Symbiosis (2013) 60:143–154
DOI 10.1007/s13199-013-0250-2

Genetic diversity of phosphate-solubilizing peanut (*Arachis hypogaea L.*) associated bacteria and mechanisms involved in this ability

María Soledad Anzuay · Ornella Frola · Jorge G. Angelini · Liliana M. Ludueña · Adriana Fabra · Tania Taurian

Received: 2 May 2013 / Accepted: 20 June 2013 / Published online: 4 July 2013

Abstract In this study, attempts were made to analyze mechanisms involved in the bacterial phosphate-solubilizing ability of peanut isolates. Bacteria were taxonomically identified by analysis of 16S rDNA sequence. Levels of soluble P released by the isolates in unbuffered or buffered with Tris–HCl or MES NBRIP-BPB medium as well as the production of D-gluconic acid were determined in their culture. Presence of two of the genes encoding the cofactor PQQ of GDH enzyme was analyzed in the genome of this bacterial collection. 16S rDNA sequence analysis indicated that isolates belong to genera *Serratia*, *Enterobacter*, *Pantoea*, *Acinetobacter*, *Bacillus* and *Enterococcus*. All bacteria showed ability to solubilize tricalcium phosphate either in unbuffered or buffered medium. Nevertheless, addition of buffer solutions reduced levels of Pi liberated by the isolates. Although almost all isolates produced detectable amounts of D-gluconic acid, no correlation with levels of P soluble released were observed. The presence of *pqq*E and *pqq*C genes was detected only in Gram negative bacteria. It was concluded from this study that the mechanism involved in phosphate solubilization is organic acids production and, presence of *pqq* genes in all Gram negative bacteria analyzed encourages to confirm their role in bacterial phosphate solubilizing ability as well to identify genes involved in this PGP trait in Gram positive bacteria.

M. S. Anzuay · O. Frola · J. G. Angelini · L. M. Ludueña · A. Fabra · T. Taurian (✉)
Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-quimicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal 3, 5800 Río Cuarto, Córdoba, Argentina
e-mail: ttaurian@exa.unrc.edu.ar

Keywords Peanut phosphate solubilizing bacteria · Phosphate solubilizing mechanisms · Effect of buffer addition · Gluconic acid · *pqq* genes

1 Introduction

Phosphorus (P), next to nitrogen, is the second essential macronutrient required for plant growth. Phosphorus exists in nature in a variety of organic and inorganic forms, mainly in either insoluble or very poorly soluble forms. Soluble forms of P fertilizers applied to the soil are easily precipitated (Podile and Kishore 2006) and converted into phosphates of Fe, Al and Mn in acid soils and phosphates of Ca or Mg in alkaline soils, which are poorly soluble and, therefore, unavailable to plants (Rodríguez and Fraga 1999). Phosphorus deficiency is one of the most important chemical factors limiting crop production in many soils worldwide (Arcand and Schneider 2006).

Within plant growth promoting bacteria (PGPB), phosphorus solubilization property is considered to be one of the most important traits associated with plant nutrition (Chen et al. 2006). Soils microorganisms are involved in a range of processes that affect phosphate transformation and thus influence the subsequent availability of phosphate to plant roots (Richardson et al. 2001). Phosphate solubilizing bacteria have been described to play an important role in plant nutrition through an increase in phosphorus uptake by plants (Rodríguez et al. 2006). Bacteria belonging to different genera are included in this group of beneficial bacteria such us *Pseudomonas*, *Bacillus*, *Rhizobium*, *Agrobacterium*,

Burkholderia, *Acromobacter*, *Micrococcus*, *Aerobacter*, *Flavobacterium*, *Erwinia*, *Pantoea*, *Acinetobacter*, *Enterococcus* and *Enterobacter* (Nahas 1996; Rodríguez and Fraga 1999; Pérez et al. 2007; Ogut et al. 2010). The bacterial release of P from insoluble organic compounds involves enzymatic processes (Rossolini et al. 1998) meanwhile mineral phosphate solubilization is widely associated with the production of low-molecular-weight organic acids, mainly gluconic and 2-cetogluconic acids (Goldstein 1995; Rodríguez and Fraga 1999; Rodríguez et al. 2006). These acids chelate the cations (Al, Fe, Ca, Mg) bound to the insoluble forms of phosphate and convert them into soluble forms with the consequent decrease in the pH of the medium (Stevenson 2005). This mechanism has been described in Gram negative bacteria, which produce organic acids in the periplasm through a non-phosphorylated direct oxidation of glucose (DOPG; Direct Oxidation Pathway of Glucose) whose physiological role remains unknown (Anthony 2004). The DOPG pathway enzymes glucose dehydrogenase (GDH) and gluconate dehydrogenase (GADH) are oriented toward the outside of the cytoplasmic membrane so that they oxidize their substrates in the periplasmic space (Anthony 2004). These enzymes require the cofactor redox pirroquinolin quinone (PQQ), whose biosynthesis involves a PQQ operon consisting of at least 5–7 genes (Biville et al. 1989; Meulenberg et al. 1992; Kim et al. 2003). The role of PQQ has been described to be essential for the phosphate solubilizing phenotype in several bacteria (Goldstein 1995; Rodríguez and Fraga 1999; Kim et al. 2003; Han et al. 2008). Although *pqq* genes are highly conserved in several bacterial species (Yang et al. 2010), the role of the protein PqqC is the only dilucidated meanwhile proposed functions have been assigned for most of the other genes (Shen et al. 2012). Adding to that, it has been described that the disruption of genes *pqq*A, B, D and E has demonstrated that they are essential for the biosynthesis of PQQ cofactor (Shen et al. 2012).

Phosphorus solubilization by bacteria depends on many factors such as nutritional, physiological and growth culture conditions. Within them, it has been described that buffering capacity of soil could limit the microbial phosphate solubilization (Gyaneshwar et al. 1998; Joseph and Jisha 2009). Therefore, it has been suggested that screening of phosphate solubilizing microbes by using buffered media may lead to the selection of more effective solubilizers (Gyaneshwar et al. 1998).

Peanut (*Arachis hypogaea L.*) is a widespread leguminous plant of great agricultural and economic significance. Argentina is one of the major peanut producers in the world along with China and USA, and about 90 % of its production takes place in the province of Córdoba (S.I.A. 2011). Nevertheless, due to the intensity of agricultural practices, peanut soils of Cordoba have decreased their availability of phosphorus (Bosch and Da Veiga 2002; Giayetto et al. 2008 personal communication). Considering this deficiency in P in Cordoba's peanut soils, we have previously obtained a collection of 433 native phosphate solubilizing bacteria from field peanut plants and the effect of their inoculation on plant growth was evaluated in microcosm assays (Taurian et al. 2010). The aim of this study was to analyze biochemical and genetic mechanisms involved in the bacterial phosphate-solubilizing ability of this bacterial collection. Approaches were focused on the analysis of levels of soluble P released in unbuffered and buffered NBRIP-BPB broth. Analysis of D-gluconic acid released in supernatans of the bacterial cultures were performed in order to evaluate if their mineralizing phosphate ability is related with the levels of this organic acid. To gain more knowledge of phosphate solubilizing mechanism of peanut associated bacteria and related to D-gluconic acid production, the presence of two of two genes (*pqq*C and *pqq*E) encoding the cofactor PQQ of glucose dehydrogenase was also analyzed in this bacterial collection.

2 Materials and methods

2.1 Bacterial growth and maintenance

Eighteen phosphate-solubilizing bacterial isolates of a collection of 433 bacterial strains isolated from peanut plants cultivated in central and southern region of Córdoba, Argentina (latitude, 32° to 34°, longitude, 63° to 65°) (Taurian et al. 2010) were used in this study: 12 bacterial strains were isolated from inside peanut nodules, 4 from stem surface (epiphytic) and 2 from inside roots (endophytic) (Table 1). The procedure used for the isolation of bacteria from nodules has been previously described (Taurian et al. 2002). Bacteria were grown in TSA (trypticase soy agar) (Britania), YEM (yeast extract mannitol) agar (Vincent 1970) or TY (Beringer 1974) media. *Pseudomonas fluorescens* PMT1 used in commercial inoculant formulation ("RIZOFOS"®-RIZOBACTER) was used as reference strain and grown in LB (Luria-Bertani) media (Miller 1972). Bacteria were maintained in 20 % glycerol (v v^{-1}) at −80 °C.

