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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).

Certain 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 (Steenhoudt 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

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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; Okunishi *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 antagonist 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_2CO_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_4Cl, 5 g/L; NaCl, 1 g/L; $MgSO_4.7H_2O$, 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 Salkowski reagent, as described elsewhere (Sarwar and Kremer, 1995). The adhesiveness of isolates to an abiotic surface was studied upon static growth in polystyrene 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 (Fahråeus, 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 10^4 conidia. The control seeds received 100 µl of sterilized deionized water. After 7 days of growth

in a greenhouse with a light:dark cycle of 16:8 h and temperatures ranging 20-24°C), the number of emerged and healthy seedlings was scored in each treatment.

Bacterial GFP tagging and analysis of rice root colonization
The isolates CT1 and CT2 were electrotransformed with *ca*. 1 µg of plasmid pBK-mini-Tn*7 gfp1* (Koch *et al.*, 2001) and plated onto NA plates containing 25 µg/ml kanamycin. After 24 h of incubation at 28°C, transformed colonies developed on the plates, and their cells fluoresced green under the UV microscope. Colonization of the CT6919 rice seedlings was studied in semisolid Farhåeus solution (0.2%, w/v agar) or in vermiculite watered with Farhåeus solution. The seedlings were inoculated for 5 days after husk removal and surface sterilization, by dripping 100 µl of a cell suspension containing 10^5 cells/ml of GFP-tagged CT1 or CT2 isolates. The inoculated seedlings were sampled at 5, 7, and 15 days after inoculation for inspection under the fluorescence microscope (Nikon Alphaphot-2 YS2, Japan). Roots were carefully separated with a scalpel, washed gently with deionized water, and deposited on slides for visualization under white or UV light using the appropriate excitation and emission filters for GFP.

Results

Bacterial mesophiles present in seeds of rice varieties cultivated in Argentina

Seedlings of the rice variety CT6919 gave rise to bacterial outgrowths upon transfer to NA plates if the seeds were surface sterilized without removing their husks (Fig. 1A). However, if the seeds were dehusked before surface sterilization, the bacterial contaminants were eliminated (Fig. 1B). This observation suggested that CT6919 grains harbor seed-borne bacteria. To explore if this was only a feature of the CT6919 seeds or a more general feature, we plated the aqueous macerates of surface-sterilized husked seeds of other rice varieties cultivated in Argentina, namely, CAMBA, IRGA 417, and El Paso 144. The number of mesophiles recovered after 24 h incubation at 28°C was comparable between the different seed varieties (2.6 to 7.6×10^6 CFU/g of husked seeds). Every rice variety contained a qualitatively different load of bacterial mesophiles in terms of the types of developed colonies (Fig. 1C). There were no residual culturable bacteria on the external seed surface when the sterilized husked seeds were plated without crushing. On the other hand, we did not recover any bacterial colonies under the growth conditions used in this study, after plating macerates from the seeds that were dehusked before surface sterilization. Thus, we conclude that seeds of the rice varieties CT6919, CAMBA, IRGA 417, and El Paso 144 contain bacterial mesophiles in the compartment delimited by the seed husk and the endosperm.

Gram staining, morphology, and molecular characterization of mesophilic isolates from rice CT6919

A total of 39 isolates were obtained from the surface-sterilized husked CT6919 seeds (Table 1). The isolates CT1 and CT2 were obtained from the colonized roots of CT6919 seedlings (Fig. 1A). The isolates CT3-CT19 correspond to the isolated colonies that showed different pigmentation and aspect under visual observation after 24 h of incubation on plates spread with serial dilutions of CT6919 seed macerates. The isolates CT20-CT39 correspond to the different colonies that developed at 48 h after plating.

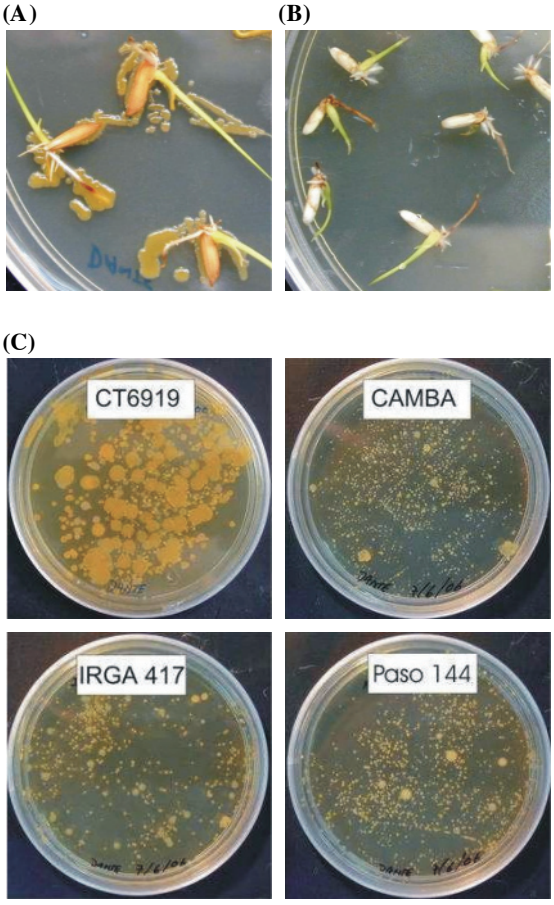


Fig. 1. Endogenous culturable mesophilic bacteria of rice seeds cultivated in Argentina. (A) Bacterial outgrowth on rice seedlings (var. CT6919). The seeds were surface sterilized without removing their seed coats (i.e., husked seeds), germinated in soft water agar for 3 days, and then transferred onto nutrient agar for incubation for 24 h. (B) Removal of seed coat before seed surface sterilization (i.e., dehusked seeds) allows axenic development of rice seedlings, as no colonies appear after the transfer of germinated seedlings to the nutrient agar. (C) Diversity of culturable mesophilic bacteria recovered from the macerates of surface-sterilized husked seeds of rice varieties CT6919, CAMBA, IRGA 417, and El Paso 144.

