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ORIGINAL PAPER

Pseudomonas spp. isolates with high phosphate-mobilizing potential and root colonization properties from agricultural bulk soils under no-till management

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Abstract Seven phosphate-mobilizing pseudomonads were isolated, identified, and characterized in terms of their biofertilizer potential and root-colonizing properties. *Pseudomonas protegens* (ex-*fluorescens*) CHA0 was used for comparative purposes. Four isolates (LF-MB1, LF-P1, LF-P2, and LF-P3) clustered with members of the "*Pseudomonas fluorescens* complex," whereas the other three (LF-MB2, LF-V1, and LF-V2) clustered with members of the "*Pseudomonas putida*/*Pseudomonas aeruginosa* complex." Assays in buffered liquid growth medium supplemented with tricalcium phosphate enabled the separation of the isolates into two groups: group A (LF-P1, LF-P2, LF-P3, and LF-V1) solubilized P from 151 up to 182 μg mL^{-1}, and group B (LF-MB1, LF-MB2, and LF-V2) solubilized less than 150 μg PmL^{-1}. All isolates displayed acid and alkaline phosphatase activities. With the exception of LF-MB2, all isolates were able to degrade phospholipids from lecithin. Additionally, all isolates exhibited extracellular protease activity, and four isolates produced hydrogen cyanide, two traits that are related to biocontrol of phytopathogens. To study root colonization in non-sterile soil, isolates were doubly tagged with *gfp* and a tetracycline resistance cassette. After 15 days of competition with the indigenous bacterial flora, all tagged isolates colonized soybean roots at counts ranging from 7.6×10^5 to 1.7×10^7 CFU g^{-1}. The results indicate that there are already efficient phosphate-mobilizing pseudomonads adapted to agricultural bulk soils under no-till management in Argentina and thus having excellent potential for use as biofertilizers.

Keywords Pseudomonads · Phosphate-mobilizing microorganisms · Tricalcium phosphate solubilization · Phosphatase activity · Phospholipase activity · Root colonization · Soil under no-till management

Leticia Fernández and Betina Agaras equally contributed to this paper. Rizobacter Argentina S.A. has priority access to the bacterial isolates reported here.

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Introduction

Phosphorus (P) is one of the main plant nutrients, the lack of which limits plant growth. Most agricultural soils contain large reserves of total P, commonly in the range of 200 to 5,000 mg Pkg^{-1} with an average of 600 mg Pkg^{-1}, due to the regular application of chemical fertilizers or sludge from wastewater treatment (Ravindra et al. 2008). Both P fixation and precipitation occur in soil because of the large reactivity of phosphate ions with numerous soil constituents. Thus, only a small proportion of P is immediately available for plant uptake (Gyaneshwar et al. 2002; De-Bashan and Bashan 2004). Plants obtain P as orthophosphate anions, predominantly in the form of HPO_4^{2-} and $H_2PO_4^{1-}$ from the

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soil solution. In most soils, the concentration of orthophosphate in solution is low and must be replenished from other pools of soil P to satisfy plant requirements (Richardson et al. 2009). In this context, microorganisms are key participants in the soil P cycle mediating the availability of P to plants (Richardson and Simpson 2011). "Phosphate mobilization" is an expression that includes two different traits of microorganisms related to changes in P availability, e.g., P solubilization from inorganic sources and P mineralization from organic sources. The inverse process, P immobilization, refers to the incorporation of inorganic phosphate from the soil solution into organic P through metabolic reactions, or into soil particles through adsorption or precipitation (Richardson and Simpson 2011). Thus, phosphate-mobilizing microorganisms, an accepted class of plant growth promoting rhizobacteria (PGPR), may be used as biofertilizers to increase P availability to plants, minimizing the use of expensive chemical P fertilizers (Lugtenberg and Kamilova 2009; Oliveira et al. 2009; Singh et al. 2011).

Phosphate-solubilizing bacteria belonging to the *Pseudomonas*, *Bacillus*, *Rhizobium*, *Agrobacterium*, *Burkholderia*, *Achromobacter*, *Micrococcus*, *Aerobacter*, *Enterobacter*, *Flavobacterium*, *Paenibacillus*, *Rahnella*, *Escherichia*, and *Erwinia* genera have been isolated from soils (Rodríguez and Fraga 1999; Kämpfer 2007). Among these, pseudomonad isolates are of interest because they usually exhibit additional plant probiotic traits such as antagonism to fungal pathogens and induced systemic resistance (Haas and Défago 2005; Picard and Bosco 2007; Barrett et al. 2011), biodegradation of pollutants and of plant growth inhibitors (Ramette et al. 2011), and biocontrol and biofertilization properties in agricultural fields (De Souza et al. 2003). In addition, members of the genus *Pseudomonas* are excellent rhizosphere colonizers (Ravindra et al. 2008; Browne et al. 2009). These characteristics make pseudomonads interesting PGPR candidates as components of agricultural inputs to stimulate plant development and/or promote crop health.

In Argentina, about 25 million hectares are currently cultivated under no-till management, with soybean covering more than 15 million hectares, and maize and wheat together covering ca. 5 million hectares (Wall 2011). The benefits of the no-till practice for these extensive crops in terms of physical and chemical soil properties have been documented (Picone et al. 2007; Ferreras et al. 2007; Cantú et al. 2007; Álvarez et al. 2009; Campitelli et al. 2010), but data is lacking on the biological indicators of soil health under this regime. Recently, a multidisciplinary consortium was created to study soil biology related to crop productivity under sustainable agricultural management (Wall 2011), one of the aims of which is to generate a collection of plant probiotic microorganisms already adapted to local soil and plant host rhizosphere conditions. Evidence points to a soluble P deficit in Argentina's pampean flatlands, and thus, interest has been focused on the possibility of inoculating phosphate-mobilizing microorganisms into the soil to promote solubilization of inorganic P and mineralization of organic P (Richardson and Simpson 2011). Here, we report the isolation, identification, and characterization of the biofertilizer potential and root-colonizing properties of phosphate-mobilizing pseudomonads from agricultural bulk soils under no-till management in Argentina.

