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Role of bacterial pyrroloquinoline quinone in phosphate solubilizing ability and in plant growth promotion on strain *Serratia* sp. S119

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Abstract The aim of this study was to analyze if cofactor pyrroquinoline quinone from *Serratia* sp. S119 is involved in the inorganic phosphate solubilization mechanism and in its ability to promote the plant growth. Site directed mutagenesis was performed to obtain a *pqq*E- minus mutant of strain *Serratia* sp. S119. The phosphate solubilization ability, gluconate and PQQ production of the mutant *Serratia* sp. RSL (*pqq*E-) was analyzed. Mutant RSL (*pqq*E-) showed significant decrease in P soluble and gluconic acid levels produced and undetectable levels of PQQ cofactor compared with wild-type strain. Complementation with synthetic PQQ cofactor restored P solubilization and gluconate production reaching the levels produced by wild-type strain. PqqE gene sequence indicated that it is highly conserved within *Serratia* strains and its product shows conserved motifs found in other PqqE proteins of several bacteria. The effect of the inoculation of the PQQ-mutant on peanut and maize plants was evaluated in pot assays. Plants growth parameters showed no differences among the different treatments indicating that PQQ from *Serratia* sp. S119 is not involved in the growth promotion of these plants. PQQ cofactor is essential for phosphate solubilization ability of *Serratia* sp. S119 but is not required for growth promotion of peanut and maize plants.

Keywords PQQ cofactor · *Serratia* sp. · Phosphate solubilization · Plant growth promotion · *pqqE* sequence

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1 Introduction

Phosphorus (P), next to nitrogen, is the second essential macronutrient required for plant growth. P exists in nature in a variety of organic and inorganic forms, mainly in insoluble or very poorly soluble forms. Soluble forms of P are easily precipitated and converted into Fe, Al or Mn phosphates in acid soils, and into Ca or Mg phosphates in alkaline soils, which are poorly soluble and, therefore, unavailable to plants (Gyaneshwar et al. 2002). P deficiency is one of the most important chemical factors limiting crop production in many soils worldwide (Arcand and Schneider 2006).

Within PGPB ("Plant Growth Promoting Bacteria"), 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 its subsequent availability to plant roots (Richardson et al. 2001). Phosphate solubilizing bacteria can play an important role in plant nutrition through an increase in P uptake (Rodriguez et al. 2006). Different bacterial genera such us *Pseudomonas, Bacillus, Rhizobium, Agrobacterium, Burkholderia, Acromobacter, Micrococcus, Aerobacter, Flavobacterium, Erwinia*, *Pantoea, Acinetobacter, Enterococcus* and *Enterobacter* are included in this group of beneficial bacteria (Nahas 1996; Kumar et al. 2001; Son et al.

2006; Pérez et al. 2007; Ogut et al. 2010; Walpola and Yoon 2013; Kaur and Sudhakara 2014).

Mobilization of insoluble inorganic phosphate by plant beneficial rhizobacteria is a key trait to the development of microbial biofertilizers. 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; Kim et al. 1997; Rodriguez et al. 2006). These acids chelate the cations (Al^{+3}, Fe^{+3}, Ca^{+2}) bound to the insoluble forms of phosphate and convert them into soluble forms with the consequent decrease in the pH of the medium (Kim et al. 1997). This mechanism has been reported in Gram negative bacteria, which produce gluconate and 2-cetogluconate through a non-phosphorylated direct oxidation of glucose (DOPG; Direct Oxidation Pathway of Glucose) whose physiological role remains unknown (Anthony 2004). Whiting et al. (1976) and Goldstein (1995) considered this oxidation as a competitive strategy to transform carbon sources in products that are less useful for other organisms. The DOPG protein glucose dehydrogenase (GDH) is oriented toward the outside of the cytoplasmic membrane so that it oxidizes its substrates in the periplasmic space (Anthony 2004). The activity of this enzyme requires the cofactor redox pyrroquinoline quinone (PQQ), whose biosynthesis involves a PQQ operon consisting of at least 5 *pqq* genes designated as *pqqABCDE* (Goosen et al. 1989; Biville et al. 1989; Meulenberg et al. 1992; Morris et al. 1994; Kim et al. 2003). Number and organization of PQQ biosynthetic genes differ from organism to organism but in most of these bacterial species studied so far PQQ biosynthetic genes are clustered in a putative *pqq*ABCDEF operon (Gliese et al. 2010). The *pqq*A gene encodes an oligopeptide of 23–24 amino acids, the putative precursor of the cofactor PQQ. The protein encoded by the *pqq*B gene is suggested to be involved in transporting PQQ across the cytoplasmic membrane into the periplasm. PqqC catalyses the last step in PQQ biosynthesis, which is oxygen dependent and the *pqq*F gene, codes for a putative peptidase. The function of the proteins encoded by the *pqq*D and *pqq*E genes is still unknown (Gliese et al. 2010). The cloning and expression of these genes has demonstrated the importance of the production of gluconic acid and 2-ketogluconic in the phosphate solubilizing ability of several bacteria (Kim et al. 2003; Rodriguez et al. 2006; Han et al. 2008).