2.2 Evaluation of phosphate solubilizing ability

In vitro inorganic phosphate-solubilizing ability was determined in NBRIP-BPB solid medium (National Botanical Research Institute's phosphate grown medium) (Mehta and Nautiyal 2001) containing tricalcium-phosphate as sole source of P. Ten microliters of fresh bacterial culture (10^8 CFU ml^{-1}) were spotted onto these plates and incubated at 28 °C during 7 days. The halo of clearance around the bacterial colony indicated solubilizing ability.

Table 1 Source of isolation of phosphate solubilizing bacteria and identification by 16S rDNA analysis

Bacteria	Source[a]	16S rDNA similarity (accession number)[b]	Gene identity (%)[c]	Taxonomical assignment	Accession number
1. J21	Nodule	*Serratia ureilytica* BM0512 (JQ680882)	97.0/99.8	*Serratia* sp	JN091871
2. J145	Nodule	*Serratia ureilytica* BM0561 (JQ680908)	99.0/99.8	*Serratia ureilytica*	JN091870
3. J260	Nodule	*Serratia marcescens* ITBB B5-1 (JN896750)	99.0/100	*Serratia marcescens*	JN969594
4. S93	Nodule	*Serratia ureilytica* BM0525 (JQ680890)	98.0/98.9	*Serratia* sp	JN173078
5. S119	Nodule	*Serratia marcescens* ITBB B5-1 (JN896750)	99.0/100	*Serratia marcescens*	GQ165511
6. J49	Nodule	*Pantoea agglomerans* HK 14–1 (AY335552)	99.0/99.9	*Pantoea agglomerans*	GQ165512
7. J157	Nodule	*Enterobacter sp* ydwj-5 (EF028156)	99.0/99.7	*Enterobacter* sp	JN104595
8. J33	Nodule	*Enterobacter cowanii* BCC 009 (EU629163)	99.0/99.8	*Enterobacter cowanii*	JQ304269
9. S57	Nodule	*Enterobacter* sp JA07 (EU260124)	99.0/99.8	*Enterobacter* sp	JN091872
10. L176	Stem Epiphyte	*Acinetobacter calcoaceticus* BA60 (FJ263920)	99.0/100	*Acinetobacter calcoaceticus*	JN173077
11. J9	Nodule	*Bacillus subtilis* I4 (JN794569)	99.0/100	*Bacillus subtilis*	JN104596
12. J225	Nodule	*Bacillus megaterium* 1287 (JN645941)	99.0/99.8	*Bacillus megaterium*	JN104592
13. J255	Nodule	*Bacillus megaterium* 1287 (JN645941)	99.0/99.8	*Bacillus megaterium*	JN969593
14. L54	Stem Epiphyte	*Bacillus subtilis* I4 (JN794569)	99.0/99.0	*Bacillus* sp	JN969591
15. L55	Stem Epiphyte	*Bacillus subtilis* RST1902 (JX185717)	99.0/99.5	*Bacillus* sp	JN969592
16. L177	Stem Epiphyte	*Enterococcus faecalis* IJ-12 (EU547777)	100.0/99.1	*Enterococcus* sp	JN104594
17. L185	Root endophyte	*Enterococcus faecium* CE3.A (JN653465)	99.0/96,1	*Enterococcus* sp	JN104593
18. L191	Root endophyte	*Enterococcus faecium* CE3.A (JN653465)	99.0/97.3	*Enterococcus* sp	JN173076

[a] All bacteria were isolated from peanut plants of the producing area of Córdoba, Argentina (Taurian et al. 2010)

[b] Sequence with highest percentage of identity observed

[c] Similarity score (percentage of identity) with NCBI/RDP analysis

2.3 Quantification of soluble phosphate released

The quantity of inorganic phosphate solubilized was determined in NBRIP-BPB broth medium (Mehta and Nautiyal 2001) following Fiske and Subbarow (1925) method. One hundred microlitres of an overnight inoculum (approximately 10^9 CFU ml^{-1}) in LB medium was transferred to 15 ml of unbuffered NBRIP-BPB medium or buffered with either Tris (2-amino-2-hydroxymethylpropanw-1,3-diol)-HCl or MES (4-morpholine-ethanesulfonic acid) at a final concentration of 100 mM and pH 7. After 24, 48, 72 h and 7 days of growth, 1,5 ml of bacterial cultures were sampled and centrifuged for 12 min at 10,000 g. The amount of soluble phosphorus released to the medium was quantified spectrophotometrically by measuring absorbance at 660 nm. At each incubation time, CFU ml^{-1} by drop plate method (Hoben and Somasegaran 1982) in TY medium and supernatants' pH of each sample were determined.

2.4 Production of gluconic acid

Detection and quantification of gluconic acid produced by bacteria was performed using the kit D-gluconic acid/D-glucono lactone (K-GATE, Megazyme) with a detection limit of 20 μg ml^{-1}. Samples of bacterial cultures in NBRIP-BPB medium were centrifuged for 12 min at 10,000 g. One hundred μl of supernatants were used to assay gluconic acid production following a colorimetric reaction and measuring absorbance at 340 nm.

2.5 Phenotypic and genotypic analysis of the bacterial isolates

Morphology and Gram stain of phosphate solubilizing bacteria were examined by light microscopy. The nucleotide sequence of the nearly full-length 16S rRNA genes were obtained by Macrogen Laboratories (Korea). Sequence analyses were performed by using the algoritm BLASTN (Altschul et al. 1997) and RDP project (Cole et al. 2009) to identify similarities and to perform alignments. The nucleotide sequence of the 16S rRNA gene of the bacteria have been deposited in NCBI gene data bank (Table 1).

2.6 Amplification of *pqq*E and *pqq*C genes

Total bacterial DNA was obtained by using the procedure described by Walsh et al. (1991). The amplification of a 700 bp *pqq*E gene fragment was assayed using the primers F317 (5′TTYTAYACCAACCTGATCACSTC3′) and R1019 (5′TBAGCATRAASGCCTGRCG3′) designed in this study by multiple alignment of sequences of gene *pqq*E obtained from NCBI gene data bank: *Pantoea agglomerans* (M94448); *Enterobacter sakazakii* ATCC BAA 894 (CP000783); *Kluyvera intermedia* (AY216683); *Rahnella*