Materials and methods

Site description and soil sampling

Samples of top soil (0–10 cm) were collected in agricultural fields located across a west–east transect in the most productive region of the Argentinean Pampas, in the vicinity of Bengolea, Córdoba Province (33°01′31″S; 63°37′53″W); Monte Buey, Córdoba Province (32°58′14″S; 62°27′06″W); Pergamino, Buenos Aires Province (33°56′36″S; 60°33′57″W); and Viale, Entre Ríos Province (31°52′59″S; 59°40′07″W). Soil samples were sieved through a 2-mm mesh to remove roots and plant detritus, and stored at 4°C until processing. The physicochemical properties of the sampled soils that gave origin to the isolates (Table 2) were (a) for LF-MB1 and LF-MB2: typical Argiudoll, silt loam, 1.7% C, 0.132% N, 20.6 mg kg^{-1} P, pH 6.2; (b) for LF-P1: typical Argiudoll, silt loam, 1.7% C, 0.153% N, 18 mg kg^{-1} P, pH 6.0; (c) for LF-P2 and LF-P3: typical Argiudoll, silt loam, 2.7% C, 0.233% N, 10.5 mg kg^{-1} P, pH 6.2; and (d) for LF-V1 and LF-V2: Argic Pelludert, silty clay, 5% C, 0.369% N, 20.2 mg kg^{-1} P, pH 6.4.

Isolation of phosphate-solubilizing pseudomonads

Bulk soil suspensions were obtained by shaking 10 g of sieved soil in 90 mL of 0.85% *w/v* NaCl for 30 min at 180 rpm at room temperature. Decimal serial dilutions were plated onto National Botanical Research Institute Phosphorus (NBRIP) agar, a minimal medium with insoluble tricalcium phosphate $Ca_3(PO_4)_2$ as the sole P source (Nautiyal 1999). The plates were incubated at 28°C for 7 days. Isolates were examined for phosphate solubilization on NBRIP agar on the basis of the formation of a clearing zone around the colony. Every phosphate-solubilizing isolate was streaked onto Gould's S1 agar (Gould et al. 1985), a selective medium for pseudomonads. S1 plates were incubated at 28°C for 3 days. Positive isolates were re-streaked on nutrient agar plates (tryptone soy agar 40 gL^{-1}, yeast extract 5 gL^{-1}) to check their purity. Unless otherwise stated, isolates were routinely grown overnight in nutrient yeast broth (nutrient broth 25 gL^{-1}, yeast extract 5 gL^{-1}) at 28°C under shaking. All isolates were stored as frozen suspensions in 20% glycerol for further studies. The biocontrol and phosphate solubilizer *Pseudomonas protegens*

(ex-*fluorescens*) strain CHA0 was used for comparative purposes (De Werra et al. 2009; Ramette et al. 2011).

Identification of phosphate-solubilizing pseudomonads

The taxonomic identity of the isolates was determined by sequencing the conserved 16S rDNA gene and the *Pseudomonas*-specific marker gene *oprF* (Bodilis et al. 2006). The 16S rDNA gene was amplified by polymerase chain reaction (PCR) with primers P0 and P6 (Picard et al. 2000), and the *oprF* gene was amplified with primers oprF-FW2 and oprF-Rev2, as described elsewhere (Agaras et al. 2011). Thermal cell lysates served as DNA source. One to five microliters of DNA template were added in a total reaction volume of 20 μl. The PCR products were loaded onto in 1% agarose gels (Ultrapure™ Agarose, Invitrogen) run at 2 V cm^{-1} for 40 min. A 100-bp ladder (PB-L, Argentina) was used as a size standard.

The 16S rDNA and *oprF* amplicons were sequenced by Macrogen Inc. (Seoul, Korea) with primers P0 for 16S rDNA and oprF-FW2 for *oprF*. The 16S rDNA sequences were used to query the Seqmatch tool of the Ribosomal database project II (Cole et al. 2009). The BlastN tool in the NCBI database was used to analyze *oprF* sequences. Phylogenetic analysis was performed on sequence concatenation of conserved regions of 16S rDNA (300 nt, from position 225 to 524 of the *Pseudomonas fluorescens* Pf-5 16S rRNA gene; AJ417072) and *oprF* (276 nt, from position 282 to 558 of the *P. fluorescens* Pf-5 *oprF* gene; NC004129). Neighbor-joining trees were inferred from evolutionary distances calculated with the Kimura 2-parameter formula, using the software MEGA v4 (Tamura et al. 2007). Confidence analyses were undertaken using 1,000 bootstrap replicates. Positions containing alignment gaps and missing data were eliminated only in pairwise sequence comparisons (pairwise deletion option).

Phosphate solubilization efficiency in agar plates

Phosphate solubilization efficiency (SE) on NBRIP agar was determined according to Ramachandran et al. (2007) as SE=(diameter of solubilization halo/diameter of colony)× 100. The isolates with the highest SE were selected for quantitative estimation of phosphate solubilization.