Strain *Serratia* sp. S119 was isolated from peanut nodules and it exhibits a strong ability to solubilize phosphate (Anzuay et al. 2013) and to promote peanut growth (Taurian et al. 2010; Anzuay et al. 2013). We hypothesize that the phosphate solubilizing activity of *Serratia* sp. S119 is correlated with the production of gluconic acid, involving processes requiring PQQ as an essential cofactor for glucose dehydrogenase. The aim of this study was to analyze if PQQ from *Serratia* sp. S119 is involved in its ability to solubilize inorganic phosphate and to promote peanut and maize growth.

2 Materials and methods

2.1 Bacterial strains, plasmids, and growth conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Serratia* sp. S119 is a native phosphate solubilizing bacteria isolated from peanut tissues (Taurian et al. 2010). *Serratia* sp. S119 and *Escherichia coli* SM10 λ pir (Simon et al. 1983) were grown on Luria-Bertani (LB) agar medium or LB broth in a culture with shaking (Sambrook et al. 1989) at 28 °C and 37 °C, respectively. When required filter-sterilized antibiotics chloramphenicol (Cm), 30 μg ml^{-1} and gentamycin (Gm), 10 μg ml^{-1} were added.

2.2 Construction of the *Serratia* sp. S119 *pqq*E mutant

DNA manipulations for cloning and subcloning were carried out as described by Sambrook et al. (1989). The genomic DNA of *Serratia* sp. S119 was isolated from an overnight bacterial culture with HiYield™ Genomic DNA Mini Kit

Table 1 Bacterial strains and plasmids used or generated in this study

Strain/plasmid	Description	Reference
Serratia sp. S119	Wild type P-solubilizing isolate, Cm^r	Taurian et al. 2010
Escherichia coli SM10 λ pir	Rec- derivative of C600 with RP4-2Tc::Mu integrated in the chromosome, Km^r	Simon et al. 1982
E. coli SM 10-pKNOCK540	*E. coli SM10* containing plasmid pKNOCK540pqqE-Gm^r	This study
pKNOCK-Gm	1.6-kb R6K, mob RP4 Gm^r	Alexeyev 1999
pKNOCK540	pKNOCK-Gm containing 540 pb *pqq*E gene fragment, Gm^r	This study
Serratia sp. RSL (*pqq*E-)	*Serratia* sp. S119 derivative with chromosomal *pqq*E gene disrupted by pKNOCK-540*pqq*EGm^r, Cm^r, Gm^r	This study

Cm Chloramphenicol, *Km* Kanamycin, *Gm* Gentamicin

(Real Genomics) and by the CTAB/NaCl method (Ausubel et al. 1991). The plasmid DNA was isolated by using NucleoSpin Plasmid (MACHEREY-NAGEL).