Among the 39 isolates, the predominant morphologies were bacilli and coccobacilli, either as single cells or as chains. Almost 60% of all the isolates were gram negative (G-). Eighty percent of the isolates obtained at the first 24 h were G-(15/19). Only the isolate CT11 grew in the *Pseudomonas*-selective medium Gould's S1, and their colonies did not release UV-fluorescent pigments. 16S rDNA sequencing confirmed that the isolate CT11 is a non-fluorescent pseudomonad, closely related to the members of the *P. mendocina* group (Table 1). Gram positives (G+) were more abundant among isolates recovered at 48 h after plating (60%; 12/20). Based on BOX-PCR fingerprinting, we could identify 14 banding patterns (A to N) among 28 isolates (Table 1; Fig. 2A). Indeed, the B pattern was the most frequent one (43%;

12/28). As the cellular morphology and colony features of the 12 isolates showing a B-pattern were quite similar (Table 1), it is likely that they represent closely related strains. Based on the microbiological characterization and the results of the BOX-PCR grouping, we selected a subgroup of isolates for 16S rDNA sequencing. Among the G- isolates, we identified members of *Pantoea* (CT1, CT2, CT10, and CT19), *Acinetobacter* (CT5), *Pseudomonas* (CT11), *Sphingomonas* (CT25 and CT33), and *Rhizobium* (CT26) (Fig. 2B). Among the G+, we identified members of *Microbacterium* (CT8, CT24, CT28, CT32, CT34, and CT39), *Curtobacterium* (CT7, CT22, and CT30), *Paenibacillus* (CT14), and *Staphylococcus* (CT21) (Table 1 and Fig. 2B).

Production of AHL-dependent quorum-sensing signals

All the G- isolates assigned to the BOX-PCR group B produced quorum-sensing signals activating both the short and long chain *N*-acyl homoserine lactone (AHL) reporters (Table

Table 1. Characterization of bacterial isolates from rice CT6919 seeds

Isolate	Gram[a]	Colony[b]	S1[c]	BOX[d]	Nearest neighbor by 16S rDNA typing[e]	sAHL[f]	lAHL[g]
CT1	CB(-)	y/s/c	-	A	*Pantoea agglomerans* Sc1 (γ/824 bp/EF585308/0.994)	-	+
CT2	B(-)	y/m/s/c	-	B	*Pantoea agglomerans* S33 (γ/1,408 bp/EF585309/0.987)*	+	+
CT3	CB(-)	y/m/s/c	-	B		+	+
CT4	B(-)	y/s/c	-	B		+	+
CT5	CB(-)	be/s/c	-	C	*Acinetobacter* sp. 4-2 (γ/878 bp/EF585312/0.979)	-	-
CT6	CB(+)	y/s/c	-	D		-	-
CT7	CB(+)	y/s/c	-	D	*Curtobacterium citreum* DSM20528 (A/875 bp/HQ112351/0.987)	-	-
CT8	CB(+)	w/r/c	-	E	*Microbacterium* sp. RE1-14b (A/837 bp/EF585317/0.894)	-	-
CT9	B(-)	be/s/c	-	B		+	+
CT10	B(-)	y/s/c	-	B	*Pantoea ananatis* BD561 (γ/625 bp/HQ112352/0.938)	+	+
CT11	B(-)	y/r/c	+	F	*Pseudomonas* sp. Aek29 (γ/942 bp/EF585313/0.831)*	-	-
CT12	B(-)	y/s/c	-	B		+	+
CT13	B(-)	y/s/c	-	B		+	+
CT14	LB(+)	y/s/c	-	G	*Paenibacillus* sp. FeL05 (F/1,293 bp/EF585318/0.959)*	-	-
CT15	B(-)	y/m/s/c	-	B		+	+
CT16	B(-)	y/m/s/c	-	B		+	+
CT17	B(-)	y/s/c	-	B		+	+
CT18	B(-)	y/s/c	-	B		+	+
CT19	B(-)	be/s/c	-	H	*Pantoea* sp. (γ/535 bp/HQ112353/0.730)	-	-
CT20	CB(-)	y/s/c	-	n.d.		-	-
CT21	C(+)	w/s/c	-	n.d.	*Staphylococcus cohnii* ATCC49330 (F/1,298 bp/HQ112354/0.993)	-	-
CT22	CB(+)	y/s/c	-	n.d.	*Curtobacterium citreum* DSM20528 (A/1,090 bp/HQ112355/0.977)	-	-
CT23	CB(-)	y/s/c	-	n.d.		-	-
CT24	CB(+)	y/s/c	-	n.d.	*Microbacterium* sp. 2761 (A/566 bp/HQ112356/0.940)	-	-
CT25	SB(-)	o/s/c	-	I	*Sphingomonas* sp. Fed32 (α/1,057 bp/EF585314/0.994)*	-	-
CT26	B(-)	be/s/c	-	J	*Rhizobium larrymoorei* SSR03 (α/680 bp/HQ112357/0.930)	-	-
CT27	CB(+)	be/s/c	-	K	*Curtobacterium* sp. Aed04 (A/1,029 bp/JN235990/0.990)	-	-
CT28	CB(+)	o/s/c	-	L	*Microbacterium* sp. 3502 (A/937 bp/HQ112358/0.991)	-	-
CT29	CB(-)	be/s/c	-	I		-	-
CT30	CB(+)	y/s/c	-	n.d.	*Curtobacterium* sp. Aed04 (A/1,125 bp/HQ112359/0.987)*	-	-
CT31	CB(+)	y/s/c	-	n.d.		-	-
CT32	CB(+)	o/s/c	-	M	*Microbacterium* sp. Fek04 (A/923 bp/EF585315/0.983)*	-	-
CT33	LSB(-)	o/s/c	-	n.d.	*Sphingomonas* sp. Fed32 (α/1,172 bp/EF585316/0.997)*	-	+
CT34	CB(+)	o/s/c	-	M	*Microbacterium* sp. 3502 (A/1,045 bp/HQ112360/0.990)	-	-
CT35	CB(+)	y/s/c	-	n.d.		-	-
CT36	CB(+)	y/s/c	-	n.d.		-	-
CT37	CB(-)	y/s/c	-	n.d.		-	-
CT38	B(-)	y/s/c	-	B		+	+
CT39	CB(+)	o/s/c	-	N	*Microbacterium* sp. SB12K-6-3 (A/655 bp/HQ154126/0.967)	-	-