Phosphate solubilization in liquid medium

Phosphate solubilization was quantified in liquid NBRIP medium with 5 gL^{-1} of $Ca_3(PO_4)_2$, modified by buffering with 100 mM Tris, and adjusting the pH to 8.0 with HCl. Triplicate cultures were incubated at 28°C with 180 rpm on a rotary shaker. Samples were withdrawn at 0 and 7 days after cultivation to assess soluble phosphate, phosphatase activity and CFU counts, and for pH determination. For quantitation of soluble phosphate, 1 mL of the cell suspension was centrifuged (Biofugepico, Germany) at 14,800×*g* for 2 min, and the P content in the supernatant was determined by the molybdenum blue method (Murphy and Riley 1962). Bacterial counts were estimated with the drop plate method (Herigstad et al. 2001) on Gould's S1 agar after incubation at 28°C for 3 days.

Phosphatase activity

Acid and alkaline phosphatase activities were quantified in triplicate cultures containing buffered liquid NBRIP medium. An aliquot of each isolate culture was assayed for the quantitative study of phosphatase activity according to the modified protocol of De Freitas et al. (1997). Briefly, 0.1 mL of culture was incubated at 37°C with 0.1 mL 0.05 M *p*-nitrophenyl phosphate and 0.4 mL modified universal buffer, pH 6.5 or pH 11, for assay of acid or alkaline phosphatase, respectively. After 1 h, the reaction was stopped by adding 0.1 mL 0.5 M $CaCl_2$ and 0.4 mL 0.5 M NaOH. Control reactions contained sterile liquid medium. The assay mixtures were centrifuged at 13,000×*g* for 2 min, and the intensity of the yellow color of *p*-nitrophenol (*p*NP) was measured in the supernatant as absorbance at 405 nm. Phosphatase activities of the isolate cultures were expressed as micrograms *p*NP produced per milliliter per hour.

Phospholipase detection on agar plates

Phospholipase release was detected qualitatively on egg yolk agar. Ten microliters of overnight culture were spot inoculated onto the plates and incubated at 28°C for 48 h. Phospholipase activities were detected as either opaque (phospholipase C) or clear (phospholipase A) zones surrounding the colonies (Sacherer et al. 1994). The relative phospholipase activity was calculated as (diameter of halo/diameter of colony)×100.

Extracellular protease activity and cyanhidric acid (HCN) production

For screening of extracellular protease activity, 10 μL of overnight culture were spot inoculated onto skim milk agar plates and incubated at 28°C for 24 h. Proteolytic activities were detected as a clear zone around the colonies (Sacherer et al. 1994). The relative extracellular protease activity was calculated as (diameter of halo/diameter of colony)×100.

Cyanhidric acid production was detected using the picrate filter paper method (Egan et al. 1998). Pieces

of picrate-saturated filter papers measuring 1 cm^2 were deposited on the lid of a Petri dish sealed with parafilm and incubated at 28°C. The change in color of the filter paper from yellow to orange was recorded at 24 h as an indication of cyanogenesis. Reactions from inoculated plates were visually compared with those from non-inoculated control plates.

Analysis of root colonization by phosphate-solubilizing pseudomonads

In order to detect and quantify root colonization in natural (non-sterile) soil, the isolates were tagged by electrotransformation with plasmid pME7402, which confers tetracycline resistance and *gfp* expression (Dubuis et al. 2006). Rhizosphere colonization was studied in 300-cm^3 pots containing natural soil (16–32 cm layer) from the experimental field of Universidad Nacional del Sur (0.97% C, 0.18% N, and extractable P, 3 mg kg^{-1}). Soybean (var. SPS 3900), wheat (var. Baguette 19), and maize (var. C3) seeds were surface disinfected with ethanol (1 min) and then with sodium hypochlorite (25 gL^{-1}; 15 min for soybean, and 10 min for wheat and maize), followed by six rinses in sterile distilled water. Surface-disinfected seeds were then inoculated by immersion in 20 mL of 0.85% NaCl containing approximately 10^8 CFU/mL of each tagged isolate. Pots were sown with inoculated soybean (5), maize (5), or wheat (10) seeds; control pots were sown with surface-disinfected but non-bacterized seeds. Five pots per treatment were set up for each plant. The experiment was carried out in a growth chamber under controlled environmental conditions: 25±3/20±3°C day/night temperature, 12-h photoperiod at 400 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD) from fluorescent lamps, and 55–60% relative humidity. Plants were watered daily with sterile distilled water.

Wheat plants were harvested at 3 weeks after sowing, maize and soybean plants at 2 weeks. The presence of colonizing cells on the roots was first examined by fluorescence microscopy under an optical microscope Nikon Eclipse 80i with a digital DXM1200F Nikon camera (blue excitation filters between 470 and 480 nm and emission filters of 510–515 nm). Roots were then thoroughly washed with sterile distilled water to remove adhered soil, cut into 1 to 5 cm pieces, and shaken vigorously in 20 mL of 0.85% NaCl. The number of CFU per gram of dry weight was determined with the drop plate method by plating serial decimal dilutions of the rhizosphere suspensions on nutrient agar containing 125 μg mL^{-1} tetracycline and 20 μg mL^{-1} chloramphenicol (for which pseudomonads are naturally resistant to).

Statistical analysis

The assays were carried out in a completely randomized design. Multiple comparisons were performed with a one-way analysis of variance. When a significant *F* value was detected, least significant difference (Fisher LSD) was used for comparison of means. Differences were considered to be significant when the *P* value was less than or equal to 0.05.

