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BRIEF COMMUNICATION

# An endophytic bacterium isolated from roots of the halophyte *Prosopis strombulifera* produces ABA, IAA, gibberellins $A_1$ and $A_3$ and jasmonic acid in chemically-defined culture medium

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**Abstract** This paper informs the characterization by 16SrDNA partial sequence analysis of an endophytic diazotrophic bacterium isolated from roots of the halophyte shrub *Prosopis strombulifera*. The bacterium produced ABA, IAA, $GA_1$, $GA_3$ and jasmonic acid in chemically-defined culture medium as assessed by GC-EIMS. The results emphasize the role of phytohormones produced by endophytic bacteria in the association host-beneficial microorganisms, especially under conditions of adverse environments.

**Keywords** Plant growth promoting bacteria · *Prosopis strombulifera* · ABA, gibberellins, jasmonic acid

## Introduction

*Prosopis strombulifera* (Lam.) Benth. (Burkart 1937) is a spiny shrub distributed in saline areas from the Arizona desert (USA) to Patagonia (Argentina), where it must tolerate extreme water shortage. Plants of *P. strombulifera* hydroponically-cultured in NaCl solutions up to 500 mmol $l^{-1}$ showed root and stem increased growth as compared with control plants placed in medium without NaCl (Reinoso et al. 2004).

On the other hand it is well established the beneficial effect of endophytic diazotrophic bacteria on several crops, especially cereals (Okon and Labanderas-González 1994). Such benefits may be explained by production of phytohormones like gibberellins (GAs, Bottini et al. 1989, 2004), IAA (Crozier et al. 1988) and ABA (Cohen et al. 2008), that either stimulate growth (Fulchieri et al. 1993) and/or ameliorate the plant status under stressful conditions (Cohen et al. 2008, 2009).

This paper informs the characterization of an endophytic bacterium isolated from *P. strombulifera* roots, and that this bacterium produced ABA, IAA, $GA_1$, $GA_3$ and jasmonic acid in chemically-defined culture medium.

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## Materials and methods

### Isolation of bacteria

Six vigorous plants of *P. strombulifera* were collected from the southwestern zone of Provincia de San Luis (near El Bebedero saline stream), Argentina, located at 33°43′S, 66°37′W. The plant roots were surface-disinfected with ethanol 95% and Na hypochloride 3%, washed with abundant running tap water, and finally with distilled sterile water. The roots were cut in 2 cm pieces and each one was macerated with a mortar and pestle in NaCl 0.85%. From the macerate serial dilutions were performed and platted in Petri dishes with a rich medium to support growth of fungi

and bacteria (glucose 1 g, peptone 0.5 g, $KH_2PO_4$ 0.1 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, dichloran 0.0002 g, Rose Bengal 50 µl, cloramphenicol 0.01 g per 100 ml) and incubated 48 h at 28°C. The colonies formed were picked-up and sub-cultured in glass jars with semisolid (0.18% agar) NFb medium pH 6.8 (Bottini et al. 1989). After 48 h at 32°C, the flasks with development of sub-superficial film (typical growth of diazotrophic bacteria in micro-aerophilia and without N, Döbereiner et al. 1993) were selected. After repeating this procedure three times, the isolates were sowed on to Petri dishes with the same medium of above but now in solid form (1.8% agar) plus yeast extract as N source. Subsequent to incubation for 72 h at 32°C, one type of colony was evident. This type of colony was sub-cultured in semi-solid NFb medium with Tween 20 0.1% and subcultured in solid NFB with 1.25% $NH_4Cl$ for better isolation of bacteria. After that the colonies were characterized by biochemical tests of growth in different sugars and organic acids with the commercial Kit API 20 NE (BioMérieux SA, Marcy l'Etoile, France).