The 700-pb *pqq*E gene fragment was amplified using the primers pqqEF-317 5′TTYTAYACCAACCTGATCACSTC3′ and pqqER-1019 5′TBAGCATRAASGCCTGRCAG3′ (Anzuay et al. 2013). This fragment was sequenced by Macrogen Inc (Seoul, Korea) and digested with *Pst*I. A *Pst*I 540-bp fragment of the *pqq*E gene was cloned into the pKNOCK-Gm plasmid (Alexeyev 1999) to give pKNOCK540. Plasmid pKNOCK540 was then introduced into chemical competent *Escherichia coli* SM10 λ pir cells (Huff et al. 1990). *Serratia* sp. S119 wt (Cm^R) site directed mutagenesis was developed by biparental mating using *E. coli* SM10 λ pir containing pKNOCK540 as donor strain. The pKNOCK-Gm^R is a suicide plasmid in enterobacterial cells, and as a result of homologous recombination between the cloned 540-bp internal region of *pqq*E gene and the chromosomal copy of the gene, the 5′ region of the gene is separated from the 3′region by insertion of the pKNOCK540 plasmid. The putative mutants were selected by their resistance to Gm and Cm, and by the absence of phosphate solubilization halo on NBRIP-BPB solid medium (National Botanical Research Institute's phosphate grown medium) (Mehta and Nautiyal 2001). The insertion of the pKNOCK540 construct into the *pqq*E gene was confirmed through PCR assays. A *Serratia* sp. S119 derivative was obtained and named *Serratia* sp. RSL (*pqqE*-).

2.3 Genotypic analysis of *pqq*E minus mutant

The insertion site and orientation of pKNOCK540 construct in the mutant were evaluated by PCR using combinations of pKNOCK primer (5′TAAGGTTTAACGGTTGTGG3′) with pqqEF or pqqER primers. Each PCR reaction contained 1 μl (10 μmol l^{-1}) of each primer, 1 μl (10X) of PCR buffer, 1 μl (2 mmol l^{-1}) of dNTPs, 2,65 μl of sterile bidistilled water, 1, 2 μl of $MgCl_2$ (25 mmol l^{-1}), 0,15 μl of Taq DNA polymerase (5 U μl^{-1}) and 2 μl of template DNA. Amplification was performed in a Mastercycler gradient block (Eppendorf, Germany). The temperature profile was: 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. *Serratia* sp. S119 wt and *Serratia* sp. RSL (*pqq*E-) mutant strains were used for ERIC and ARDRA fingerprints analyses. The sequences of ERIC (Enterobacterial Repetitive Intergenic Consensus) primers E1 (5′-ATGTAAGCTCCTGGGGATTCAC-3′)/E2 (5′-AAGTAAGTGACTG GGGTGAGCG-3′) used in this study were described by de Bruijn (1992). The ERIC-PCR was performed in 12 μl reaction mixture containing 1x PCR buffer, 1.5 mmol l^{-1} $MgCl_2$, 200 μmol l^{-1} of each nucleotide (Promega, USA), 0.3 μmol l^{-1} of each primer, 1 U of Taq DNA polymerase (Promega, USA) and 3.6 μl of template DNA solution. The temperature profile was as follows: initial denaturation at 95 °C for 1 min, 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 65 °C for 8 min and a final extension step at 68 °C for 16 min. PCRs were performed in a Mastercycler gradient block (Eppendorf, Germany). The ERIC amplification products in 12 μl sub-samples were separated according to molecular size by horizontal electrophoresis on 2.5 % (w/v) agarose gels and stained with SYBR-Green.

FD1 and RD1 universal primers (Laguerre et al. 1994) were used for amplification of a fragment of the 16S rRNA gene. The reactions were carried out in a 20 μl reaction mixture containing 1 μl (10 μmol l^{-1}) of each primer, 1,2 μl (10X) of buffer, 1,2 μl (2 mmol l^{-1}) of dNTPs, 3,03 μl of sterile bidistilled water, 1,44 μl of $MgCl_2$ (25 mmol l^{-1}), 0,13 μl of Taq DNA polymerase (5 U μl^{-1}) and 2 μl of template DNA. Amplification was performed in a Mastercycler gradient block (Eppendorf, Germany). The temperature profile for PCRs was: an initial cycle at 95 °C for 3 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. The amplification products were separated according to molecular size by horizontal electrophoresis on 2.5 % (w/v) agarose gels and stained with SYBR green. Amplicons were digested with the endonuclease enzymes *Cfo*I and *Msp*I (Promega); 6,3 μl reaction mixtures contained 5 μl of PCR product, 0,7 μl buffer B 1X, 0,5 μl enzyme (3U), and 0,1 μl BSA (bovine serum albumine). RFLPs were resolved by electrophoresis in 2 % agarose gels with 0.5X TBE buffer. Gels were stained with SYBR green.