Results

Isolation and screening of phosphate-solubilizing pseudomonads

Of the 357 colonies that grew and displayed a phosphate solubilization halo in NBRIP plates, 80 isolates (22%) retained this ability upon subculture in NBRIP plates. The collection comprised isolates from agricultural bulk soils with a documented history of no-till management of at least 10 years, from different geographical locations: 14 isolates from Bengolea, 16 from Monte Buey, 30 from Viale, and 20 from Pergamino. Forty-six isolates were able to grow in the *Pseudomonas* selective medium S1, and all of them developed diffusible pigments that fluoresced with varying intensity and color under UV light. Finally, seven bacterial strains with phosphate solubilization efficiencies ranging from 133% to 200% were selected for further studies (Table 1).

Identification of phosphate-solubilizing pseudomonads

All of the isolates (Table 1) were positive with PCR primers for the *Pseudomonas*-specific marker gene *oprF*. This result confirmed their genus assumption based on their ability to grow on S1 medium (Agaras et al 2011). Sequence of the 16S rDNA and *oprF* genes allowed us to determine the phylogenetic position of the isolates among the pseudomonads (Table 2 and Fig. 1). Four isolates (LF-MB1, LF-P1, LF-P2, and LF-P3) clustered with members of the "*P. fluorescens* complex," whereas the other three (LF-MB2, LF-V1, and LF-V2) clustered with members of the "*Pseudomonas putida*/*Pseudomonas aeruginosa* complex" (Fig. 1).

Phosphorus mobilization ability of the isolates

Based on the inorganic phosphate-solubilizing activity in liquid medium that was initially buffered with 0.1 M Tris at pH 8.0, the isolates could be separated into two groups: group A included isolates that solubilized from 151 up to 182 μg mL^{-1} and group B those that solubilized less amounts of P (<150 μg P mL^{-1}; Table 1). The P solubilization correlated with the extent of culture acidification, as the pH of group A dropped from 8 to 4.25–4.87, and that of

Table 1 Phosphate-mobilizing properties of pseudomonad isolates from agricultural bulk soils under no-till management in Argentina

Strain/isolate	Group[a]	SE (%)	Solubilization activity in buffered liquid medium[b]			Phosphatase activity (μg*p*NP mL^{-1} h^{-1})[d]		PL (%)
			Final pH	Soluble P (μg mL^{-1})[c]	Surviving bacteria (CFU/mL)	Acid	Alkaline	
CHA0	A	160	4.25	189±12	1.2×10^6	105±17 a	62±7.7 a	188
LF-V1	A	200	4.66	178±19	3.2×10^4	58±2.3 b	61±6.8 ab	118
LF-P2	A	171	4.39	167±11	1.4×10^7	47±2.8 cd	51±3.1 c	133
LF-P3	A	133	4.79	151±13	9.6×10^6	60±1.9 b	54±0.7 bc	128
LF-P1	A	142	4.87	182±40	1.2×10^5	45±2.3 cd	48±4.4 c	148
LF-MB2	B	160	5.55	149±11	5.0×10^6	40±1.7 d	49±1.7 c	0
LF-V2	B	166	5.50	149±20	3.8×10^6	57±4.1 bc	59±3.1 ab	133
LF-MB1	B	150	5.84	128±45	5.5×10^6	42±5.7 d	47±1.4 c	139

SE solubilization efficiency, *PL* phospholipase activity

[a] Group A includes strains which solubilized more than 150 μg P mL^{-1}, and group B includes strains which solubilized less than 150 μg P mL^{-1}

[b] pH, soluble P, and bacterial count were determined 7 days after medium inoculation. The soluble P concentration in non-inoculated medium was <5 μg mL^{-1}

[c] Results are mean of three replicates±its standard deviation. ANOVA revealed that there were no significant statistical differences among means ($P\leq0.05$)

[d] Results are mean of three replicates±its standard deviation. Means with different letters in the same column differ significantly at $P\leq0.05$ according to the Fisher LSD

group B isolates dropped from 8 to 5.50–5.84. The non-inoculated medium had <5 μg mL^{-1} of soluble P. Linear regression between growth medium pH and level of solubilized phosphate was statistically significant (R^2 of 0.82). Thus, 82% of phosphate solubilization can be attributed to the drop in the pH of the medium. At the time of culture harvest, the number of viable cells ranged from 3.2×10^4 to 1.4×10^7 CFU mL^{-1} (Table 1). No significant correlation was detected between the amount of solubilized phosphate and the survival rate of the isolates. All isolates displayed acid and alkaline phosphatase activities. Alkaline phosphatase activities ranged from 48 to 61 μg*p*NP mL^{-1} h^{-1}, whereas acid phosphatase activities varied from 40 to 60 μg *p*NP mL^{-1} h^{-1} (Table 1). Finally, all isolates, but LF-MB2, were able to hydrolyze lecithin in an agar plate assay (Table 1).