## Characterization of phytohormones from chemically-defined culture

One of the isolates purportedly characterized as *Arthrobacter* sp. (see Results) was cultivated in 250 ml Erlenmeyer flasks containing 50 ml of specific and selective chemically-defined NFb medium plus $NH_4Cl$ (1.25 g $l^{-1}$) as N source. The flasks were incubated in a water bath with orbital shaking (Shaker Pro, Viking, BIO-CONTROL, Buenos Aires, Argentina) at 120 r.p.m., in darkness and 30°C, until stationary phase as determined by $OD_{620}$ (biomass production), and further processed as previously described in Cohen et al. (2008) with modifications. The bacterial culture was then sonicated twice for 6 min and centrifuged 10 min at 10,000×g and 4°C. The cells were discarded and the supernatant was adjusted to pH 3.0 with acetic acid and partitioned three times with equal volume of ethyl acetate (saturated with 1% acetic acid) pH 2.8–3.0. The ethyl acetate fraction was evaporated and the residue dissolved in one mL of a mixture of methanol: water: acetic acid (79/20/1; v/v/v), filtrated and submitted to HPLC purification with a µBondapack $C_{18}$ reverse phase (Waters Associates, Parker Ltd., Milford, MA, USA) column. Elution was performed with a KONIK 500 apparatus (Konik Instruments, Barcelona, Spain) at a flow rate of 2 ml $min^{-1}$ using the following gradient: from 0 to 10 min 10% methanol in 1% acetic acid, from 10 to 40 min 10–73% methanol in 1% acetic acid, from 40 to 50 min with 73% methanol in 1% acetic acid, from 50 to 60 min with 100% methanol. The fraction between 20 and 30 min was used for GA and IAA characterization, while the 30–45 min one was used for ABA and JA identification.

After solvent evaporation in vacuum at room temperature the samples were derivatized for capillary gas chromatography-electron impact mass spectrometry (GC-EIMS) analysis. For methyl-ester (Me) derivatization 20–40 µl of methanol and 50–100 µl of fresh $CH_2N_2$ were added, and left for 30 min at room temperature. After solvents had been eliminated under $N_2$ the extract was dissolved in 5 µl hexane and 1 µl was injected in the split less mode in a GC-EIMS system (PerkinElmer Clarus 500, Atlanta, GA, USA). The GC column was a PerkinElmer Elite-5MS, cross-linked methyl silicone capillary column (30 m length, 0.25 mm internal diameter, and 0.25 µm film thickness) eluted with He (1 ml $min^{-1}$). The GC temperature program was 100–190°C at 15°C $min^{-1}$, then from 190 to 260°C at 5°C $min^{-1}$. IAA, JA and ABA characterization was performed in full scan mode and the spectra obtained matched with authentic standards (Sigma Chem. Co., St Louis, MO, USA) with matching score higher than 900.

After IAA, ABA and JA characterization the 20–30 min samples were further derivatized with BSTFA 1% TCMSi (Pierce Chem. Co.) in dry pyridine (1:1 ratio, v/v) in order to obtain the trimethyl sylilated GA derivatives. One µL was injected in the same equipment and with the same conditions of above. $GA_1$ and $GA_3$ were detected and their full mass spectra matched with those of authentic standards (Sigma Chem. Co., St Louis, MO, USA).

## Extraction and purification of total genomic DNA

Genomic DNA was extracted from 1.5 ml of a bacterial culture in early stationary phase (48–72 h) centrifuged 4 min at 14,000 rpm. The pellet was re-suspended in 1 ml NaCl 1 M and let stand 10 min in ice and centrifuged again. To this pellet 1 ml Tris-EDTA (TE) was added and centrifuged. The new pellet was re-suspended in 0.5 ml TE with lisozime (2 mg $ml^{-1}$ in TE) and incubated 1 h at 37°C. The DNA was extracted with 0.5 ml of a mixture of phenol, chloroform and isoamilic alcohol (25:24:1, v/v), re-suspended and centrifuged 4 min at 14,000 rpm. The supernatant was precipitated in two volumes of ethanol 96%, and the DNA finally re-suspended in sterile distilled water.

