim *et al*., 2012). Of the remaining 32 sequences, 10 were identified at the taxonomic level of gender, with a percentage of similarity greater than 95% (Bou *et al*., 2011) and 22 at the species level, with a percentage of similarity greater than 98.7% (Kim *et al*., 2014), as shown in Table 3.

Table 3. Bacterial identification according to the EZTaxon database.

Strain	Identification	Nearest sequence (access number)	Coverage (%)	Similarity (%)
P1	*Pseudomonas koreensis*	*Pseudomonas koreensis* (AF468452)	99.52	99.7
P2	*Bacillus subtilis* subsp. inaquosorum	*Bacillus subtilis* subsp. *inaquosorum* (AMXN01000021)	100	100
P3	*Paenibacillus lautus*	*Paenibacillus lautus* (D78473)	95.8	99.42
P4	*Bacillus*	*Bacillus aryabhattai* (EF114313)	100	95.23
P5	*Paenibacillus*	*Paenibacillus harenae* (AY839867)	100	96.22
P6	*Bacillus subtilis* subsp. inaquosorum	*Bacillus subtilis* subsp. *Inaquosorum* (AMXN01000021)	99.55	100
P7	*Bacillus subtilis* subsp. inaquosorum	*Bacillus subtilis subsp. Inaquosorum* (AMXN01000021)	100	99.65
P8	*Brevibacterium*	*Brevibacterium frigoritolerans* (AM747813)	100	95.77
P9	*Bacillus velezensis*	*Bacillus velezensis* (AY603658)	95.4	99.14
P11	*Staphylococcus*	*Staphylococcus pasteuri* (AF041361)	96.2	96.85
P12	*Stenotrophomonas rhizophila*	*Stenotrophomonas rhizophila* (CP007597)	99.2	100
P13	*Bacillus siamensis*	*Bacillus siamensis* (AJVF01000043)	100	99.55
P14	*Bacillus subtilis* subsp. inaquosorum	*Bacillus subtilis* subsp. *Inaquosorum* (AMXN01000021)	100	99.86
P15	*Bacillus aryabhattai*	*Bacillus aryabhattai* (EF114313)	100	99.65

Strain	Identification	Nearest sequence (access number)	Coverage (%)	Similarity (%)
P16	*Pseudomonas*	*Pseudomonas mediterranea* (AUPB01000004)	100	98.65
P18	*Pseudomonas*	*Pseudomonas koreensis* (AF468452)	97.4	99.7
P22	*Cutibacterium*	*Cutibacterium acnes* (AWZZ01000008)	100	97.89
P23	*Bacillus subtilis* subsp. inaquosorum	*Bacillus subtilis* subsp. *Inaquosorum* (AMXN01000021)	100	99.57
P25	*Bacillus*	*Bacillus wiedmannii* (LOBC01000053)	100	97.57
P26	*Bacillus*	*Bacillus velezensis* (AY603658)	95.4	99.56
P28	*Bacillus*	*Bacillus velezensis* (AY603658)	95.4	97.98
P29	*Bacillus tequilensis*	*Bacillus tequilensis* (AYTO01000043)	100	99.51
P31	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia* (JALV01000036)	100	99.57
P32	*Pseudomonas granadensis*	*Pseudomonas granadensis* (LT629778)	100	99.08
P34	*Bacillus velezensis*	*Bacillus velezensis* (AY603658)	95.4	98.72
P35	*Bacillus*	*Bacillus subtilis* subsp. *Inaquosorum* (AMXN01000021)	100	98.13
P36	*Stenotrophomonas*	*Stenotrophomonas maltophilia* (JALV01000036)	100	95.17
P37	*Bacillus subtilis* subsp. inaquosorum	*Bacillus subtilis* subsp. *Inaquosorum* (AMXN01000021)	100	99.93
P38	*Bacillus velezensis*	*Bacillus velezensis* (AY603658)	95.4	99.36
P39	*Bacillus subtilis* subsp. inaquosorum	*Bacillus subtilis* subsp. *Inaquosorum* (AMXN01000021)	100	99.3
P40	*Bacillus paramycoides*	*Bacillus paramycoides* (MAOI01000012)	100	99.66
P41	*Pseudomonas*	*Pseudomonas geniculata* (AB021404)	97.1	98.45

In the four cases in which two strains were selected per ribotype, both isolates from each pattern produced similar results. The phylogenetic relationships of the representatives of our collection and the type strains of each assigned genus or species are shown in Figure 1.