Screening phosphate-mobilizing pseudomonad isolates for other plant probiotic attributes

All of the isolates produced clear halos around the colonies on skim milk agar plates, which is indicative of extracellular

Table 2 Phylogenetic assignment of phosphate-mobilizing isolates from agricultural bulk soils under no-till management in Argentina

Isolate	Origin	Closest pseudomonad relative based on 16S rDNA sequence[a]	16S accession	Closest pseudomonad relative based on *oprF* sequence[a]	*oprF* accession
LF-MB1	Monte Buey	*Pseudomonas koreensis* Ps 9-14 (AF468452)/665 bp/100%	JN411630	*P. fluorescens* Pf0-1 (CP000094)/ 587 bp/96%	JN411580
LF-MB2	Monte Buey	*Pseudomonas monteilii CIP 104883* (AF064458)/521 bp/100%	JN411633	*P. putida* W619 (CP000949)/ 589 bp/95%	JN411583
LF-P1	Pergamino	*P. koreensis Ps 9-14* (AF468452)/703 bp/99%	JN411634	*P. fluorescens* Pf0-1 (CP000094)/ 670 bp/96%	JN411584
LF-P2	Pergamino	*Pseudomonas vancouverensis ATCC* 700688 (AJ011507)/495 bp/99%	JN411636	*P. fluorescens* Pf0-1 (CP000094)/ 620 bp/97%	JN411586
LF-P3	Pergamino	*Pseudomonas moraviensis* CCM 7280 (AY970952)/702 bp/99%	JN411637	*P. fluorescens* Pf0-1 (CP000094)/ 597 bp/96%	JN411587
LF-V1	Viale	*Pseudomonas asplenii* ATCC 23835T (AB021397)/626 bp/99%	JN411638	*P. putida* W619 (CP000949)/ 605 bp/97%	JN411588
LF-V2	Viale	*P. asplenii* ATCC 23835 T (AB021397)/705 bp/99%	JN411639	*P. putida* W619 (CP000949)/ 600 bp/97%	JN411589

[a] The GenBank accession number of the closest pseudomonad isolate or strain is indicated between brackets, followed by the length (base pair) of the obtained sequence and the nucleotide identity (percent) based on BlastN comparison

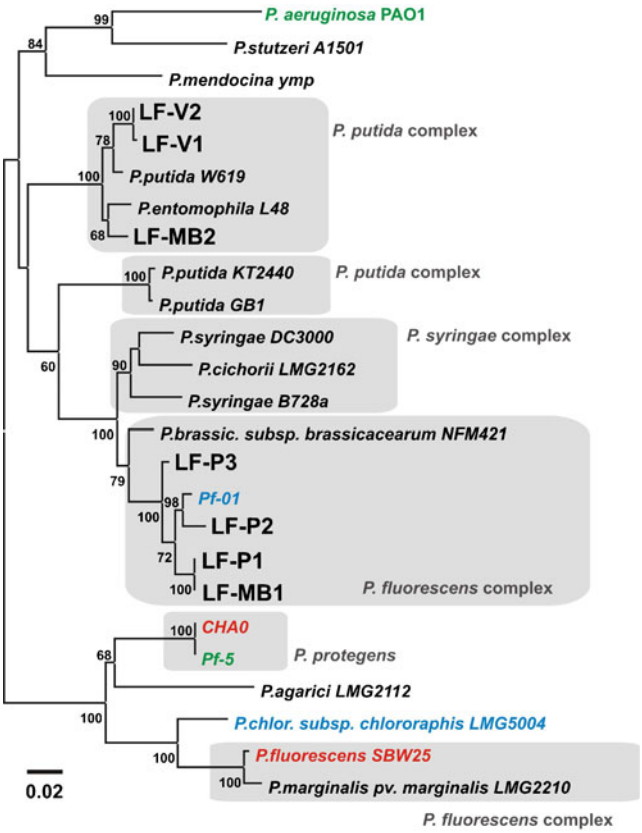

Fig. 1 Phylogenetic position of the phosphate-mobilizing isolates among the pseudomonads. Phylogenetic analysis of concatenated 16S rRNA-*oprF* sequences from the isolates (detailed in Table 2) and reference strains. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown at the nodes when values are higher than 50%. The *scale bar* represents 0.02 nt substitutions per site. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Taxonomic grouping was based on the work of Ramette et al. (2011). Colored strains or isolates follow the definition of Meyer et al. (2011), with low (*blue*), medium (*green*), or strong (*red*) phosphate solubilization activity on NBRIP medium

protease activity. As observed for the reference strain CHA0, the extracellular protease halos extended from 3 to 6 mm out of the colonies. Of the seven selected isolates, four produced sufficient HCN to be detected by the picrate-impregnated filter paper. Isolates LF-P2, LF-MB1, and LF-P3 developed a strong color development, whereas isolate LF-P1 produced a weaker signal.

Rhizosphere colonization ability in natural soil

The results of root colonization experiments for five of the isolates and the reference strain CHA0 are presented in Table 3. For unknown reasons, it was not possible to obtain tagged transformants from isolates LF-P2 and LF-P3; as a consequence, the root-colonizing potential could not be studied in these isolates. In this experiment, surface-disinfected seeds were bacterized with the tagged isolates and planted into pots containing natural soil with its own bacterial flora. Native pseudomonads population in the soil was $<10^3$ CFU g^{-1}, but upon planting maize, soybean, or wheat seeds, we could recover $1–5\times10^6$ CFU g^{-1} of pseudomonads after plating rhizosphere suspensions on S1 medium without antibiotics. Thus, we were able to evaluate the competitive colonizing ability of the tagged isolates in the presence of indigenous flora.

