Characterization and Screening of Plant Probiotic Traits of Bacteria Isolated from Rice Seeds Cultivated in Argentina

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Many seeds carry endophytes, which ensure good chances of seedling colonization. In this work, we have studied the seed-borne bacterial flora of rice varieties cultivated in the northeast of Argentina. Surface-sterilized husked seeds of the rice cultivars CT6919, El Paso 144, CAMBA, and IRGA 417 contained an average of 5×10^6 CFU/g of mesophilic and copiotrophic bacteria. Microbiological, physiological, and molecular characterization of a set of 39 fast-growing isolates from the CT6919 seeds revealed an important diversity of seed-borne mesophiles and potential plant probiotic activities, including diazotrophy and antagonism of fungal pathogens. In fact, the seed-borne bacterial flora protected the rice seedlings against Curvularia sp. infection. The root colonization pattern of 2 Pantoea isolates from the seeds was studied by fluorescence microscopy of the inoculated axenic rice seedlings. Both isolates strongly colonized the site of emergence of the lateral roots and lenticels, which may represent the entry sites for endophytic spreading. These findings suggest that rice plants allow grain colonization by bacterial species that may act as natural biofertilizers and bioprotectives early from seed germination.

Keywords: rice, seed, plant probiotic bacteria, diversity, root colonization, Pantoea

Plants interact with a variety of microorganisms in both aerial and belowground tissues and establish different kinds of relationships that may be detrimental (pathogenic), neutral, or beneficial for the host plant. Prokaryotes that improve plant growth and/or prevent diseases are collectively referred to as plant growth-promoting bacteria (PGPB) (Bashan and Holguin, 1998) or as plant probiotic bacteria (PPB) (Haas and Keel, 2003). The mechanisms by which PPB enhance plant growth or promote plant health include facilitation of nutrient acquisition (e.g., nitrogen fixation or inorganic phosphate solubilization), synthesis of phytohormones or modification of phytohormone balance, antagonism of phytopathogens, and induction of systemic resistance (Lugtenberg and Kamilova, 2009).

Recent PPB are highly specific to their plant hosts, as in the case of root-nodule forming rhizobia for legumes and actinomycetes of the genus Frankia for actinorhizal plants (Pawlowski and Bisseling, 1996). In other cases, the association is more promiscuous as it occurs with Azospirillum species that colonize cereal and grass tissues (Steinhoudt and Vanderleyden, 2000). Although PPB have been found living on plant surfaces as epiphytes, within plant organs or tissues as endophytes, or in the thin soil zone under the direct influence of root exudates as rhizospheric bacteria, traditionally, most of the PPB isolates have been obtained from belowground tissues and from the rhizosphere where PPB counts are higher than in aboveground tissues (Rosenblueth and Martinez-Romero, 2006). This is consistent with the fact that plants need to gather most nutrients from the soil. Upon seed germination, the radicle encounters soil microbial populations that will have the opportunity to colonize the root tissues. This is the reason why seeds are bacterized with inoculants before planting; this is to ensure adequate contact with the desired microorganism and reduce the chance of colonization of the indigenous soil flora (Brown, 1974). The isolation and identification of bacterial strains from plants and the demonstration of plant-beneficial effects when they are inoculated in the original host under controlled conditions has boosted the development of inoculant technology in agriculture and horticulture for reducing the utilization of chemicals and minimizing pollution and consumption of renewable energy sources (Bashan, 1998). Most inoculants contain specific rhizobial strains to promote legume symbiotic nitrogen fixation and to reduce chemical nitrogen fertilization, or Azospirillum brasilense for plant growth promotion due to phytohormone production (Berg, 2009). The development of novel inoculant formulations based on other plant probiotic effects (e.g., solubilization of mineral phosphates and biocontrol of phytopathogens) will rely on the knowledge about the interactions between target crops and their associated microorganisms (Berg, 2009).

What if seeds already harbor microorganisms? Seed-borne endophytes may represent a strong competitive population for soil indigenous bacteria as well as for bacteria massively introduced as inoculants (Bacilio-Jiménez et al., 2001). Certainly, the study of seed bacterial flora has received less attention than that of vegetative tissues. This is not a minor...
issue, as seed-borne endophytes are better positioned than bulk soil bacteria to interact with the young developing root after seed imbibition and germination (Nelson, 2004). Seed-borne endophytes have been reported in wheat, alfalfa, buckwheat, sugar beet, barley, cotton, broadleaf weeds, and in some rice varieties cultivated in the East (Cottyn et al., 2001; Nelson, 2004; Okanishi et al., 2005; Mano et al., 2006). In Argentina, several rice varieties are cultivated towards the northeastern region of the country. The cultivated area comprises about 164,000 ha, with an average yield of 6,000 kg/ha (Pozzolo and Ferrari, 2008). The purpose of this study was to isolate and characterize bacteria that naturally colonize seeds of rice varieties cropped in Argentina. Within a set of 39 bacterial isolates, we found an important diversity in terms of microbiological, molecular, and plant probiotic traits, including their antagonistic effects against well-known plant pathogens. Finally, the root colonization pattern of 2 isolates was analyzed by fluorescence microscopy. This study contributes to a better understanding of the role of rice seed-borne bacteria and provides a source of isolates with promising traits as PPB for inoculant development.