2.4 Phosphate solubilization ability measurements

In vitro inorganic phosphate-solubilizing ability was determined in NBRIP-BPB solid medium 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 2–7 days. The halo of clearance around the bacterial colony indicated P solubilizing ability. Each treatment was replicated 3 times and the assay was repeated twice.

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 NBRIP-BPB medium pH 7. After 2, 6, 8, 10, 12, 24, 30, and 48 h of growth, 1.5 ml of bacterial cultures were sampled and centrifuged for 12 min at 10,000 g. The amount of soluble P released to the medium was quantified spectrophotometrically by measuring absorbance at 660 nm. At each incubation time, supernatants' pH of each sample and CFU ml^{-1} in LB medium by using drop plate method (Hoben and Somasegaran 1982) were determined.

2.5 PQQ analysis

To measure PQQ production, bacteria were grown for 48 h at 28 °C in NBRIP-BPB medium. One volume of cell culture was diluted with nine volumes of methanol and the precipitated materials were removed by using nylon filters of 0.22 μm pore size. After evaporation of the methanol, the samples were lyophilized and this product was resuspended in 10 ml of mobile phase. Reverse phase HPLC was performed using a Perkin Elmer 200 Series HPLC System equipped with autosampler and fluorescence detection. Fluorescence was monitored at e_x 360 and e_m 480 nm. A Phenomenex LUNA RP C-18 column (250 mm × 4,5 ID, 5 μm pore size) (Phenomenex Inc., Torrance, CA, USA) was used for analytical separation fitted with a C18 guard column using a gradient mode. RP-HPLC was performed as described previously by Stites et al. (2000) with modifications. The initial mobile phase for the HPLC protocol consisted of 30 % methanol and 70 % 0.06 M phosphoric acid. A linear gradient was applied from 5 to 30 min with a final concentration of 70 % methanol and 30 % 0.06 mol l^{-1} phosphoric acid.

2.5.1 PQQ derivatization

PQQ was derivatized with acetone to form the acetone adduct (5-acetonyl-PQQ) to aid in identity and validation [18]. PQQ (200 nmol, 0.2 ml) was derivatized in 0.1 M sodium carbonate (pH 9.2, 0.1 ml) with the addition of 16 % acetone (v/v, 0.1 ml) at 37^{3}C for 30 min (Stites et al. 2000).

2.6 Gluconic acid production

Gluconic acid production was determined in bacterial supernatant by HPLC. Briefly, *Serratia* sp. S119 wt and RSL (*pqq*E-) mutant were grown in LB medium for 48 h at 28 °C. Five hundred ml of culture were sampled and 0.5 ml of absolute ethanol was added to the sample. The mixture was maintained at 4 °C 24 h and then, centrifuged at 10,000 rpm for 5 min. Supernatant was filtered using nylon filters of 0.22 μm pore size. HPLC assays were performed in Center for Research and Development of Industrial Fermentations (CINDEFI, CONICET-UNLP). Gluconic acid was determined by High Performance liquid chromatography (HPLC) with a Hamilton PRP-X300 (250 × 4,1 mm ID, 7 μm pore size) column equipped with Waters 717 plus autosampler and UV detector. UV absorption was monitored at 210 nm. The mobile phase was H_2SO_4 1 mN applied at a flow rate of 2.0 ml.min^{-1}. Column temperature was maintained at 25 °C.

2.7 Complementation assays with synthetic PQQ cofactor

Complementation assays was done adding exogenous synthetic pyrroloquinoline quinone (PQQ) (Sigma, product number D7783) to *Serratia* sp. RSL (*pqq*E-) mutant solid and liquid culture media (Fender et al. 2012; Guo et al. 2009) to a final concentration of 3.03 μmol $^{-1}$. *Serratia* sp. S119 wt and *Serratia* sp. RSL (*pqq*E-) mutant were initially grown overnight in 15 ml LB broth media. RSL (*pqq*E-) LB broth was supplemented with 15 μl of synthetic PQQ from the beginning of the incubation period. Afterwards, 10 μl of these bacterial cultures were placed in NBRIP-BPB plates and, after 2 to 7 days of incubation at 28 °C, the diameter of halo of clearance around the bacterial colony was analyzed. Simultaneously, 50 μl of RSL (*pqq*E-) mutant and wt strains LB cultures were transferred from these to tubes containing 15 ml NBRIP-BPB medium with and without synthetic PQQ cofactor. After 10, 24 and 48 h of growth a 28 °C at 150 rpm, 1.5 ml of each culture was sampled to determine levels of soluble P, pH of supernatant and viability of bacteria as described above.