Table 3 Soybean root colonizing ability of phosphate-mobilizing pseudomonad isolates in natural soil

Strain/isolate	CFU g^{-1}
CHA0	$5.0\pm4.4\times10^8$ a
LF-MB1	$1.7\pm0.7\times10^7$ b
LF-MB2	$5.6\pm3.8\times10^6$ c
LF-P1	$1.0\pm0.2\times10^6$ d
LF-V1	$4.8\pm1.3\times10^6$ c
LF-V2	$7.6\pm0.6\times10^5$ d

Results are mean of three replicates±standard deviation. CFU values were $\log_{10}$-transformed prior to statistical analysis. Means with different letters in the same column differ significantly at $P\leq0.05$ according to the Fisher LSD. The plant roots from non-inoculated seeds did not develop any colonies

Fifteen days after inoculation, all tagged isolates colonized soybean roots at counts ranging from 7.6×10^5 to 1.7×10^7 CFU g^{-1} (Table 3). Maize roots showed similar levels of colonization (1.2×10^6 to 1.6×10^8 CFU g^{-1}). For wheat, only isolates LF-MB1, LF-MB2, and LF-P1 were found colonizing roots at levels above 10^6 CFU g^{-1}, whereas isolate LF-V1 could not be detected on wheat roots. Root washes from non-bacterized control plants did not develop any colonies. Representative images of cells from isolates colonizing the roots of maize, wheat, and soybean plants are shown in Fig. 2. Under our experimental conditions, isolate LF-MB1 (group B) was the best colonizer among the tested isolates, followed by isolates LF-MB2 (group B) and LF-V1 (group A), which were able to reach colonization levels of 10^6 CFU g^{-1} for soybean and wheat (except LF-V1), and of 10^7 CFU g^{-1} for maize. Isolates LF-P1 (group A) and LF-V2 (group B) proved to be the least competitive (Table 3).

Discussion

Although phosphate-solubilizing capabilities appear to be widespread within bacterial taxa (Kämpfer 2007), it is of particular interest to detect this potential in genera, which have other plant probiotic attributes like the pseudomonads (Trujillo et al. 2007; Martín et al. 2007; Selvakumar et al. 2011).

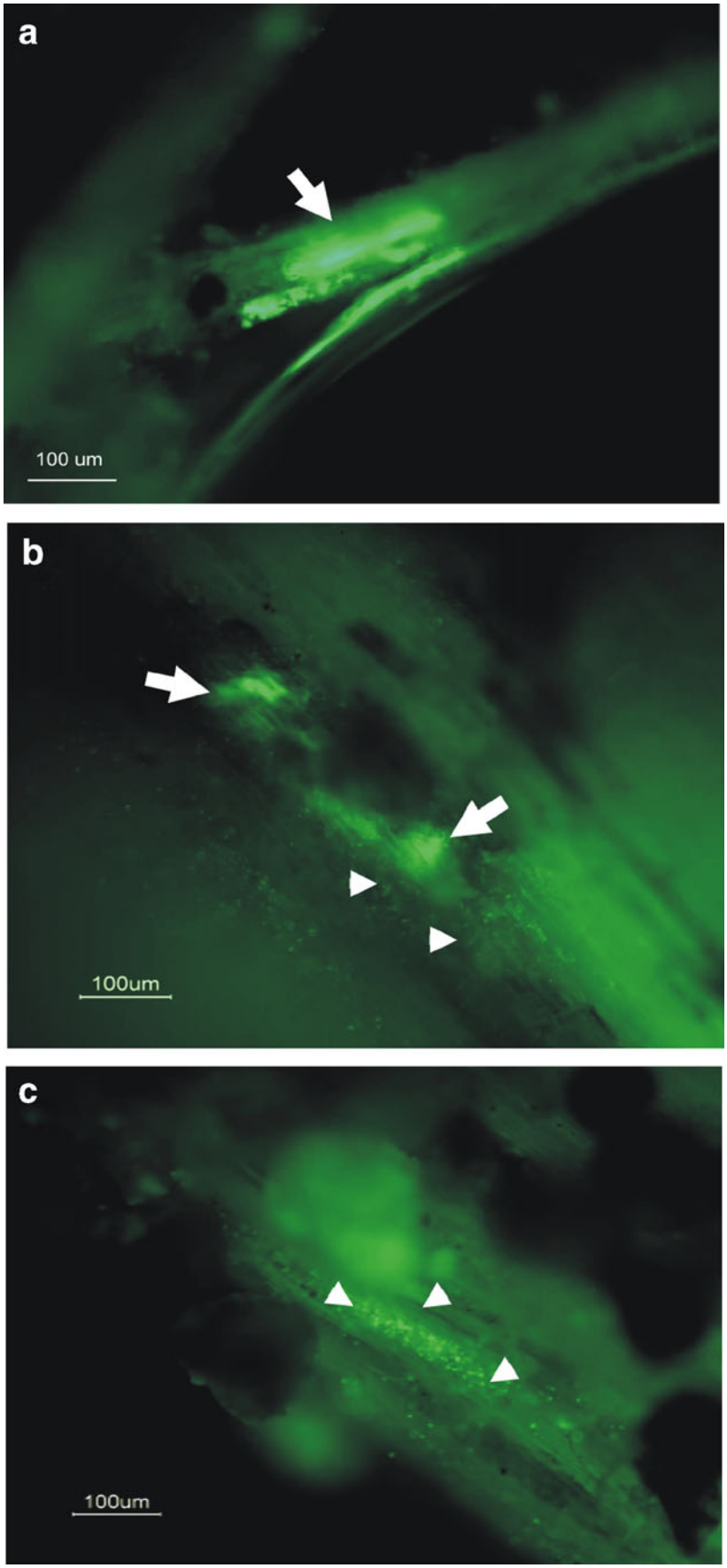

Fig. 2 Phosphate-mobilizing pseudomonads colonizing roots in natural soil. Representative images of *gfp*-tagged isolates as detected on the surface of wheat (**a**), soybean (**b**), and maize (**c**) roots growing in natural, non-sterilized soil. *Arrowheads* point to single cells found on the root surface, and *arrows* indicate densely colonized foci

Fluorescent pseudomonad strains such as *P. fluorescens*, *Pseudomonas chlororaphis*, *Pseudomonas savastanoi*, *Pseudomonas pickettii*, *Pseudomonas corrugata*, *P. putida*, and *Pseudomonas plecoglossicida* (Ravindra et al. 2008) and a list of more than 30 other taxa reviewed by Kämpfer (2007) have been reported to be phosphate solubilizers. In this work, we report the isolation of phosphate-solubilizing pseudomonads from agricultural bulk soils under no-till management in Argentina, which show high potential for mobilizing phosphate from inorganic and organic substrates and are able to colonize roots of maize, wheat, and soybean plants.