[a] Major cellular morphotype and Gram stain. B, bacilli; C, cocci; CB, coccobacilli; LB, long bacilli; LSB, long streptobacilli; SB, streptobacilli.
[b] Colony pigmentation and aspect. be, beige; c, convex; m, mucoid; o, orange; r, rough; s, smooth; w, white; y, yellow.
[c] Growth on *Pseudomonas* selective medium Gould's S1.
[d] Phylogenetic group according to BOX A1R PCR fingerprinting. Isolates assigned the same letter shared the same PCR banding pattern. n.d., not determined.
[e] The closest phylogenetically related isolate as revealed by 16S rDNA comparison. The major phylogenetic lineage (A, Actinobacteria; F, Firmicutes; α, alpha-proteobacteria; γ, gamma-proteobacteria), the number of 16S rDNA base pairs obtained, the GenBank accession number, and the S_ab similarity score reported by the Seqmatch tool (RDP II, release 10, update 17), are indicated between parentheses. *, isolated from rice seeds by other groups.
[f] Induction of violacein production in a *Chromobacterium violaceum* CV026 bioassay for detection of short chain acyl homoserine lactone production (sAHL).
[g] Induction of *lacZ* expression in an *Agrobacterium tumefaciens* bioassay for detection of long chain acyl homoserine lactone production (lAHL).