aquatilis Hx2 (FJ868974); *Kleibsiella pneumoniae* (X58778) and *Serratia marcescens* (DQ8685361). The amplification of a ~140 bp fragment of *pqq*C gene was assayed using the primers *pqq*CF (5′GYGTSCGBTTY GCVGTBGA3′) and *pqq*CR (5′TARTGYTGSGG CCARCTGT3′) designed in this study by multiple alignment of sequences of gene *pqq*C obtained from NCBI gene data bank: *S. marcesens* (DQ868536.1), *Erwinia billingiae* Eb661 (NC_014306.1), *P. ananatis* SC17(GU58089), *P. ananatis* LMG 20103 (NC_013956.2), *R. aquatilis* HX2 (FJ868974.1), *K. pneumoniae* NTUH-K2044 (AP006725.1), *Pseudomonas putida* KT2440 (NC_002947.3), *P. putida* DOT-T1E (GQ506557.1), *P. vagans* C9-1(NC_014562.1), *Burkholderia cepacia* (NC_008392.1), *K. pneumoniae* NTUH-K2044 (AP006725.1), *P. fluorescens* F113 (GQ202029.1), *P. fluorescens* Pf-5 (NC_004129.6), *Pantoea* sp. At-9b (CP002433.1), *Azotobacter vinelandii* DJ (NC_012560.1). Each PCR reaction (20 μl) contained 2 μl (10 μM) of each primer, 2 μl (10X) of buffer, 2 μl (2 mM) of dNTPs, 7,4 μl of sterile bidistilled water, 2 μl of $MgCl_2$ (50 mM), 0,2 μl of *Taq* DNA polymerase (5 U/μl) and 2,4 μl of template DNA. Amplification was performed in a DNA thermalcycler (Mastercycler Eppendorf). The temperature profile for PCR-*pqq*E were: An initial cycle at 95 °C for 1 min, followed by 35 cycles at 94 °C for 1 min, at 55 °C for 1 min and at 72 °C for 2 min, and a final step of 72 °C for 10 min. For PCR-*pqq*C temperature profile was as follows: One cycle at 94 °C for 3 min, 96 °C for 30 s, 65 °C for 30 s, 72 °C for 30 s, 2 cycles at 96 °C for 20 s, 62 °C for 30 s, 72 °C for 35 s, 3 cycles at 96 °C for 20 s, 59 °C for 30 s, 72 °C for 40 s, 4 cycles at 96 °C for 20 s, 56 °C for 30 s, 72 °C for 45 s, 5 cycles at 96 °C for 20 s, 53 °C for 30 s, 72 °C for 50 s, followed by 25 cycles at 94 °C for 20 s, 50 °C for 30 s, 72 °C for 1 min and a final elongation step of 72 °C for 5 min. Ten μls PCR products were separated by horizontal electrophoresis on 1,5 % (w v^{-1}) agarose gels and visualized by ethidium bromide staining. In this assay there were included reference strains that were previously analyzed in our laboratory in their phosphate solubilization ability. The phosphate solubilizing strains used were: *Pseudomonas* sp. NVAM 24, *Enterobacter* sp. NONC 13; *Klebsiella* sp NT31, *Ps.* sp NCHA33, *K.* sp TT001 (Ibañez et al. 2009) and *P.fluorescens* CTR212 (Latour et al. 1996). Reference strains that showed no halo of clearance in NBRIP-BPB medium were: *Bradyrhizobium* sp (*Arachis hypogaea L.*) SEMIA 6144 (IPAGRO, Brasil), *B. japonicum* USDA 110 (Taurian et al. 2002), *Azospirillum brasilense* Cd (EMBRAPA, Brasil), *Escherichia coli* HB101, and *Ps.corrugata* Pc5 (Achouak et al. 2000).

In-silico bio-informatic analyses of *pqq*E and *pqq*C sequences from several bacteria were performed by using the algorithm BLASTN (Altschul et al. 1997).

2.7 Data analysis

Data were subjected to analysis of variance (ANOVA) and differences among treatments were detected by Tukey test ($P<0,05$). Pearson correlation coefficients between soluble P released by bacteria and pH as well as with levels of gluconic acid produced by them were calculated. In all cases Infostat software was used.

3 Results

3.1 Morphological and genetic characteristics of the bacterial isolates

Ten of the analyzed isolates were Gram negative bacilli and 8 of them were Gram positive bacilli (5) or cocci (3). For genus assignment it was considered a 16S rDNA sequence similarity with reference sequences higher than 95 % and all the sequences obtained showed similarities higher than 97 % (Table 1). In those sequences in which a similarity score higher than 99.0 and 99.5 was determined with NCBI and RDP analysis, respectively, it was suggested species assignment. Alignment analysis indicated that isolates belong to different genera *Serratia*, *Enterobacter*, *Pantoea* (Enterobacteriales), *Acinetobacter*, *Bacillus* and *Enterococcus* and that species found in this bacterial collection corresponded to *S. ureilytica*, *S. marcescens*, *P. agglomerans*, *E. cowanii*, *A. calcoaceticus*, *B. subtilis*, and *B. megaterium* (Table 1). Isolates showed highest identity with references strains that are environmental, epiphytic bacteria and in most of the cases, plant growth promoting bacteria such as phosphate solubilizers and biocontrol bacteria.

3.2 Phosphate solubilizing ability

It was evaluated considering the diameter of solubilization halo (halo of clearance) produced by the bacteria in NBRIP-BPB solid medium. *Acinetobacter* sp. L176, *Serratia* sp. S119, *Serratia* sp. J260, *Serratia* sp. S93 and *Enterobacter* sp. J33 produced the greatest halos (from 12 to 17 mm diameters) together with *Pseuodmonas fluorescens* PMT1 (15 mm diameter), while isolates *Bacillus* sp. L54, *Bacillus* sp. L55, *Serratia* sp. J145, *Serratia* sp. J21 and *Enterobacter* sp. S57 produced the lowest ones (from 5 to 6 mm diameters) (Table 2, Fig. 1).

The concentration of soluble phosphorus released by the isolates in liquid medium NBRIP ranged from 12.9 to 764.7 μg ml^{-1} (Table 2). *P. fluorescens* PMT1 and isolates *Enterobacter* sp. J33, *Serratia* sp. J260 and *Acinetobacter* sp. L176 secreted the highest amounts of available phosphorus into the medium (764.7, 636.3, 605.2 and 492.7 μg ml^{-1},

Table 2 Phosphate solubilizing halo, maximum amounts of P-liberated, pH, time of growth, colony forming units and gluconic acid production in NBRIP media and presence of *pqq*E and *pqq*C genes in the peanut associated native bacteria and *Pseudomonas fluorescens* PMT1

Strain	Phosphate solubilizing halo (mm)[a]	P-liberated (μg ml^{-1})[b]	Time of growth (h)[c]	pH[d]	CFU ml^{-1}[e]	Gluconic acid (μg ml^{-1})	*pqq*E/*pqq*C-PCR
Bacillus sp. L55	5.3±0.3	12.9±1.0^{A}	24	6.2±0.1	4.10^{7}	90.92±15.08^{AB}	−/−
Serratia sp. J145	5.3±0.3	300.6±51.4^{CD}	24	3.6±0.0	5.10^{8}	146.86±27.27^{AB}	+/+
Enterobacter sp. J157	8.7±1.2	220.6±50.4^{ABC}	24	5.1±0.2	4.10^{9}	1077.85±50.49^{D}	+/+
Serratia sp. S93	15.0±1.1	232.0±8.7^{BC}	24	3.9±0.1	1.10^{5}	964.53±80.30^{D}	+/+
Enterobacter sp. J33	16.0±2.5	636.3±32.8^{FG}	24	4.1±0.1	14.10^{8}	240.88±50.43^{ABC}	+/+
Serratia sp. J21	5.7±1.3	272.1±6.7^{CD}	48	4.6±0.2	4.10^{8}	263.53±19.0^{ABC}	+/+
Bacillus sp. J255	9.0±1.7	82±14.7^{AB}	48	5.9±0.2	4.10^{7}	280.27±107.13^{ABC}	−/−
Enteroccocus sp. L177	9.3±1.8	145.4±17.9^{ABC}	48	5.0±0.1	21.10^{7}	ND	−/−
Bacillus sp. J9	10.3±2.7	202.7±12.6^{ABC}	48	4.3±0.1	24.10^{8}	556.06±47.91^{C}	−/−
Enteroccocus sp. L191	11.0±1.0	237.1±20.7^{BC}	48	4.0±0.0	7.10^{7}	41.07±6.33^{AB}	−/−
Bacillus sp. L54	5.3±1.2	26.8±2.5^{A}	48	5.7±0.0	1.10^{8}	ND	−/−
Acinetobacter sp. L176	11.7±3.7	492.7±33.9^{EF}	48	4.2±0.1	6.10^{6}	1181.00±51.20^{D}	+/+
Enterobacter sp. S57	6.0±0.3	227.0±2.3^{BC}	72	4.0±0.0	7.10^{8}	376.85±107.27^{BC}	+/+
Bacillus sp. J225	6.7±0.9	107.3±19.1^{ABC}	72	6.2±0.2	15.10^{7}	82.57±50.95^{AB}	−/−
Enteroccocus sp. L185	7.7±0.9	378.9±36.5^{CDE}	168	4.4±0.2	13.10^{7}	383.44±169.98^{BC}	−/−
Pantoea sp. J49	10.0±3.0	385.4±41.4^{DE}	168	3.8±0.2	17.10^{7}	1213.57±27.67^{D}	+/+
Serratia sp. J260	14.0±2.0	605.2±15.4^{F}	168	3.8±0.2	7.10^{8}	253.0±7.9^{ABC}	+/+
Serratia sp. S119	13.7±2.7	362.0±27.8^{CDE}	168	4.5±0.1	2.10^{7}	304.35±54.96^{ABC}	+/+
Ps. fluorescens PMT1	15.0±3.2	764.7±37.4^{G}	168	3.7±0.1	2.10^{7}	244.90±71.1^{ABC}	+/+