Based on a combination of plating on NBRIP medium followed by selection on *Pseudomonas* S1 medium, we have isolated a group of phosphate-mobilizing pseudomonads whose 16S rDNA and *oprF* sequences allowed us to position the isolates as members of the *P. fluorescens* and *P. putida* complexes (Table 2 and Fig. 1). These two phylogenetic clusters have been reported to include isolates with strong inorganic phosphate solubilization activity in vitro on NBRIP plates (Browne et al. 2009; Meyer et al. 2011).

Inorganic P in soil occurs as either phosphate anions that are adsorbed to soil particles of varied composition (Fe and Al oxides, silicates and Ca carbonates), or depending on pH, as poorly soluble precipitates of Ca–P in neutral to alkaline soils, and Fe–P and Al–P in acid soils (Richardson 2001). Tricalcium phosphate is usually utilized for measuring the potential of microbial P solubilization. However, bacterial strains that solubilize phosphate from $Ca_3(PO_4)_2$ are not necessarily capable to dissolve more resistant compounds, such as Fe–P or Al–P species (Chung et al. 2005; Collavino et al. 2010). In this study, we have analyzed the P solubilization potential of pseudomonad isolates in NBRIP medium containing $Ca_3(PO_4)_2$ because the soils sampled for isolation show pH values near neutrality, and it is presumed that the abundance of Al–P and Fe–P species is low. Thus, the solubilization efficiency measured in the qualitative NBRIP plate assay with $Ca_3(PO_4)_2$ may be used as a first criterion to screen isolates. The solubilization efficiency of our isolates (Table 1) was comparable to those reported by Ramachandran et al. (2007). However, the plate assay is not considered a reliable technique for determining the phosphate-solubilizing potential of a given strain. Pure cultures must be tested in liquid medium, though the amount of in vitro solubilized phosphate is not always a good indicator of phosphate-solubilizing ability in soil (Rodríguez and Fraga 1999). Gyaneshwar et al. (1998) demonstrated that some bacteria capable of solubilizing phosphate under laboratory conditions were unable to do so in soils. The authors suggested that this inability could be due to the buffer capacity of soils. With this in mind, we used $Ca_3(PO_4)_2$ at a concentration of 5 gL^{-1} as the insoluble P source with 0.1 M Tris–HCl at pH 8.0 to simulate the buffering conditions provided by soils (Gyaneshwar et al. 1998). Even with such buffering that is expected to reduce the P released from tricalcium phosphates (Joseph and Jisha 2009), the isolates achieved soluble P levels that ranged from 128 to 182 μg PmL^{-1}. These in vitro solubilization results are remarkable because they are quantitatively higher than the solubilizing ability reported for other isolates that were studied in the same liquid medium but without buffering. For instance, *Enterobacter* isolates from non-rhizospheric soil attained phosphate solubilization levels of 60 to 80 μg PmL^{-1} after 10 days of incubation (Deepa et al. 2010). Jha et al. (2009), who characterized pseudomonads strains from rhizosphere soil of a rice field in India, reported values of 33 μg PmL^{-1} for *P. aeruginosa* strain BFPB9, 74 μg PmL^{-1} for *P. plecoglossicida* strain FP12, and 63 μg PmL^{-1} for *Pseudomonas mosselii* strain FP13. Similarly, Chung et al. (2005) reported levels from 96 to 142 μg PmL^{-1} for a series of 13 enterobacterial strains isolated from the rhizosphere of crop plants in Korea. Release of P from mineral phosphate species is related to the production of organic acids, mainly gluconic acid (Lugtenberg and Kamilova 2009). In fluorescent pseudomonads, gluconic acid production is catalyzed by periplasmic oxidation of glucose by a membrane-bound glucose dehydrogenase and its cofactor, pyrroloquinoline quinone (De Werra et al. 2009; Meyer et al. 2011). All phosphate-mobilizing pseudomonads isolated in this work decreased the pH of the medium in the quantitative solubilization assay (Table 1), most probably due to gluconic acid production. The isolates should be also evaluated using insoluble P forms, like Fe–P or Al–P (Chung et al. 2005; Collavino et al. 2010).