Table 1. Continued

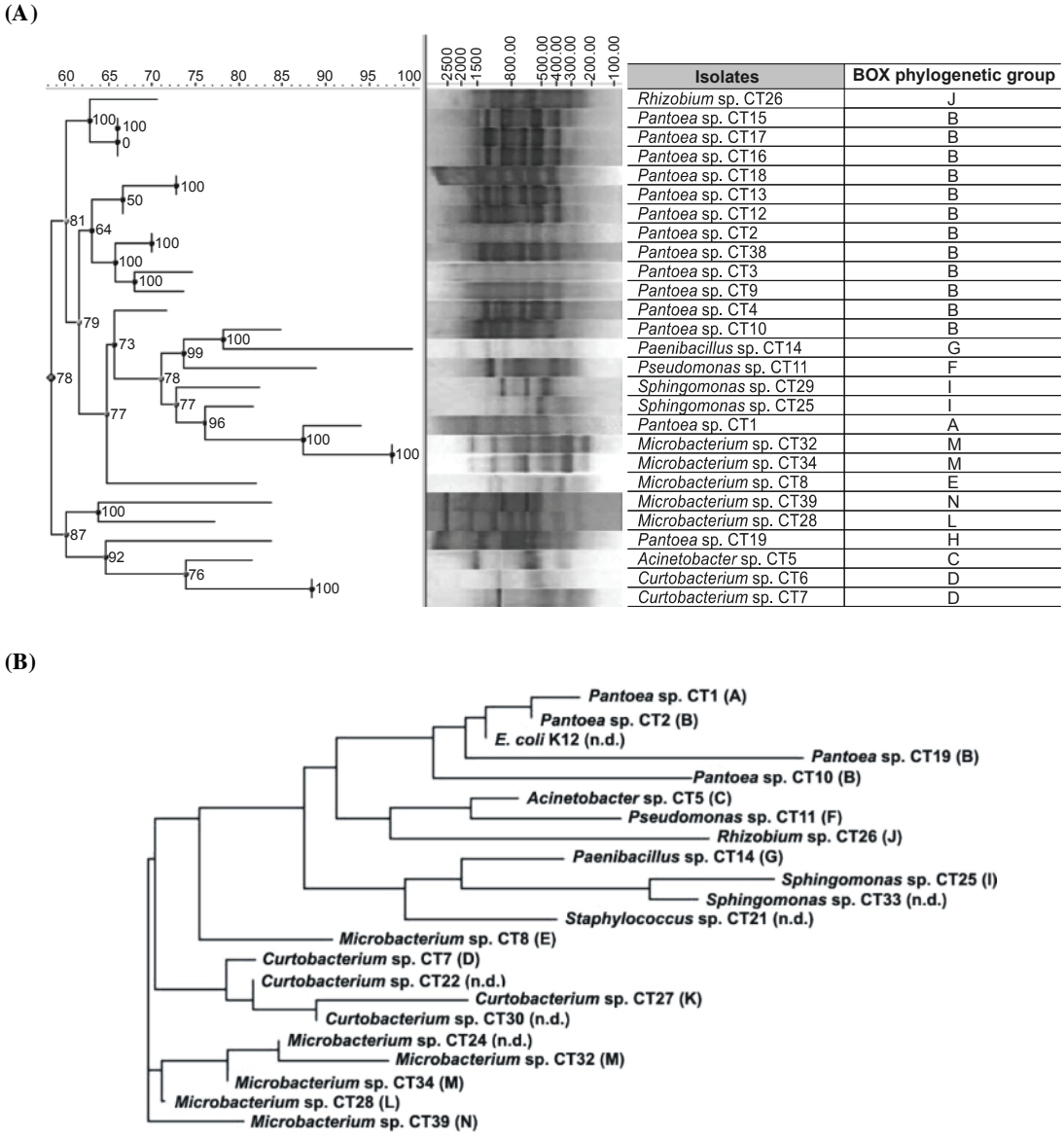
Isolate	NFb[h]	P-solub[i]	IAA[j]	Anti-*Curvularia* sp.[k]	Anti-FORL[l]	Anti-Pu[m]	Adhesiveness[n]	Rice germination[o]
CT1	+	3.5	>40	-	-	+	<5.0	87%
CT2	+	2.5	33	+	+	-	<5.0	93%
CT3	+	3.0	18	+	+	-	<5.0	53%
CT4	+	2.0	29	+	+	-	<5.0	47%
CT5	+	1.0	26	-	-	-	<5.0	60%
CT6	-	1.0	5	-	-	-	<5.0	73%
CT7	-	1.0	24	-	-	-	<5.0	80%
CT8	-	2.0	<5	-	-	-	<5.0	100%
CT9	+	3.5	31	+	-	+	<5.0	47%
CT10	+	2.0	28	+	+	+	9.0	80%*
CT11	+	1.4	20	-	-	-	<5.0	80%
CT12	+	2.0	37	+	+	-	22.3	87%
CT13	+	2.0	17	+	+	-	5.9	67%*
CT14	-	1.4	17	+	-	-	<5.0	0%
CT15	+	2.0	20	+	-	-	6.0	100%
CT16	+	2.0	23	-	-	-	6.1	87%
CT17	+	2.5	35	+	+	-	5.3	80%
CT18	+	2.5	28	+	+	-	<5.0	87%
CT19	-	1.0	9	-	-	-	42.5	93%
CT20	-	1.0	<5	-	-	-	<5.0	93%
CT21	-	1.0	<5	-	-	-	100.0	73%
CT22	-	1.0	<5	-	-	-	13.7	100%
CT23	-	1.0	<5	-	-	-	<5.0	87%
CT24	-	1.0	13	-	-	-	<5.0	80%
CT25	+	1.0	32	-	-	-	<5.0	93%
CT26	-	1.0	22	-	-	-	6.2	87%
CT27	-	1.0	5	-	-	-	<5.0	87%
CT28	-	1.0	7	-	-	+	<5.0	80%
CT29	-	1.0	<5	-	-	-	5.6	93%
CT30	-	1.0	5	-	-	+	<5.0	73%
CT31	-	1.0	<5	-	-	-	9.7	80%
CT32	+	1.0	7	-	-	-	9.3	73%
CT33	+	1.0	>40	-	-	-	<5.0	67%
CT34	-	1.0	9	-	-	-	12.0	93%
CT35	-	1.0	<5	-	-	-	6.7	80%
CT36	-	1.0	<5	-	-	-	<5.0	87%
CT37	-	1.0	5	-	-	-	<5.0	60%
CT38	+	1.8	22	+	+	+	<5.0	73%
CT39	-	1.0	10	-	-	+	15.4	53%

[h] Growth in semisolid nitrogen-free NFb medium as indication of nitrogen fixation ability.
[i] Mineral phosphate solubilization index (diameter of $CaHPO_4$ solubilization halo/colony diameter).
[j] Indole acetic acid production as determined by a colorimetric assay. The data are expressed as μg/ml IAA.
[k] Antagonism to *Curvularia* sp. in dual culture plates.
[l] Antagonism to *Fusarium oxysporum* var. *radicis-lycopersici* strain 22 in dual culture plates.
[m] Antagonism to *Pythium ultimum* isolate 67 in dual culture plates.
[n] Adhesiveness to polystyrene wells in ELISA plates. The results are expressed as % of the maximum adhesiveness shown by isolate CT21 ($A_{650}/OD_{650}=0.35\pm0.04$).
[o] Percentage of the germinated rice CT6919 seeds after bacterization with each isolate and incubation in soft water agar for 5 days at 28°C. *, All germinated seedlings showed reduced growth compared to non-inoculated controls.

1), whereas 2 isolates gave a positive result only in the long chain AHL bioassay (Table 1), i.e., CT1 (*Pantoea* sp.) and CT33 (*Sphingomonas* sp.). Co-culture of each of the 39 isolates and of *P. aeruginosa* PAO1 (producer of C4-AHL and C12-AHL) did not reveal interference with *P. aeruginosa* AHL-dependent induction of violacein synthesis in the *C. violaceum* bioassay.