Constructed with the maximum likelihood method (-ln= 15 318.8991), using the Jukes-Cantor nucleotide substitution model (Ts/Tv= 0.5). The 16S rRNA gene sequence from *Thermoplasma acidophilum* was used as the *outgroup*. Bootstrap analysis was done with 1 000 randomized replicates and only values ≥50% are shown. The scale bar indicates 5% estimated divergence between the sequences.

The isolated strains were mainly assigned to the genera *Bacillus* (21 strains) and *Pseudomonas* (6 strains), although other genera such as *Paenibacillus*, *Brevibacterium*, *Staphylococcus*, *Cutibacterium* and *Stenotrophomonas* were also found less abundantly. This agrees with previous findings, where it was reported that *Bacillus* and *Pseudomonas* are the predominant genera in the rhizosphere of the cactus *Pachycereus pringlei*, *Stenocereus thurberi*, *Mammillaria fraileana* and *Opuntia cholla* (Bashan and de-Bashan, 2010).

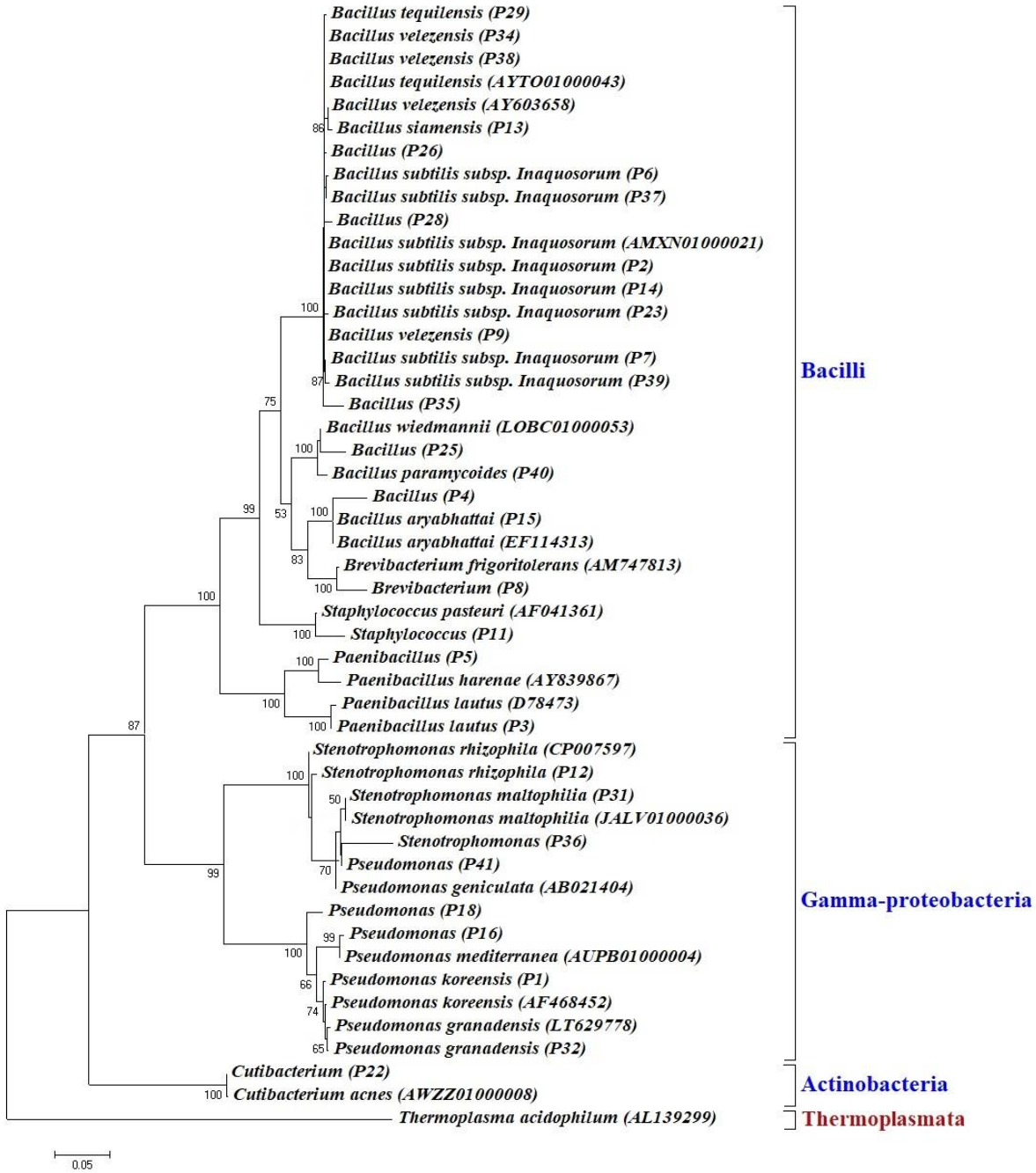

Figure 1. Phylogenetic tree of the cultivable bacterial community associated with *Echinocactus platyacanthus*.

Furthermore, representatives of the genus *Bacillus* have been isolated from the rhizospheres of *Mammilaria* spp. and *Coryphanta* radians, native to the semi-desert of Querétaro (Chávez-Ambríz, 2016). These bacterial genera have also been found associated with the rhizospheres of *Mammillaria carnea*, *Opuntia pilifera* and *Stenocereus stellatus*, three cactus native to the Tehuacán-Cuicatlán Valley (Aguirre *et al*., 2012).

In a recent work, Sarria-Carabali *et al*. (2019), where they also used the rhizosphere of cultivated *E*. *platyacanthus* as a model, reported an abundance of sequences related to the genus *Bacillus*, in response to soils contaminated with different doses of zinc.

Conclusions