The organic fraction of soil P is an important reservoir of immobilized P that accounts for 20–80% of the total soil P. The presence of organic phosphorus mineralizing bacteria has been considered to be the major source of phosphatases (phosphomonoesterase and phosphodiesterase) in soils which can release P from organic compounds and as a consequence provide available P to plants (Nannipieri et al. 2011). The relative low number of reports on mineralization of organic phosphate by bacteria (Abd-alla 1994; De Freitas et al. 1997; Tao et al. 2008; Malboobi et al. 2009; Oliveira et al. 2009; Viruel et al. 2011) highlights the relevance of this study. The alkaline and acid phosphomonoesterase activities of our isolates (Table 1) are comparable with those reported by other authors (De Freitas et al. 1997; Tao et al. 2008; Oliveira et al. 2009). Degradation of phospholipids into phosphate and diacylglycerol, and its subsequent turnover into fatty acids by the combined action of phospholipases (phosphodiesterase), may also contribute to P recycling in soil (Richardson et al. 2009). Most of the isolates reported here were able to degrade phospholipids (Table 1). Thus, most of the pseudomonads isolates from bulk soil samples solubilized phosphate from $Ca_3(PO_4)_2$, produced extracellular phosphomonoesterases and phospholipase(s), which implies that they have a high potential to mobilize P from inorganic and organic substrates (Table 4).

Table 4 Phosphate-mobilizing potential and plant probiotic traits of pseudomonad isolates from agricultural bulk soils under no-till management in Argentina

Strain/isolate	Solubilization efficiency[a]	Solubilization activity[b]	Acid phosphatase activity[c]	Alkaline phosphatase activity[d]	Phospholipase activity[e]	Root colonization[f]	HCN production[g]	Protease activity[h]
CHA0	++	++	++	++	++	+++	++	+
LF-MB1	++	+	+	+	+	+++	++	+
LF-MB2	++	+	+	+	−	++	−	+
LF-P1	+	++	+	+	+	+	+	+
LF-P2	++	++	+	++	+	ND	++	+
LF-P3	+	++	++	++	+	ND	++	+
LF-V1	++	++	++	++	+	++	−	+
LF-V2	++	+	+	++	+	+	−	+

ND not determined

[a] <150%, +; ≥150%, ++

[b] Soluble P (micrograms per milliliter) <150, +; ≥150, ++

[c] Micrograms *p*NP per milliliter per hour: <50, +; ≥50, ++

[d] Micrograms *p*NP per milliliter per hour: <50, +; ≥50, ++

[e] <150%, +; ≥150%, ++

[f] CFU per gram soybean roots: $\leq 10^5$, +; 10^5 to 10^6, ++; $\geq 10^6$, +++

[g] No reaction, same as negative control, −; weak positive reaction, +; strong positive reaction, ++

[h] Without halo, −; with halo, +

Several traits associated with survival, tolerance, competition with indigenous rhizospheric microorganisms, and root colonization are critical for the successful establishment of the inoculated bacteria in the rhizosphere. The failure of PGPR to produce the desired effects in the field after seed inoculation is frequently associated with their poor root-colonizing ability (Ahmad et al. 2011; Barrett et al. 2011). The study of root colonization by inoculated bacteria therefore provides an important criterion of strain selection for the formulation of efficient PGPR inoculants. Here, we have analyzed root colonization in natural soil using isolate derivatives doubly labeled with a selective marker (tetracycline resistance) and the *gfp* reporter gene, thus facilitating selective counting and visualization on the inoculated roots (Table 3 and Fig. 2). The results shown in Table 3 indicate that most studied phosphate-mobilizing pseudomonad isolates were able to establish themselves in the rhizosphere of maize, wheat, and soybean roots in the presence of indigenous bacteria, in some cases reaching colonization densities higher than 10^6 CFU g^{-1}. These values are comparable to root colonization levels reported for pseudomonads in natural soil. For instance, *P. putida* strain GR7.4*lux* achieved a mean of 4.8 $\log_{10}$CFU g^{-1} of soybean roots (Beauchamp and Kloepper 2003), whereas several rhizospheric pseudomonad isolates colonized soybean roots in natural soil at levels ranging from 3.9 to 5.7 $\log_{10}$CFU g^{-1} (Polonenko et al. 1987). In maize roots, strains *P. fluorescens* ANP15 and *P. aeruginosa* 7NSK2 achieved 6.7 and 6.8 $\log_{10}$CFU g^{-1}, respectively (Devliegher et al. 1995). Furthermore, the root-colonizing potential of a single isolate like LF-MB1 (Table 3) is comparable to the fluorescent pseudomonad counts detected in the rhizosphere of maize (5.4 to 5.8 $\log_{10}$CFU g^{-1}) (Picard et al. 2000).

Bacterial plant growth promotion is a well-established and complex phenomenon and is often achieved by various probiotic traits exhibited by the associated bacterium, such as antagonism against phytophatogenic fungi (Haas and Defago 2005). Microbial production of extracellular proteases and of volatile cyanhidric acid has been demonstrated to contribute to the biocontrol of root pathogens (Ramette et al 2003; Haas and Defago 2005; Hayat et al. 2010). It has recently been reported that the inorganic phosphate solubilization potential of pseudomonads is often combined with the production of other metabolites taking part in the biological control of soil-borne phytopathogens (Vassilev et al. 2006; Jha et al. 2009). Our results indicate that all of the phosphate-mobilizing pseudomonads isolated in this work had protease activity, and most of them produced HCN (Table 4).

Conclusions

Table 4 summarizes the findings of the present study on the P-mobilizing potential and plant probiotic traits of phosphate-mobilizing pseudomonad isolates. Based on the phosphate

solubilization and mineralization results and those of root colonization of different plants, the data suggest that there are efficient phosphate-mobilizing pseudomonads already adapted to agricultural soils under no-till management in Argentina with excellent potential for use as biofertilizers. The production of hydrogen cyanide and of extracellular protease by some of the isolates prompts us to consider these strains as putative biocontrol agents. The biofertilizer ability of the phosphate-mobilizing pseudomonads isolates should be subjected to appropriate tests under greenhouse and field conditions.

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