Growth in nitrogen-free medium, $CaHPO_4$ solubilization, and IAA production

As the isolates obtained in this study from the rice CT6919 seeds may influence seed germination, development, and/or health of the rice seedling, we characterized different PPB phenotypes (Table 1). Almost half of the isolates (18/39) were able to grow in nitrogen-free medium NFb. With the exception of the isolate CT32 (*Microbacterium* sp.), all the nitrogen



Isolates	BOX phylogenetic group
Rhizobium sp. CT26	J
Pantoea sp. CT15	B
Pantoea sp. CT17	B
Pantoea sp. CT16	B
Pantoea sp. CT18	B
Pantoea sp. CT13	B
Pantoea sp. CT12	B
Pantoea sp. CT2	B
Pantoea sp. CT38	B
Pantoea sp. CT3	B
Pantoea sp. CT9	B
Pantoea sp. CT4	B
Pantoea sp. CT10	B
Paenibacillus sp. CT14	G
Pseudomonas sp. CT11	F
Sphingomonas sp. CT29	I
Sphingomonas sp. CT25	I
Pantoea sp. CT1	A
Microbacterium sp. CT32	M
Microbacterium sp. CT34	M
Microbacterium sp. CT8	E
Microbacterium sp. CT39	N
Microbacterium sp. CT28	L
Pantoea sp. CT19	H
Acinetobacter sp. CT5	C
Curtobacterium sp. CT6	D
Curtobacterium sp. CT7	D

Fig. 2. Diversity of bacterial mesophiles isolated from rice CT6919 seeds. (A) Clustering of isolates based on BOX-PCR banding pattern. The analysis was performed with GelCompar (similarity coefficient: Jaccard; dendogram type: neighbor joining optimization: 2%; position tolerance: 2%). (B) Phylogenetic relationships among isolates based on partial 16S rDNA sequences (Table 1). The dendrogram tree was constructed after multiple sequence alignment in the EBI CLUSTAL W2 server (http://www.ebi.ac.uk/Tools/msa/clustalw2/), using default parameters. The BOX-PCR pattern assigned to every sequenced isolate is indicated between parentheses; n.d., 16S rDNA sequence not determined.

fixers were G- and comprised representatives of *Pantoea* sp. (13 isolates), *Sphingomonas* sp. (2 isolates), *Acinetobacter* sp. (CT5), and *Pseudomonas* sp. (isolate CT11). Forty-one percent of the isolates (16/39) were able to grow in a defined medium containing $CaHPO_4$ and produced a clarification halo around the colony indicative of $CaHPO_4$ solubilization. This feature was mainly associated with isolates of the BOX-PCR group B. Finally, based on the concentration of IAA and reactive related compounds detected in cultures grown in a minimal medium containing tryptophan, the isolates could be categorized into 3 groups: strong (>30 μg/ml; 18% of the isolates), intermediate (10-30 μg/ml; 38% of the isolates), and weak IAA producers (<10 μg/ml; 44% of the isolates). Within the first group, we found 5 *Pantoea* sp. isolates and the 2 *Sphingomonas* sp. isolates. Both genera have been reported to synthesize auxins at levels comparable to those detected in this study (Brandl and Lindow, 1996; Tsavkelova *et al.*, 2005).

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Adhesiveness to polystyrene

The ability to develop biofilms on the root surface may confer a competitive advantage and facilitate seedling bacterial colonization during its early development. We tested this capacity indirectly by measuring the adhesiveness of the isolates to

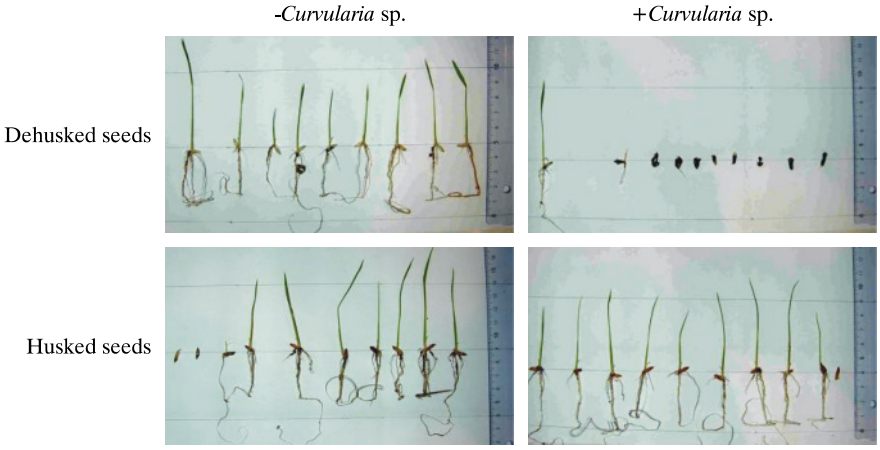

Fig. 3. The endogenous microorganisms of rice CT6919 seeds allow germination and seedling development in the presence of the fungal pathogen *Curvularia* sp. Rice CT6919 seeds were surface sterilized, either with (husked) or without their seed coats (dehusked), planted in flasks containing vermiculite, and inoculated with 10^4 *Curvularia* sp. conidia (+*Curvularia* sp.). Control flasks were amended with the same amount of sterile deionized water (-*Curvularia* sp.).

a polystyrene surface in ELISA plates. We found that most of the isolates were not able to develop tightly adhered biofilms onto polystyrene (Table 1). Only 2 isolates, CT19 (*Pantoea* sp.) and CT21 (*Staphylococcus* sp.), appeared as pellicle-forming isolates (Table 1). The mucoid aspect observed for some isolates on the NA plates did not correlate with their adhesiveness to polystyrene (Table 1).