The community of cultivable rhizospheric bacteria associated with wild *E*. *platyacanthus* is more diverse than that of cultivated plants, presenting a greater number of ribotypes (genera or species), although the community structure is less homogeneous since only two patterns; P1 and P2 (*Pseudomonas koorensis* and *Bacillus subtilis*, respectively) have 54.6% of the isolates. While the community of cultivable bacteria associated with cultivated plants has only 23 genera, but the relative abundances of each one is more homogeneous, varying between 2.3 and 13.6%.

Of the bacteria representing the 41 ribotypes obtained by RFLP, 32 strains could be taxonomically identified, 13 were identified at the genus level and 19 at the species level. The cultivable fraction of the bacterial community associated with sweet biznaga is mainly made up of members of the genera *Bacillus*, *Pseudomonas*, *Paenibacillus*, *Brevibacterium*, *Staphylococcus*, *Cutibacterium* and *Stenotrophomonas*. The predominant genus in the rhizosphere of *E*. *platyacanthus* is *Bacillus*, since 19 of the strains characterized by molecular techniques belong to this genus.

Perspectives

There are reports of bacteria belonging to the genera found, mainly *Bacillus* (Zhao *et al*., 2015; Gao *et al*., 2016; Pin *et al*., 2016; Liu *et al*., 2017; Zhang *et al*., 2017) and *Pseudomonas* (Reetha *et al*., 2014; Kamble and Galerao, 2015; Abed *et al*., 2016; Buono and Ulla, 2016; Li *et al*., 2017), which demonstrate that they are capable of establishing beneficial relationships for plants, since they are classified within the Plant Growth Promoting Rhizobacteria (PGPR) group.

For this reason, microbiological characterization studies of the collection of bacteria associated with sweet biznaga should be continued, focusing on those isolates that may have one or more mechanisms for promoting plant growth.

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