2.8 *PqqE* gene sequencing and data analysis

The nucleotide sequence of full-length *pqq*E gene of strain *Serratia* sp. S119 was obtained by PCR amplification. For this, two pair of specific primers were designed, Fw1pqqFSerratia/Rv1pqqESerratia (5′TCTGCGCTTACTTCGAAGAAGA3′/5′ACCTGCCAGTTCTACGGCT3′) and Fw2pqqEserratia/Rv2pqqDserratia (5′ACTGTGACAGGGTAACGCCAT3′/5′ATCATCGCCCAGTTGAACG3′) that amplify ~830 pb and ~660 pb products respectively. These primers were designed from the sequence of *pqqF, pqqE y pqqD* genes of *Serratia marcescens* SM39 (AP013063.1). Amplification products obtained were sequenced and alienated in order to generate consensus full-length *pqq*E sequence.

Phylogenetic analysis was performed using BioEdit (Hall 1999) and MEGA version 4 (Tamura et al. 2007) softwares. Full-length sequences of *pqqE* gene of several bacterial strains were used for phyologenetic analysis: *S. marcescens* subsp. *marcescens* Db11 (HG326223.1), *S. marcescens* SM39 (AP013063), *S. marcescens* subsp. *marcescens* ATCC13880 (JMPQ01000044.1), *S. marcescens* WW4 (NC_020211.1), *S. marcescens* FG194 (NC_020064.1), *S. plymuthica* 4Rx13 (CP006250.1), *S. ureilytica* LG59 (JSFB01000001.1), *S. odorifera* DSM4582 (ADBY01000052.1), *S*. sp. FS14 (CP005927.1), *S. marcescens* RM66262 (NZ_JWLO01000001.1), *S. nematodiphila* DSM21420 (NZ_JPUX01000002.1), *S*. sp. M24T3 (NZ_AJHJ01000004.1), *S.* sp. SCBI (CP003424.1), *S. marcescens* YDC563 (JPOB01000002.1), *S. marcescens* UC188 (NZ_KQ089789.1), *S. marcescens* CAV1492 (NZ_CP011642.1), *Pantoea ananatis* LMG20103 (CP001875.2), *Enterobacter aerogenes* CDC UA0804-01 (JOUC01000005.1), *Kluyvera intermedia* (AY216683.1), *Rahnella aquatilis* HX2 (FJ868974.1), *Klebsiella pneumonia* SB3193 (LK022716.1) *Pseudomonas fluorescens* F113 (CP003150.1) and *Pseudomonas* sp QAU92 (KM251436.1)

2.9 *Rpo*B gene sequencing and data analysis

The nucleotide sequence of partial *rpo*B gene of *Serratia* sp.S119 was directly obtained by Macrogen Laboratories (Korea) by using universal primers rpoBF/rpoBR (Ko et al. 2002) and sequence analysis was performed by using softwares decribed above.