Each value is a mean ± S.E. of two to six independent replicates (n=3–10). Different letters indicate differences among isolates

ND not detectable, + amplification PCR product, − no amplification PCR product

[a] Diameter of solubilizing halo in NBRIP-BPB medium

[b] Maximum levels of soluble phosphorus released

[c] Time of growth (h) in which maximum levels of soluble P were released

[d] Lowest culture pH reached during incubation time

[e] Colony-forming units at time of maximum levels of soluble P released by each bacterium

respectively) while the lowest amounts of soluble phosphorus were liberated by isolates *Bacillus* sp. L55, *Bacillus* sp. L54 and *Bacillus* sp. J255 (12.9, 26.8 and 82 μg ml^{-1}, respectively). Isolates that released the highest levels of available P (>400 μg ml^{-1}) are all Gram negative bacteria whereas those that released lowest levels (<200 μg ml^{-1}) are all Gram positive bacteria. Considering growth time in which highest amounts of soluble phosphorus were secreted, isolates were separated in two groups. One of them, which contained the 78 % of bacteria, included those that showed the highest levels of soluble phosphorus released within the 3 first days of growth (Fig. 2a), while the second one included bacteria that reached the highest phosphate solubilization ability at 7 days of growth (Fig. 2b). Viability of isolates was not affected along the assay until the end of the experiment (Table 2). Irrespective of amounts of phosphate released, pH of all isolates cultures dropped from 7 to values within a range of 6.0–3.5 (Table 2). Phosphate solubilization activity of bacteria growing in NBRIP medium buffered by the addition of either Tris–HCl or MES was evaluated. Times of evaluation were choosen considering those of maximum phosphate solubilization in unbuffered NBRIP-BPB medium. The levels of soluble phosphorus released in medium containing Tris–HCl varied from 6.5 to 510 μg ml^{-1} (Fig. 3a). These values were significantly lower than those produced in unbuffered medium for the 53 % of the isolates. However, as it was also noted in unbuffered conditions, all

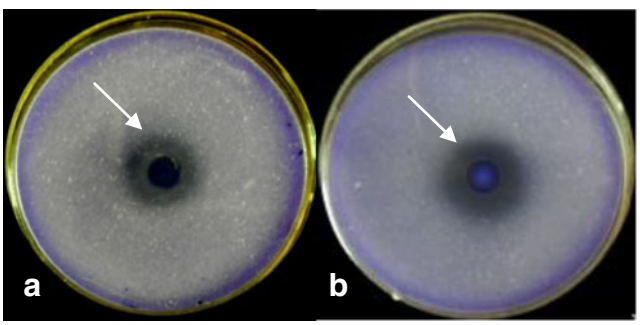

Fig. 1 Phosphate solubilization halo produced by **a** *Enterococus* sp L185 and **b** *Serratia.* sp. S119 in NBRIP-BPB medium

bacteria acidified the medium (Fig. 3b). In medium supplemented with buffer MES, the amount of phosphorus solubilized by all bacteria decreased significantly compared with unbuffered medium, reaching values ranging from 3 to 74.5 μg ml^{-1} (Fig. 3a). Under these conditions, the pH of the culture medium remained almost constant with respect of the initial along the time, being statistically different to values determined in unbuffered media (Fig. 3b). Correlation analysis showed a negative relationship between soluble P secreted by bacteria and supernatants'pH either in unbuffered (r=−0,72) (Fig. 4a) and buffered media (Tris-Cl r=−0,71; MES r=−0,78), (Fig. 4b and c, respectively).

3.3 Production of gluconic acid

The concentration of gluconic acid produced by each isolate was determinated at the time in which maximum soluble phosphorus levels were detected in NBRIP-BPB medium (Table 2). With the exception of *Enterococcus* sp L177 and *Bacillus* sp L54, all bacteria showed to produce detectable amounts of gluconic acid in NBRIP-BPB medium. The quantities of gluconate produced ranged from 40 to 1,200 μg ml^{-1}. As shown in Fig. 5, no correlation were observed between levels of gluconic acid secreted and those of soluble P detected in bacterial culture (r=0,09).

3.4 Detection of *pqq*E and *pqq*C genes

The presence of two of the genes encoding the cofactor PQQ of GDH enzyme, responsible for gluconic acid production, was evaluated in the isolates. All Gram-negative isolates and *Pseudomonas fluorescens* PMT1 exhibited a band of the expected size corresponding to the fragment of the *pqq*E (700 pb) and *pqq*C (~140 pb) genes (Table 2) while no amplification products were obtained in *Bacillus* and *Enterococcus* strains analyzed. When presence of these

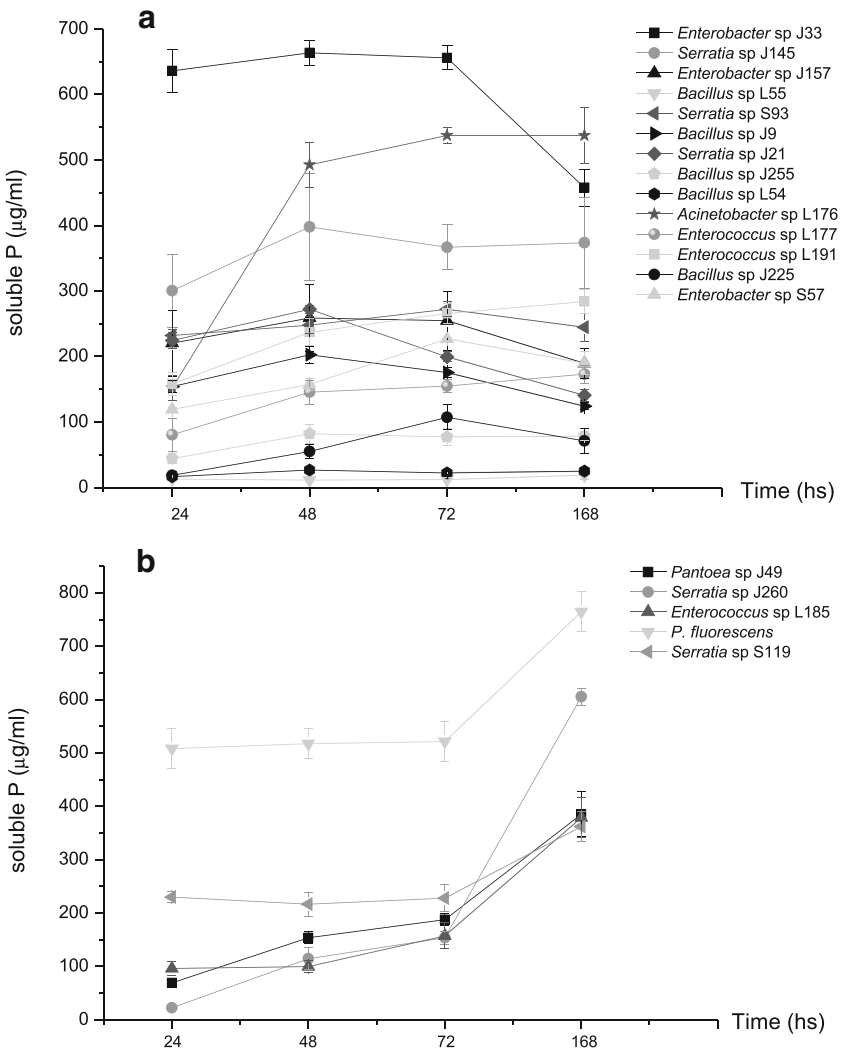

Fig. 2 Levels of soluble P of supernatants of "early solubilizers" (**a**) and "late solubilizers" isolates (**b**) growing in NBRIP broth at 30 °C after 1, 2, 3 and 7 days of growth. Each value is a mean ± S.E. of 3–6 independent replicates (n=10)