## Amplification of 16S rRNA

The PCR reactions were carried out with 10 ng of genomic DNA. The reaction were performed in 20 µl reaction volume by mixing DNA with the polymerase reaction buffer (10 mM Tris-HCl pH 8.0, 50 mM KCl, 1.5 mM MgCl2, 0.01% Tween-20, 0.01% Triton X-100), 100 µM of each dNTP, 1 unit of Taq DNA Polymerase (Promega, Madison, WI) and 0.2 µM of each primer. For amplification of the

16S rDNA the universal eubacterial primers 27f and 1525r were used.

Amplifications were performed in a PTC-100 MJ Research (Watertown, MA) thermocycler, programmed for a first denaturation step of 3 min at 94°C followed by 30 cycles of 94°C for 30 s, 55°C for 30 s, and 72°C for 2 min and a final extension step of 72°c for 5 min. PCR products were separated on a 1.2% agarose gel and were stained with ethidium bromide and observed in an UV transiluminator.

### Direct sequencing and phylogenetic analysis of the 16S rDNA genes

The PCR products were purified with a Qiaquick PCR purification kit (Qiagen, Santa Clarita, CA). PCR primers 27f and 1525r were used by the Biotechnology Center of the University of Wisconsin (Madison, WI) to sequence the 3 and 5′ end of the 1,500 bp PCR fragment. Partial sequences data from both strands were used to search for related sequences in the NCBI database using the BLASTN program. Multiple alignment of the 16S rDNA sequences was carried out using the ClustalW system (Higgins et al. 1994), as implemented in MEGA version 4.0 (Tamura et al. 2007).

## Results and discussion

From the different bacterial isolates one showed development of sub-superficial film in semi-solid medium without N indicating it is diazotrophic. By microscopical observation this microorganism shows a coco-bacilar morphology, and the biochemical probes indicated an *Azospirillum* sp. like bacteria. However, the 16SrDNA partial sequence analysis (825 bp) after search in the GenBank showed 97% matching with *Arthrobacter koreensis*, an arthrobacteria found in saline environments. Therefore, the physiological traits of the plant-microorganism relationship indicate characteristics suggesting that the isolated bacterium may be preliminary classified as a new species of *Arthrobacter* adapted to saline environments and with the ability to fix $N_2$.

As well, by GC-EIMS it was determined that the isolated bacteria from roots of *P. strombulifera* produces $GA_1$, $GA_3$, IAA, ABA and jasmonic acid in chemically-defined culture medium. The identity was established by comparison of capillary GC retention times and full scan mass spectra with authentic standards (Table 1).

The results indicate that roots of *P. strombulifera* are colonized by at least one diazotrophical endophytic microorganism. This microorganism is able to produce phytohormones *per se*. Although this capacity regarding production of GAs and IAA (Bottini et al. 1989, Bastián et al. 1998) and ABA (Cohen et al. 2008) by microorganisms was already known, for the first time jasmonic acid is reported as produced by bacteria. Considering the environments in which *P. strombulifera* grows and previous results (Cohen et al. 2008, 2009), it is feasible that these phytohormones may cooperate with plant growth under conditions of excess of salt. More generally, production of phytohormones by bacteria confirms its importance in the association host-beneficial microorganisms.

**Table 1** Characterization by GC-EIMS of Me-TMSi-$GA_1$, Me-TMSi-$GA_3$, Me-IAA, Me-ABA and Me-JA in chemically-defined culture of the isolated bacteria from roots of *P. strombulifera*

| Compound | Characteristic ions and % of their relative abundance (in parenthesis) |
|---|---|
| Me-IAA | 189(64), 172(5), 149(10), 130(100), 103(15) |
| Me-ABA | 190(100), 162(55), 147(13), 134(62), 125(49) |
| Me-JA | 224(66), 193(27), 151(100), 133(50), 121(32) |
| Me-TMSi-$GA_1$ | 506(100), 491(8), 448(20), 377(26), 313(17) |
| Me-TMSi-$GA_3$ | 504(100), 489(12), 475(7), 445(10), 414 (7) |

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