Antagonism to phytopathogens

During our preliminary tests to germinate the CT6919 rice seeds, we isolated a *Curvularia* sp. strain. This fungal genus is commonly encountered during rice seed health testing (Mew and Misra, 1994). Inoculation of dehusked and surface-sterilized CT6919 seeds with a *Curvularia* spore suspension completely inhibited germination (Fig. 3). However, if the seeds were surface sterilized without removing their seed coats, the pathogenic effect of *Curvularia* sp. was eliminated (Fig. 3). This suggests that the seed endogenous microorganisms contribute to protect the seedling against *Curvularia* sp. infection. We then tested the ability of the CT6919 bacterial isolates to inhibit the growth of *Curvularia* sp. and 2 other unrelated plant pathogens, *Fusarium oxysporum* var. *radicis-lycopersici* strain 22 and *Pythium ultimum* strain 67-1. Several isolates were able to antagonize these phytopathogens *in vitro* (Table 1). Again, the G- isolates belonging to the BOX-PCR group B were the more active strains (12/16 antagonistic isolates), with some differences in their activity spectrum (Table 1). Other *in vitro* antagonists were the G+ isolates *Paenibacillus* sp. CT14, *Curtobacterium* CT30, and *Microbacterium* sp. CT28 and CT39 (Table 1).

Effect of isolates on seed germination and development

Finally, considering that all characterized isolates originated from the interior of the husked CT6919 seeds, we evaluated their individual effect on the germination rate of the surface-sterilized CT6919 seeds. A few isolates, namely, CT10, CT13, and CT14, resulted detrimental for seed germination and/or development (Table 1). Another group of isolates (12/39; 30%) reduced the germination rate to <80%, whereas the majority of the isolates (24/39; 62%) did not show important effects on bacterized CT6919 seeds (Table 1).

Colonization of rice seedlings by the *Pantoea* sp. isolates CT1 and CT2

The bacterial isolates described in this study are potential colonizers of rice tissues, a trait that may facilitate invasion and spreading. We studied the colonization pattern of the first isolates from CT6919 seeds, *Pantoea* sp. CT1 and CT2 (Table 1). These strains belong to one of the two most prevalent bacterial groups (*Pantoea* spp.) in CT6919 seeds, and they are easier to transform than the other prevalent group of isolates (*Microbacterium* and *Curtobacterium* spp.). To this end, we generated the GFP-tagged derivatives CT1-*gfp* and CT2-*gfp*. We observed yellowish and light-green autofluorescence in the borders of epidermal and outer cortical cells, and red autofluorescence in the inner cortex and in the vasculature (Fig. 4A). However, this autofluorescence did not interfere with the detection of the GFP-tagged strains. Seven days after inoculation, CT1-*gfp* preferentially colonized the base of the lateral roots and lenticels, with very low or null colonization of root apices and of root hairs (Fig. 4B). CT2-*gfp* also preferably colonized the zone of lateral root emergence and lenticels, but opposite to CT1-*gfp*, CT2-*gfp* profusely colonized root hairs (Fig. 4C). For both the strains, we observed single cells present in the intercellular spaces of the root epidermis (Fig. 4D). These cells were tightly adhered as they remained attached after 30 min of thorough root washing with saline solution at 250 rpm. We detected less colonizing cells at 5 or at 15 days after inoculation, as compared to 7 days after inoculation. The matrix used for seedling growth (soft agarized medium or vermiculite) did not affect the colonization pattern of CT1-*gfp* and CT2-*gfp*. We tried to recover CT1-*gfp* and CT2-*gfp* cells from the macerates of the surface-sterilized seedlings used for the colonization studies, but we did not

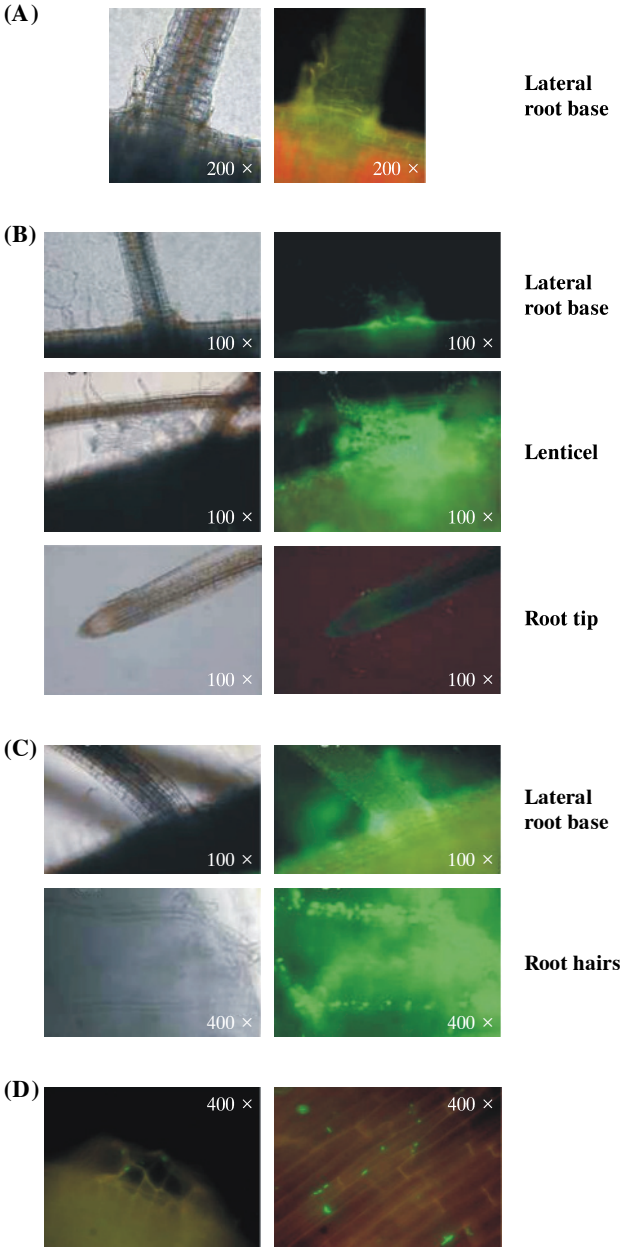

Fig. 4. Colonization pattern of rice CT6919 roots by GFP-tagged isolates CT1 and CT2 (*Pantoea* sp.). (A) Non-inoculated control seedlings. The roots show yellowish autofluorescence in the epidermal and outer cortical layers, and reddish autofluorescence at the inner cortical and vascular tissues. (B) Seedlings inoculated with *Pantoea* sp. CT1-*gfp*. (C) Seedlings inoculated with *Pantoea* sp. CT2-*gfp*. For A, B, and C, the left panels are micrographs of roots examined with white light; the right panels are micrographs of the same root section examined with UV light for GFP detection. (D) Tightly adhered cells of *Pantoea* sp. CT1-*gfp* at the site of emergence of a lateral root (left) and at the intercellular spaces between epidermal cells (right).