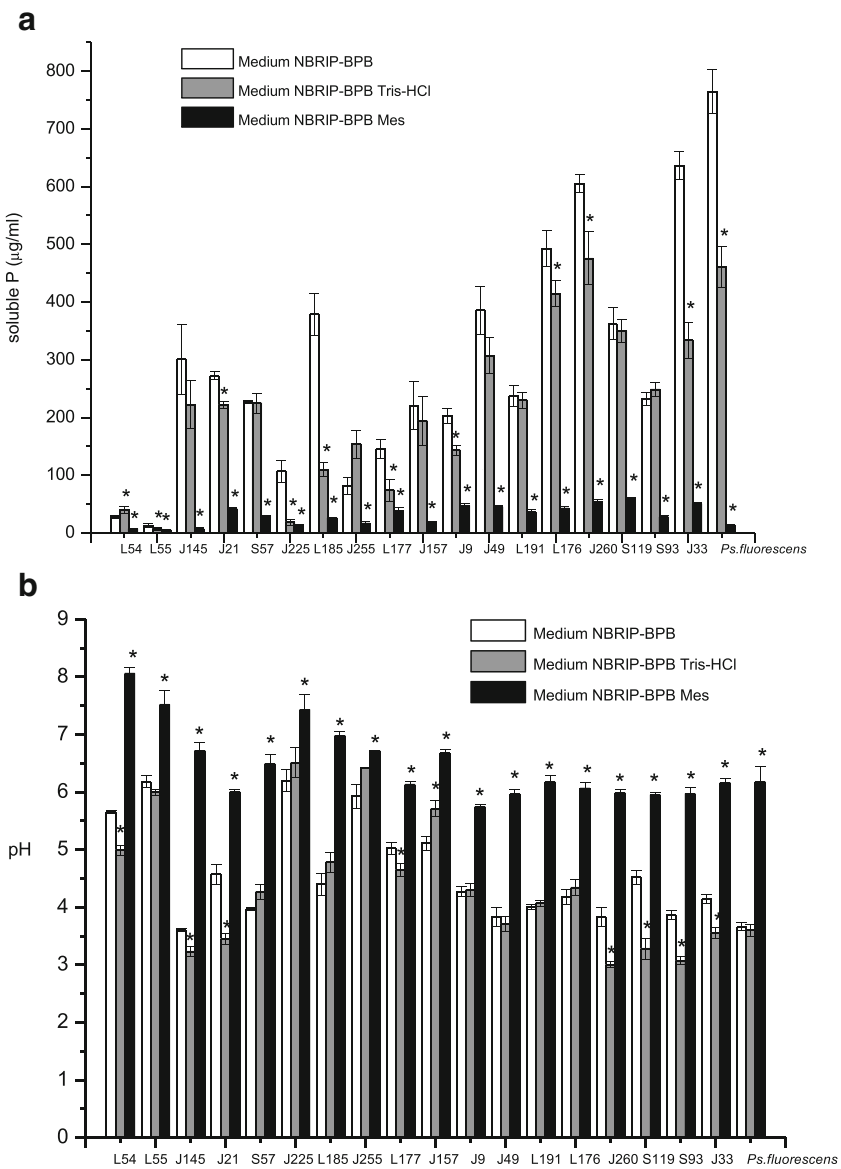

Fig. 3 Highest levels of soluble P released by the isolates and *P. fluorescens* PMT1 **a** and pH values **b** in NBRIP unbuffered medium (*white*), supplemented with Tris–HCl (*grey*) and MES (*black*). Each value is a mean of 3–6 independent replicates $n=10$, $p<0{,}05$

genes were analyzed in the genome of reference strains it was possible to observe that all non-phosphate solubilizing bacteria did not show expected PCR products. On the other hand, in phosphate solubilizing reference bacteria, with the exception of *Klebsiella* sp TT001, it was possible to observe the expected PCR product for both *pqq* genes. *Klebsiella* sp showed inespecific banding pattern in *pqq*C-PCR reaction (data not shown).

4 Discussion

In the present study, the phosphate solubilizing capacity of a bacterial collection able to promote peanut growth (Taurian et al. 2010) was studied. Several studies report about populations of phosphate solubilizing bacteria isolated mainly from soil and rhizosphere (Collavino et al. 2010; Rodríguez et al. 2006). However, studies on these PGPB isolated from inside root tissues are scarce, mostly those involving Gram positive bacteria. Analysis of 16S rRNA sequence of the isolates indicated that they belong to genera *Serratia*, *Enterobacter*, *Pantoea*, *Acinetobacter*, *Bacillus* and *Enterococcus*. Members of these genera have been previously described as P solubilizers (Seong et al. 1996; de-Bashan and Bashan 2004; Chung et al. 2005; Pérez et al. 2007). Although *Serratia* sp, *Enterobacter* sp and *Pantoea* sp have been previously described as plant endohytes, few works reports their isolation from inside nodules (Ibañez et al. 2009). Isolation of *Bacillus* sp from inside legume nodules has also been reported by other authors (Bai et al. 2002; Podile and Kishore 2006) and a cooperative effect with the rhizobia have been assigned to them (Sturz et al.