**Materials and Methods**

**Rice varieties, seed sterilization, and germination**

Seeds of the rice varieties CT6919, El Paso 144, CAMBA, and IRGA 417 were kindly provided by Estación Experimental INTA Mercedes (Entre Ríos, Argentina). Seeds, with or without the husk, were surface sterilized by treatment with an alkaline sodium hypochlorite solution (fresh commercial bleach, 25%; Na$_2$CO$_3$, 1 g/L; NaCl, 30 g/L; and NaOH, 1.5 g/L) with strong agitation for 40 min, followed by exhaustive washes with autoclaved deionized water (Hurek et al., 1994). Absence of the residual mesophilic bacteria on the seed surface was verified by incubation on nutrient agar (NA) (blood agar base, 40 g/L; yeast extract, 5 g/L; agar, 1.5% w/v) at 28°C for 48 h. Unless otherwise stated, the surface-sterilized seeds were germinated on soft water agar plates (0.7%, w/v agar) at 28°C in the dark for 3 days.

**Isolation of rice seed-borne bacteria**

Isolates CT1 and CT2 were obtained from CT6919 seedling roots that showed profuse outgrowth of yellow colonies after incubation of germinated husked seeds onto NA plates for 48 h at 28°C. The rest of the CT6919 seed isolates (CT3-CT39) were obtained by plating dilutions of husked seed macerates on NA. After 24 h of incubation at 28°C, 17 colonies with distinct growth features were isolated (CT3-CT19). Another 20 colonies were isolated after 48 h (CT20-CT39). Culture purity was confirmed by Gram staining. Isolates were grown in nutrient yeast broth (NYB) (nutrient broth, 25 g/L; yeast extract, 5 g/L) with shaking at 28°C for 24-48 h and preserved at -130°C as saturated cultures containing 20% (w/v) glycerol.

**Characterization of isolates**

Every isolate was restreaked on NA plates and grown for 48 h to register the aspect of individual colonies under a magnifier. Isolates were tested for their ability to grow in the *Pseudomonas* spp-selective medium Gould’s S1 at 28°C after 48 h (Gould et al., 1985). The production of short chain and long chain N-acyl homoserine quorum-sensing signals was evaluated in plate bioassays (McCLean et al., 1997; Cha et al., 1998). The DNA fingerprints of isolates were generated by PCR with the primer BOXA1R (von der Weid et al., 2000). Cell lysates served as DNA templates for gram-negative isolates, whereas phenol extracts of lysozyme-treated cells were used for gram-positive isolates. The 16S rRNA gene was PCR amplified with primers P0 and P6 (Picard et al., 2000) and sequenced at Macrogen Inc. (Korea). The sequences were used to query the Seqmatch tool of the Ribosomal Database Project II (release 10, update 17) (Cole et al., 2009).

**Screening of plant probiotic activities**

The ability of isolates to fix atmospheric N$_2$ was tested in screw-cap test tubes containing nitrogen-free semisolid NFb medium (Okon et al., 1977). After incubation for 7-10 days at 28 °C, those isolates showing a growth halo at variable deepness under the surface medium were scored positive. Solubilization of mineral phosphate was detected in agar medium containing inorganic phosphate (glucose, 10 g/L; NH$_4$Cl, 5 g/L; NaCl, 1 g/L; MgSO$_4$·7H$_2$O, 1 g/L; CaHPO$_4$, 0.8 g/L; pH 7.2) as a clear halo around the bacterial colonies after 48 h at 28°C (Kuklinsky-Sobral et al., 2004). Indoleacetic acid (IAA) production was analyzed in the supernatant of a tryptophan-amended medium by colorimetry with the Salkowsky reagent, as described elsewhere (Sarwar and Kremer, 1995). The adhesiveness of isolates to an abiotic surface was studied using static growth in poly styrene ELISA microplates and staining of the adhered cells with crystal violet. The amount of the surface-attached cells was proportional to the A$_{650}$ of the solution obtained after dissolving bound crystal violet with ethanol (Jackson et al., 2002). The antagonistic activity of isolates against phytopathogens was investigated in dual plate assays (Ongena et al., 1999). A piece of agar with *Fusarium oxysporum* var. *radicis-lycopersici* strain 22 mycelium or with *Curvularia* sp. mycelium isolated from the CT6919 rice seeds was deposited on the center of potato dextrose plates. After 3 days of incubation at room temperature in the dark, each bacterial isolate was streaked on the opposite edges of the plate (1 isolate per plate) at ca. 3 cm away from the fungal inoculum. For antagonism to *Pythium ultimum* strain 67-1, malt agar plates were first streaked with each isolate, incubated for 3 days at room temperature in the dark, and then a piece of malt agar containing the *P. ultimum* mycelium was deposited on the center. The inhibition of phytopathogen growth was determined visually after 7 days of further incubation and scored positive if the bacterial isolate determined a growth inhibition zone due to diffusion of extracellular metabolites. Plates without any bacterial culture served as the control.

**Effect of isolates on rice germination rate**

Dehusked and surface-sterilized CT6919 seeds were immersed for 5 min in a suspension of washed bacterial cells prepared from saturated cultures. The bacterized seeds were deposited onto soft agar plates (0.7%, w/v agar) containing mineral Farhåeus solution (Farhåeus, 1957) and incubated at 28°C in the dark. After 5 days, the percentage of germinated seeds was scored. Dehusked and surface-sterilized seeds, but not bacterized, served as the germination control.

**Protection of rice seedlings against Curvularia attack**

The protective activity of the bacterial flora already present in the rice CT6919 seeds was analyzed in 250-ml Erlenmeyer flasks containing ca. 100 cm$^3$ of autoclaved vermiculite watered with mineral Farhåeus solution. Two surface-sterilized CT6919 seeds (either husked or dehusked) were planted in each flask (5 flasks per treatment). The *Curvularia* sp. inoculum was applied right above the planted seeds as 100 μl of a suspension containing 10$^5$ conidia. The control seeds received 100 μl of sterilized deionized water. After 7 days of growth