succeed in obtaining kanamycin-resistant and fluorescent colonies (data not shown). This suggests that either the label is lost or the sterilization procedure applied on the colonized roots reached internal tissues, or the colonization does not progress internally under the experimental conditions used during the short period of interaction between CT1 or CT2 cells and the root (15 days).

Discussion

For rice, a main source of world population food, the microbial inoculant technology is not yet developed. Thus, isolation of PPB from rice and the demonstration of their ability to recolonize and promote plant growth and/or health would be an important step forward in the selection of strains to formulate rice inoculants. Both aerial and belowground rice tissues contain endophytes from different phylogenetic groups (Rosenblueth and Martinez-Romero, 2006; Sun *et al.*, 2008; Hardoim *et al.*, 2011), whereas the seeds of rice varieties cultivated in Eastern countries also have been reported to harbor bacteria (Cottyn *et al.*, 2001, 2009; Okunishi *et al.*, 2005; Mano *et al.*, 2006). In this work, we have explored the diversity of culturable mesophilic bacteria present in rice seeds cultivated in Argentina, in particular, for the rice variety CT6919, and characterized plant probiotic activities in order to get insights into the putative benefits of their presence for rice seeds.

We found that seeds of the rice cultivars CT6919, CAMBA, IRGA 417, and El Paso 144, contain a variety of mesophilic culturable bacteria beneath their seed coats, averaging 6×10^6 CFU/g of seeds (Fig. 1). The bacterial load differed qualitatively for each rice cultivar (Fig. 1C), which may reflect the plant specific selection of endophytes, as recently suggested by molecular studies of rice root endophytes (Hardoim *et al.*, 2011). The use of rich medium, the incubation temperature (28°C), and the short incubation period (24-48 h) most probably resulted in an underestimation of the amount and diversity of culturable bacteria present in the rice varieties studied here (Okunishi *et al.*, 2005). We selected 39 isolates of relatively fast growing mesophilic and copiotrophic bacteria from macerate platings of surface-sterilized husked seeds of rice CT6919 (Table 1). Their microbiological and molecular characterization revealed a diversity of G- and G+ isolates, including members of α- and γ-proteobacteria, Firmicutes and Actinobacteria (Table 1 and Fig. 2). The prevalent genus was the enterobacterial *Pantoea* (at least 14 out of 39 isolates; 36%), probably due to its higher seed load and/or its higher growth rate. Members of the Enterobacteriaceae were also the most frequently isolated bacteria from the seed crushings of rice varieties cultivated in the Philippines (Cottyn *et al.*, 2001). *Pantoea* is a heterogeneous genus whose phylogenetic relationships have been recently approached by means of molecular methods (Rezzonico *et al.*, 2009). Endophytic *Pantoea* strains have been recovered from soybean, pea, maize, sugarcane, sweet potato, and rice (Elvira-Recuenco and Van Vuurde, 2000; Mondal *et al.*, 2001; Feng *et al.*, 2003; Asis and Adachi, 2004; Loiret *et al.*, 2004; Rosenblueth and Martinez-Romero, 2006), with different plant probiotic activities, including nitrogen fixation, mineral phosphate solubilization, auxin production, and chitinase secretion (Mondal *et al.*, 2001; Loiret *et al.*, 2004; Ovadis *et al.*, 2004; Dastager *et al.*, 2009). Some antibiotic- producing *Pantoea* strains have become promising biocontrol tools (Wright *et al.*, 2001; Bonaterra *et al.*, 2003; Rezzonico *et al.*, 2009). The *Pantoea* strains isolated from rice CT6919 seeds showed

slightly different profiles of plant probiotic activities (i.e., CT1, CT2, and CT15), with some of the isolates (CT4 and CT13) displaying detrimental effects on the germination of rice seeds when present as a single bacterial species (Table 1).

Apart from *Pantoea* spp., we found close relatives to the G- *Sphingomonas* sp., *Acinetobacter venetianus*, *Pseudomonas* sp., and *Rhizobium larrymoorei*, and to the G+ *Curtobacterium* sp., *Microbacterium* sp., *Paenibacillus* sp., and *Staphylococcus cohnii* (Table 1 and Fig. 2). The actinobacterial *Microbacterium* was the prevalent G+ genus among the isolates from the rice CT6919 seeds (Table 1), as reported for mature seeds of rice varieties cultivated in Philippines and Japan (Mano *et al*., 2006; Cottyn *et al*., 2009). However, in contrast to what has been reported for mature Nipponbare rice grains, we could recover both G- as well as G+ from mature CT6919 seeds (Table 1), suggesting that there might be slight differences in the population structure of bacterial species harbored in rice seeds from different varieties and geographical locations (Fig. 1) (Hardoim *et al*., 2011).