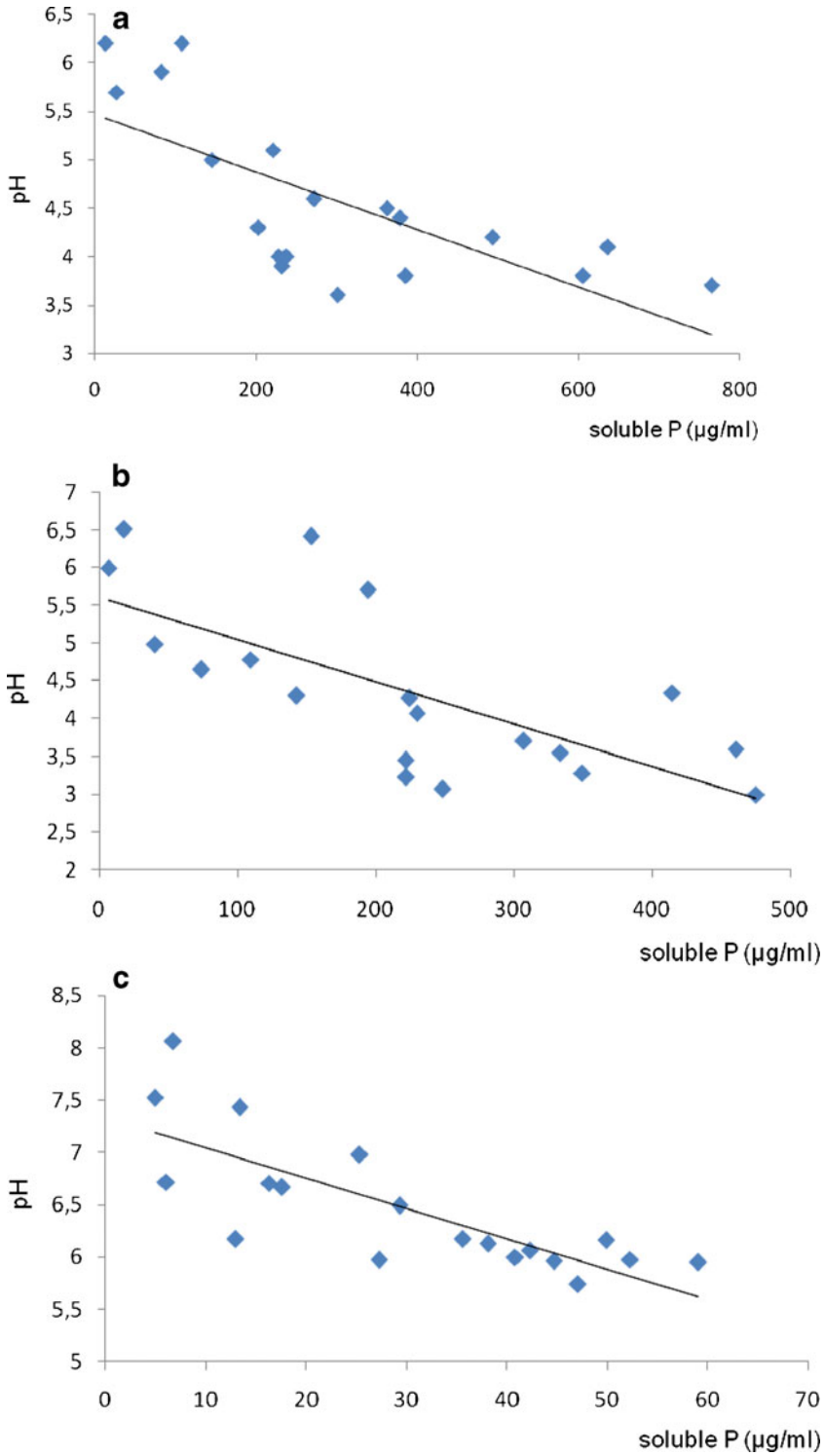

Fig. 4 Correlations between soluble P (µg ml^{-1}) and pH released by the peanut associated native isolates and *P. fluorescens* PMT1 **a** in unbuffered NBRIP-BPB medium (r=−0,72), **b** supplemented with Tris-Cl (r=−0,71) and **c** supplemented with MES (r=−0,78)

1997). Several studies have demonstrated that simultaneous infection of rhizobia with other rhizospheric bacteria increase nodule number and plant growth in a variety of legumes, including peanut (Grimes and Mount 1984; Ibañez et al. 2009). On the other hand, it is the first time that isolates from genera *Enterococcus* were detected inside root tissues. Considering the alkaline calcareous nature of soils from the peanut cultivation area of Cordoba, analysis of phosphate solubilizing ability in selected bacteria were performed using tricalcium phosphate as P source. Analysis of phosphate solubilizing ability in solid media indicated that all bacteria maintained this property even after

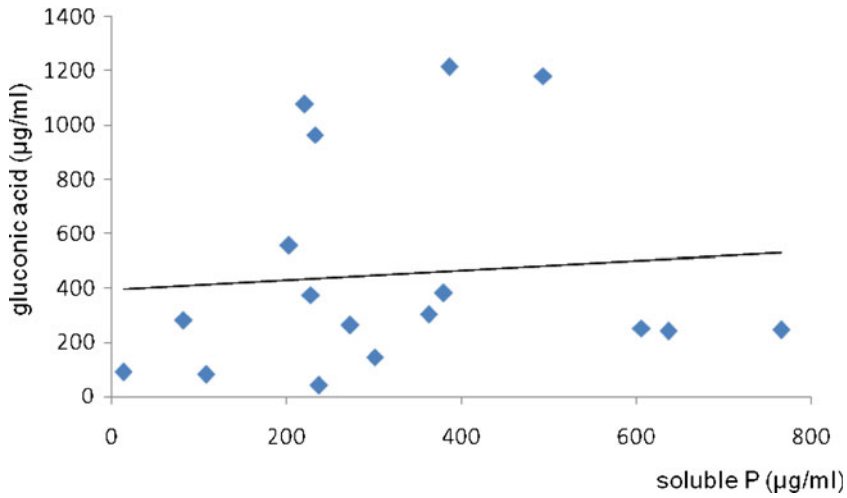

Fig. 5 Correlation between soluble P (µg ml^{-1}) and gluconic acid (µg ml^{-1}) released by the native isolates associated with peanut and *P. fluorescens* PMT1 in NBRIP-BPB medium (r=0,09)

ten subcultures, supporting the idea that it is a stable property in most strains (Das and Mukherjee 1994; Rodríguez and Fraga 1999; Taurian et al. 2010). Bacteria analyzed showed differences in the levels of soluble phosphorus released in NBRIP broth. These results are in agreement with those reported by other authors describing important differences in the bacterial kinetics and ability of $Ca_3(PO_4)_2$ solubilization (Delvasto et al. 2006; Pérez et al. 2007). Considering phosphate solubilization kinetics, isolates were separated in two groups (Collavino et al. 2010): one of them contained more than 75 % of bacteria and included isolates that showed the highest levels of soluble phosphorus released within the 3 first days of growth while the second one included bacteria that reached the highest phosphate solubilization ability at 7 days of growth. No relationship was found between kinetic or levels of soluble P released and the genus to which each isolate belong. On the other hand, amounts of soluble phosphorus released by each isolate in liquid medium showed no correlation with the diameter of halo of clearance produced in solid medium, indicating that halo formation is not a suitable criterion for selection of efficient phosphate solubilizers. Similarly, other authors have found contradictory results when analyzing diameter of solubilizing halos and amounts of Pi released in liquid media (Nautiyal 1999). Moreover, isolates with phosphate solubilizing ability on different insoluble P sources, were unable to produce solubilizing halo in solid media (Nautiyal 1999; Pérez et al. 2007).

Even when most of the studies regarding phosphate solubilizing bacteria have been developed in unbuffered conditions (Chen et al. 2006; Pérez et al. 2007; Collavino et al. 2010), it is well known that soils have a buffering capacity, mainly those alkaline or harboring calcium phosphate complexes (Ae et al. 1996) as peanut cultivated soils from Cordoba. Addition of buffer Tris–HCl to NBRIP decreased significantly the quantity of soluble phosphate produced by nearly 50 % of the isolates including *P. fluorescens* strain PMT1, meanwhile acidification of media were determined in all cultures. Isolates whose solubilizing capacity was not changed by the presence of Tris–HCl buffer (*Pantoea* sp. J49, *Enterococcus* sp. L191, *Serratia* spp. S119, S93 and J145) should be considered as the most efficient and potential candidates to assay in field experiments. On the other hand, when buffer MES was added to the medium, all bacterial cultures showed a drastic reduction in their phosphate solubilization capacity and a softly decrease in medium pH. Therefore, it is possible to speculate that in unbuffered medium, acidification could be a consequence of the production of organic acids and consequently it would be the mechanism involved in phosphate solubilization.

The negative correlationship observed between levels of soluble P released by bacteria and supernatants'pH either in unbuffered and buffered medium, suggests that acidification of medium due organic acid production would facilitate the bacterial P solubilization. This observation is consistent with earlier reports which have shown that mineral phosphate solubilization is accompanied by a decrease in pH (Hwangbo et al. 2003; Tripura et al. 2007). A major well-characterized mechanism involved in mineral phosphate solubilization in Gram negative bacteria is gluconic acid secretion. This acid results from the extracellular (periplasmic space) oxidation of glucose via the quinoprotein GDH (Goldstein 1996) that requires the cofactor PQQ, whose biosynthesis involves the *pqq* genes (Meulenberg et al. 1992; Kim et al. 2003). The chelation property of gluconic acid enables it to form insoluble complex with Ca^{+2} liberating phosphates (Li et al. 2008). Gluconic acid production has been described in *Pseudomonas* sp (Illmer and Schinner 1992), *Erwinia herbicola* (Liu et al. 1992); and *Burkholderia cepacia* (Rodríguez and Fraga 1999). In order to examine whether the phosphate solubilization trait of the isolates is associated with gluconic acid release its

production was analyzed in all bacteria of the collection. As expected, most of Gram positive bacteria analyzed only secreted traces of this acid while Gram negative showed significant higher levels. It is possible that, as it was determined by other authors (Rodríguez and Fraga 1999), the Gram positive bacteria analyzed in this study produce other acids rather than gluconic acid such as citric, lactic, propionic acids. Nevertheless, gluconic acid was produced in detectable amounts by four Gram positive isolates (*Bacillus* sp. J255, *B.* sp. J9, *B.* sp. L55 and *Enterococcus* sp. L185). Among them, the quantity secreted by *Bacillus* sp. J9 was higher than 500 μg ml^{-1}. It has been described that the production of 1.9 mg ml^{-1} of organic acids is sufficient to allow significant phosphate solubilization (Ahuja et al. 2007). In this study, levels of gluconic acid detected in supernatants from isolates cultures were lower than 1.9 mg-ml^{-1}. Other organic acids such as citrate, oxalic acid, etc. (Chen et al. 2006) would probably be involved in the high phosphate solubilization ability showed by some isolates in this study. Chen et al. (2006) reported that phosphate solubilizing *Serratia marcescens* synthesized citric acid as the major compound, in combination with other acids. Although low amounts of gluconic acid were synthesized by the isolates analyzed, they did significantly acidify the culture supernatants, producing in some cases, high amounts of soluble phosphorus. Some authors have related the levels of gluconic acids produced with the amount of P released when bacteria were grown in the presence of tricalcium phosphate (Ogut et al. 2010). On the other hand, absence of correlation between levels of solubilized P and gluconic acid production has been described by other authors (Illmer and Schinner 1992). Indeed, Gyaneshwar et al. (1998) and Halder et al. (1990) suggested that the quality (type of acid) rather than quantity of organic acid released by bacteria is more determinant for phosphate solubilizing activity. In this study, no correlation was found between levels of this acid produced and amounts of soluble P released by the isolates. Nevertheless, a tendency of low gluconic acid production was observed when small amounts of soluble P were liberated. In those isolates that released high amounts of soluble P but produced intermediate values of this acid, suggests that probably other organic acids are produced by these bacteria. Adding to that, Chen et al. (2006) analyzing phosphate solubilizing bacteria belonging to different genera (*Serratia*, *Bacillus*, *Rhodoccocus*, *Arthrobacter*, *Delftia*,etc.) indicated that levels of soluble P released by bacteria were independent of type of acid secreted and with its genetic identity.

Genes encoding PQQ cofactor are involved in the expression of phosphate solubilizing phenotype through the activation of the direct oxidation pathway of glucose (DOPG) that leads to the production of gluconic and 2- ketogluconic acids. Several studies using bacterial PQQ^- mutants have demonstrated the intimate relationship between GDH cofactor and phosphate solubilization processes (Kim et al. 2003; Han et al. 2008). In this work, the presence of *pqq*E and *pqq*C genes in the bacterial genome was evaluated in order to establish a possible relationship with the ability of the isolates to produce gluconic acid. Primers used in this study were designed by multiple alignement of *pqq* sequences of several bacterial genera that belong, in the case of those that amplify *pqq*E gene fragment, exclusively to order *Enterobacteriales*. Nevertheless, in silico PCR analysis demonstrated that they amplified *pqq* genes of the genome of other bacterial genera from other taxonomic orders such as *Pseudomonas*, *Acinetobacter*, etc. Adding to that, this was confirmed when PCRs were performed with DNA template of phosphate solubilizing reference strains belonging also to phylogenetic distantly related genera. These prompted us to employ these primers in this study. In all phosphate solubilizing Gram-negative bacteria it was found both expected amplification products while no bands were detected in PCR assays of Gram positive bacteria. The fact that *pqq* genes were not amplified from the DNA of some Gram positive isolates able to produce gluconic acid, may be due to a fail in the primers annealing, or assuming the PCR was optimized properly and inhibition is ruled out, then these genes likely aren't present in those bacteria assuming the involvement of a different gluconic acid biosynthetic pathway in these bacteria.

It was concluded from this study that endophytic and epiphytic phosphate solubilizing bacteria associated to peanut plants from the producing area of Cordoba include bacteria from Enterobactereacea, Bacillacea, and Enterococacea families. Although levels of soluble P released by bacteria varied, in all cases it was observed a drop in cultures'pH suggesting that production of organic acids could be their phosphate solubilizing mechanism. The production of gluconic acid, the main acid involved in mineral phosphate solubilization, was found in almost all bacteria. Nevertheless, the absence of correlation between levels of P and gluconic acid released by the bacteria suggests that the production of other organic acids are also involved. This conclusion is reinforced by data from Gram positive bacteria revealing the lowest levels of gluconic acid in their supernatants culture and absence of *pqq* genes in their genome. The presence of both *pqq*C and *pqq*E genes in all phosphate solubilizing Gram negative bacteria analyzed, including native isolates and reference strains that belong to different genera, encourages to suggest these genes as potential molecular markers for this bacterial group. Results obtained in this study confirm the great potential of native peanut bacteria to solubilize tricalcium phosphate under field conditions, an insoluble P source common in peanut cultivation area of Córdoba, Argentina. This beneficial property is very important to consider these bacteria to be used in microcosm assay

and in a longer future as an alternative to phosphorus fertilizers application.

Acknowledgments This research was supported by Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto (SECYT-UNRC), CONICET, Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) and Fundación Maní Argentino. María Soledad Anzuay and Liliana M. Ludueña are fellowships from CONICET, Tania Taurian, Jorge G. Angelini and Adriana Fabra are members of research career of CONICET, Argentina.

References

Achouak W, Thiéry M, Roubaud P, Heulin T (2000) Impact of crop management on intraspecific diversity of Pseudomonas corrugate in bulk soil. FEMS Microbiol Ecol 31:11–19

Ae N, Otani T, Makino T, Tazawa J (1996) Role of cell wall of groundnut roots in solubilizing sparingly soluble phosphorus in soil. Plant Soil 186:197–204

Ahuja A, Ghosh SB, D'Souza SF (2007) Isolation of a starch utilizing, phosphate solubilizing fungus on buffered medium and its characterization. Bioresour Technol 98:3408–3411

Altschul SF, Madden TL, Schäffer AA, Zhang J, Zhang Z, Millar W, Lipman DJ (1997) Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucl Acids Res 25:3389–3402

Anthony C (2004) The quinoprotein dehydrogenases for methanol and glucose. Arch Biochem Biophys 428:2–9

Arcand MM, Schneider KD (2006) Plant-and mirobial-based mechanisms to improve the argonomic effectiveness of phosphate rock: a review. An Acad Bras Cienc 78:791–807

Bai BP, Charles TC, Trevor C, Smith D (2002) Co-inoculation dose and root zone temperature for plant growth promoting rhizobacteria on soybean (*Glycin max* L.) growth in soil-less media. Soil Biol Biochem 34:1953–1957

Beringer JE (1974) R factor transfer in *Rhizobium leguminosarum*. J Gen Microbiol 84:188–198

Biville F, Turlin E, Gasser F (1989) Cloning and genetic analysis of six pyrroloquinoline quinone biosynthesis genes in *Methylobacterium organophilum* DSM760. J Gen Microbiol 135:2917–2929

Bosch EN, da Veiga A (2002) Pérdida de fertilidad de un suelo agrícola. INTA, Buenos Aires

Chen YP, Rekha AB, Arun FT, Shen W, Lai A, Young CC (2006) Phosphate solubilising bacteria from subtropical soil and their tricalcium phosphate solubilising abilities. Appl Soil Ecol 34:33–41

Chung H, Park M, Madhaiyan M, Seshadri S, Song J, Cho H, Sa T (2005) Isolation and characterization of phosphate solubilizing bacteria from the rhizosphere of crop plants of Korea. Soil Biol Biochem 37:1970–1974

Cole JR, Wang Q, Cardenas E, Fish J, Chai B, Farris RJ, Kulam-Syed-Mohideen AS, McGarrell DM, Marsh T, Garrity GM, Tiedje JM (2009) The Ribosomal Database Project: improved alignments and new tools for rRNA analysis. Nucleic Acids Res 37(Database issue):D141–145. doi:10.1093/nar/gkn879

Collavino MM, Sansberro PA, Mroginski LA, Aguilar OM (2010) Comparison of in vitro solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote *Phaseolus vulgaris* growth. Biol Fertil Soils 46:727–738

Das AC, Mukherjee D (1994) Effect of insecticides on the avalaibility of nutrients, nitrogen fixation, and phosphate solubility in the rhizosphere soil of rice. Biol Fertil Soils 18:37–41

de-Bashan LE, Bashan Y (2004) Recent advances in removing phosphorus from wasterwater and its future use as a fertilizer (1997–2003). Water Res 38:4222–4246