Diverse diazotrophic endophytes have been isolated from the roots and shoots of wild-type and cultivated rice varieties, including *Herbaspirillum*, *Ideonella*, *Enterobacter*, *Pantoea*, *Sphingomonas*, *Serratia*, *Burkholderia*, *Citrobacter*, and *Azospirillum* species (Elbeltagy *et al.*, 2001; Gyaneshwar *et al.*, 2001; Rangarajan *et al.*, 2002; Tripathi *et al.*, 2002; Feng *et al*., 2003; Li *et al.*, 2006; Singh *et al.*, 2006; Sun *et al*., 2008). Almost half of the isolates from the CT6919 seeds could grow in a nitrogen-free medium (Table 1). Although this phenotype must be confirmed with a quantitative acetylene reduction assay and the detection of *nif* genes or nitrogenase protein, the fact that the same phylogenetic groups have been previously isolated as endophytic nitrogen fixers from rice supports the diazotrophic character of the CT6919 isolates. Several *Pantoea* spp. isolates that solubilized $CaHPO_4$ in a plate assay (Table 1) may contribute to rice P nutrition in the rhizosphere. Under appropriate stimulating culture conditions, an important group of isolates produced moderate (10-30 μg/ml) or high (>30 μg/ml) levels of auxin-like compounds (Table 1). The production of this phytohormone in the rhizosphere may positively influence rice root development and increase rice absorption capability. Bacterial auxin supply does not always represent a benefit for host plants (Xi *et al.*, 1999), so further tests are required to evaluate the relevance of IAA production by these isolates on rice seedlings development.

The microbial flora present in rice CT6919 seeds conferred protection to the seedlings against infection by the fungal isolate *Curvularia* sp. (Fig. 3). This biocontrol effect may result from the additive and/or synergistic activity of several antagonistic bacterial species present in the seeds and may constitute a mechanism to increase seedling survival and establishment in the soil. Consistent with this observation, several isolates were able to individually inhibit the *in vitro* growth of *Curvularia* sp. and of 2 other broadly recognized phytopathogens (*F. oxysporum* and *P. ultimum*) (Table 1). Notably, bacterization of axenic dehusked CT6919 seeds with certain single isolates resulted in inhibited germination or perturbed seedling development (Table 1). Such a detrimental effect is certainly neutralized by other microorganisms present in the seeds, either by competitive or by regulatory interactions, as husked and surface-sterilized CT6919 seeds do germinate normally (Fig. 3). This observation reflects the necessity to approach the study of bacterial-plant interactions considering the existence of multiple bacterial strains. To summarize, we have characterized some putative plant probiotic activities in a set of mesophilic isolates obtained from rice CT6919 seeds, which might have been evolutionarily selected and propagated by the plant according to the conferred benefits, to ensure their vertical transmission to the seedling. Isolates like CT2, CT12, and CT17, which combine nitrogen fixation, $CaHPO_4$ solubilization, auxin production, and phytopathogen antagonism, deserve further studies to evaluate rice growth promotion and biocontrol of phytopathogens when present, singly or as a combination, in an inoculant formulation.

How rice seed bacteria colonize the grain is not yet understood. One possibility is by lateral transmission through water, wind, or biological vector dispersion, which facilitates microorganism movement to inflorescences (Robertson and Alexander, 1994; Mundt *et al.*, 1999). Otherwise, bacteria may migrate from the colonized root system to aerial tissues and inflorescences during grain filling (Chi *et al.*, 2005). Thus, seed loading with PPB would ensure their presence from the very early stages of seedling development to improve germination success and establishment in the soil (Rosenblueth and Martinez-Romero, 2006). We have analyzed the root colonization pattern of 2 rice CT6919 isolates (CT1 and CT2), representatives of the most frequent type of seed colonizers recovered in this study (Table 1). Although these 2 strains are indeed phylogenetically related (as judged by their 16S rDNA partial sequence), they markedly differ in other properties (e.g., cell shape, exopolysaccharide production and colony mucoidy, AHL signal production, phosphate solubilization index, IAA production, and antifungal inhibition pattern) (Table 1). Both GFP-tagged isolates showed preference by the site of emergence of lateral roots and lenticels (Figs. 4B and C), but only isolate CT2 colonized the root hairs (Fig. 4C). Thus, these 2 phylogenetically related strains also showed differential behavior in terms of root colonization. Both CT1 and CT2 cells were visualized firmly adhered in the intercellular spaces of epidermal cells and in the proximity of lateral roots (Fig. 4D). A comparable root colonization pattern has been reported for other rice endophytes (Mondal *et al*., 2001; Verma *et al.*, 2004). Cracks in the rice root epidermis represent excellent entry points for the internal colonization of the root system and further spreading, as demonstrated for *Herbaspirillum seropedicae* (James *et al.*, 2002) and endophytic rhizobia (Chi *et al*., 2005). However, we could not recover CT1 and CT2 as the root endophytes upon surface sterilization of the young seedling roots used for colonization studies. Further experiments and the use of confocal fluorescence microscopy are required to determine the internal colonization habit of these isolates and their tissue distribution within rice plants along different stages of development.

In conclusion, from this and other studies (Mano *et al*., 2006; Cottyn *et al*., 2009), it comes out that rice seeds contain an important diversity of seed-borne bacteria. This figure may even turn more complex with the utilization of other growth conditions and with the application of culture-independent methods to analyze seed microbial populations. Seed-borne rice bacteria may favor germination, early seedling develop-

ment, and plantlet establishment in the soil. At the same time, seed endophytes may outcompete strains introduced in commercial inoculants (Bacilio-Jiménez *et al*., 2001), highlighting the need to investigate the composition and properties of seed-borne flora for a better selection of novel inoculant strains.

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