Delvasto P, Valverde A, Ballester A, Igual JM, Muñoz JA, González F, Blázquez ML, García C (2006) Characterization of brushite as a re-crystallization product formed during bacterial solubilization of hydroxyapatite in batch cultures. Soil Biol Biochem 38:2645–2654

Fiske EH, Subbarow Y (1925) The solubilization of phosphate: the action of various organic compounds on dicalcium and tricalcium phosphate. N Z J Sci Technol 33:436–444

Goldstein AH (1995) Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilisation by gram negative bacteria. Biol Agric Hort 12:185–193

Goldstein AH (1996) Involvement of the quinoprotein glucose dehydrogenase in the solubilization of exogenous phosphates by Gram-negative bacteria. In: Torriani-Gorini A, Yagil E, Silver S (eds) Phosphate in microorganisms: cellular and molecular biology. ASM Press, Washington, DC, pp 197–203

Grimes HD, Mount MS (1984) Influence of *Pseudomonas putida* on nodulation of *Phaseolus vulgaris*. Soil Biol Biochem 16:27–30

Gyaneshwar P, Naresh Kumar G, Parekh LJ (1998) Effect of buffering on the phosphate-solubilizing ability of microorganisms. World J Microbiol Biotechnol 14:669–673

Halder AK, Misra AK, Bhattavharya P, Chakrabartty PK (1990) Solubilization of rock phosphate by *Rhizobium* an *Bradyehizobium*. J Gen Appl Microbiol 36:81–92

Han SH, Kim CH, Lee JH, Park JY, Cho SM, Park SK, Kim KY, Krishnan HB, Kim YC (2008) Inactivation of *pqq* genes of *Enterobacter intermedium* 60-2G reduces antifungal activity and induction of systemic resistance. FEMS Microbiol Lett 282:140–146

Hoben HJ, Somasegaran P (1982) Comparison of the pour, spread, and drop plate methods for enumeration of *Rhizobium* spp. in inoculants made from presterilized peat. Appl Environ Microbiol 44:1246–1247

Hwangbo H, Park R, Kim Y, Rim Y, Park K, Kim T, Suh J, Kim K (2003) 2-ketogluconic acid production and phosphate solubilization by *Enterobacter intermedium*. Curr Microbiol 47:87–92

Ibañez F, Angelini J, Taurian T, Tonelli ML, Fabra A (2009) Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. Syst Appl Microb 32:49–55

Illmer P, Schinner F (1992) Solubilization of inorganic phosphates by microorganisms isolated from forest soil. Soil Biol Biochem 24:389–395

Joseph S, Jisha MS (2009) Buffering reduces phosphate solubilizing ability of selected strains of bacteria. World J Agric Sci 5:135–137

Kim CH, Han SH, Kim KY, Cho BH, Kim YH, Koo BS, Kim YC (2003) Cloning and expression of pyrroloquinoline quinone (PQQ) genes from a phosphate-solubilizing bacterium *Enterobacter intermedium*. Curr Microbiol 47:457–461

Latour X, Corberand T, Laguerre G, Allard F, Lemanceau P (1996) The composition of fluorescent pseudomonad populations associated with roots is influenced by plant and soil type. Appl Environ Microbiol 62:2449–2456

Li JH, Wang ET, Chen WF, Chen WX (2008) Genetic diversity and potential for promotion of plant growth detected in nodule endophytic bacteria of soybean grown in Heilongjiang province of China. Soil Biol Biochem 40:238–246

Liu TS, Lee LY, Tai CY, Hung CH, Chang YS, Wolfram JH, Rogers R, Goldstein AH (1992) Cloning of an *Erwinia herbicola* gene necessary for gluconic acid production and enhanced mineral phospahte solubilization in *Escherichia coli* HB101: nucleotide sequence and probable involvement in biosynthesis of the coenzyme Pyrroloquinoline. J Bacteriol 174:5714–5819

Mehta S, Nautiyal CS (2001) An efficient method for qualitative screening of phosphate-solubilizing bacteria. Curr Microbiol 43:51–56

Meulenberg JJM, Sellink E, Riegman NH, Postma W (1992) Nucleotide sequence and structure of the *Klebsiella neumoniae pqq* operon. Mol Gen Genet 232:284–294

Miller JH (1972) Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbory, NY, p 433

Nahas E (1996) Factors determining rock phosphate solubilization by microorganisms isolated from soil. World J Microbiol Biotechnol 12:567–572

Nautiyal CS (1999) An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. FEMS Microbiol Lett 170:265–270

Ogut M, Er F, Kandemir N (2010) Phosphate solubilization potentials of soil *Acinetobacter* strains. Biol Fertil Soils 46:707–715

Pérez E, Sulbarán M, Ball MM, Yarzábal LA (2007) Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the southeastern Venezuelan region. Soil Biol Biochem 39:2905–2914

Podile AR, Kishore GK (2006) Plant growth-promoting rhizobacteria. In: Gnanamanickam SS (ed) Plant-associated bacteria, part 2. Springer, pp 195–230

Richardson AE, Hadobas PA, Hayes JE, O'Hara CP, Simpson RJ (2001) Utilization of phosphorus by pasture plants supplied with *myo*-inositol hexaphosphate is enhanced by the presence of soil microorganisms. Plant Soil 229:47–56

Rodríguez H, Fraga R (1999) Phosphate solubilizing bacteria and their role in plant growth promotion. Biotech Adv 2:71–76

Rodríguez H, Fraga R, Gonzales T, Bashan Y (2006) Genetics of phosphate solubilization and its applications for improving plant growth-promoting bacteria. Plant Soil 287:15–21

Rossolini GM, Schippa S, Riccio ML, Berlutti F, Macaskie LE, Thaller MC (1998) Bacterial nonspecific acid phosphohydrolases: physiology, evolution and use as tools in microbial biotechnology. Cell Mol Life Sci 54:833–850

Seong JY, Yun JC, Hae KM, Kwang KC, Jin WK, Sang CL, Yeon HJ (1996) Isolation and identification of phytase-producing bacterium, Enterobacter sp. 4, and enzymatic properties of phytase enzyme. Enzym Microb Tech 18:449–454

Shen SH, Wertz DL, Klinman JP (2012) Implication for functions of the ectopic adipocyte copper amine oxidase (AOC3) from purified enzyme and cell-based kinetic studies. PLoS One 7(1):e29270

Sistema de Información Agroeconómica (2011) Informe ESPECIAL Maní, Bolsa de Cereales de Córdoba 2011

Stevenson FJ (2005) Cycles of soil: carbon, nitrogen, phosphorus, sulfur, micronutrients. Wiley, New York

Sturz AV, Chistie BR, Matheson BG, Nowak J (1997) Biodiversity of endophytic bacteria which colonize red clover nodules, roots, stems and foliage and their influence on host growth. Biol Fertil Soil 25:13–19

Taurian T, Aguilar OM, Fabra A (2002) Characterization of nodulating peanut rhizobia isolated from a native soil population in Córdoba, Argentina. Symbiosis 33:59–72

Taurian T, Anzuay MS, Angelini JG, Tonelli MA, Ludueña L, Pena D, Ibañez F, Fabra A (2010) Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities. Plant Soil 329:421–431

Tripura C, Sashidhar B, Podile AR (2007) Ethyl methanesulfonate mutagenesis-enhanced mineral phosphate solubilization by groundnut-associated *Serratia marcescens* GPS-5. Curr Microbiol 54:79–84

Vincent JM (1970) A manual for the practical study of root nodule bacteria, IBP Handbook N°15. Blackwell, Oxford

Walsh PS, Metzger DA, Higuchi R (1991) Chelex 100 as a medium for a simple extraction of DNA for PCR-based typing from forensic material. BoiTechniques 10:506–513

Yang XP, Zhong GE, Lin JP, Mao DB, Wei DZ (2010) Pyrroloquinoline quinone biosynthesis in *Escherichia coli* through expression of the Gluconobacter oxydans pqqABCDE gene cluster. J Ind Microbiol Biotechnolag 37:575–580