2.10 Plant growth promotion and bacterial survival measurements

2.10.1 Plant growth promotion assay

Ability of *Serratia* sp. RSL (*pqq*E-) mutant to promote growth of peanut (*Arachis hypogaea* L.) *and* maize (*Zea mays* L.) plants was analyzed. Seeds of *Arachis hypogaea* L cv. Granoleico were surface disinfected in 96 % ethanol for 30 s, rinsed in sterile water, dipped in H_2O_2 15 % during 10–15 min, and washed 5 times in sterile water (Anzuay et al. 2013). Seeds of Maize NK 910 TDMax (Syngenta) were surface disinfected in 96 % ethanol for 30 s, rinsed in sterile water, dipped in 6 % sodium hypochlorite for 10 min, and washed 6 times in sterile water (Pereira et al. 2011). Seeds were germinated at 28 °C in sterilized Petri dishes with one layer of Whatman N°1 filter paper and moist cotton, until the radicle reached approximately 2 cm length (Taurian et al. 2002). Seedlings were transferred to sterilized plastic pots that contained a mixture of sterile vermiculite and sand (2:1) supplemented with $Ca_3(PO_4)_2$ (2 g l^{-1}) as unique source of P. The following treatments were used: (a) Uninoculated peanut/maize plants (control); (b) Uninoculated peanut/maize plants supplemented once a week with KH_2PO_4 (20 mmol l^{-1}) (phosphorus fertilized plants); (c) Plants inoculated with wild type strain *Serratia* sp. S119, (d) Plants inoculated with mutant *Serratia* sp. RSL (*pqq*E-). Bacterial inoculums were obtained by harvesting 3 ml (10^9 CFU ml^{-1}) of stationary phase LB cultures supplemented with appropriate antibiotics and incubated at 28 °C and 120 rpm. Inoculums were deposited in the crown of the root. Plants were grown under controlled environmental conditions (light intensity of 200 μR/m^{-2} sec −1,16-h day/8-h night cycle, at a constant temperature of 28 °C and a relative humidity of 50 %), watered twice a week with sterilized tap water and once a week with the nutrient solution described by Hoagland and Arnon (1950) devoid of soluble phosphate. Plants were harvested at 21 and 40 days post inoculation for maize and peanut, respectively, and their aerial and root length, shoot and root dry and fresh weight were determined. In maize plants, P content in the mixture of sterile vermiculite and sand (rhizospheric substrate) they were grown (Fiske and Subbarow 1925) and in the aerial tissues (Murphy and Riley 1962) were determined.

2.10.2 Determination of the number of viable bacteria after harvest

To determine rhizosferic, rhizoplane and endophytic populations of inoculated bacteria samples were obtained at the end of the experiments. For each tissue/substrate three samples were combined to determine the average of bacterial colonization. Serial dilutions of 1 g of plant growth substrate was done in tubes containing 9 ml of phosphate buffered saline (PBS) and then, plated on LB supplemented with the appropriate antibiotics. After incubation of plates at 28 °C during 24 h, CFU.g^{-1} substrate was determined. Isolation of epiphytic and endophytic bacteria from plant tissues (leaves, stem and roots) were performed as described by Kuklinsky-Sobral et al. (2004). For the isolation of epiphytic bacteria 3 g of tissue were placed in 500 ml Erlenmeyer flask containing 25 g of glass beads with a diameter of 0.1 cm and 50 ml of phosphate buffered saline (PBS) and agitated at 150 rpm for 1 h. To isolate endophytic bacteria, plant tissues were surface-disinfected by serial washing in 70 % ethanol for 1 min, 2 % sodium hypochlorite for 3 min, 70 % ethanol for 30 s and two rinses in sterilized distilled water. The process was checked by plating aliquots of the wash water used in the final rinse onto LB and incubating the plates at 28 °C. After disinfection process, leaves and stems were macerated individually in mortars containing 10 ml physiologic solution. The tissue was then treated in flasks as above. Appropriate dilutions (10^{-1}–10^{-3}) of the flask contents were plated onto LB supplemented with appropriate antibiotics and the plates incubated at 28 °C for 24 h. CFU ml^{-1} were determined by the microplate method (Somasegaran and Hoben 1994).

2.11 Statistical analysis

Data analysis was carried out using the Infostat software by ANOVA and differences among treatments were detected by LSD or Tukey tests ($p < 0.05$).

3 Results

3.1 Genotypic and phenotypic analysis of *pqq*E- minus mutant

Site directed mutagenesis experiments of *pqq*E gene generated 19 phosphate solubilizing deficient transformants. From these bacteria, only *Serratia* sp. S119 RSL (*pqq*E-) showed not to be affected in its growth in NBRIP-BPB and LB media, reaching similar values of CFU than the wt strain (Fig. 1a). Analysis of genomic (ERIC) and ARDRA profiles indicated that it is isogenic with the wild type strain (data not shown).

The simple recombination event produced to interrupt the chromosomal copy of *pqq*E gene generates two possible

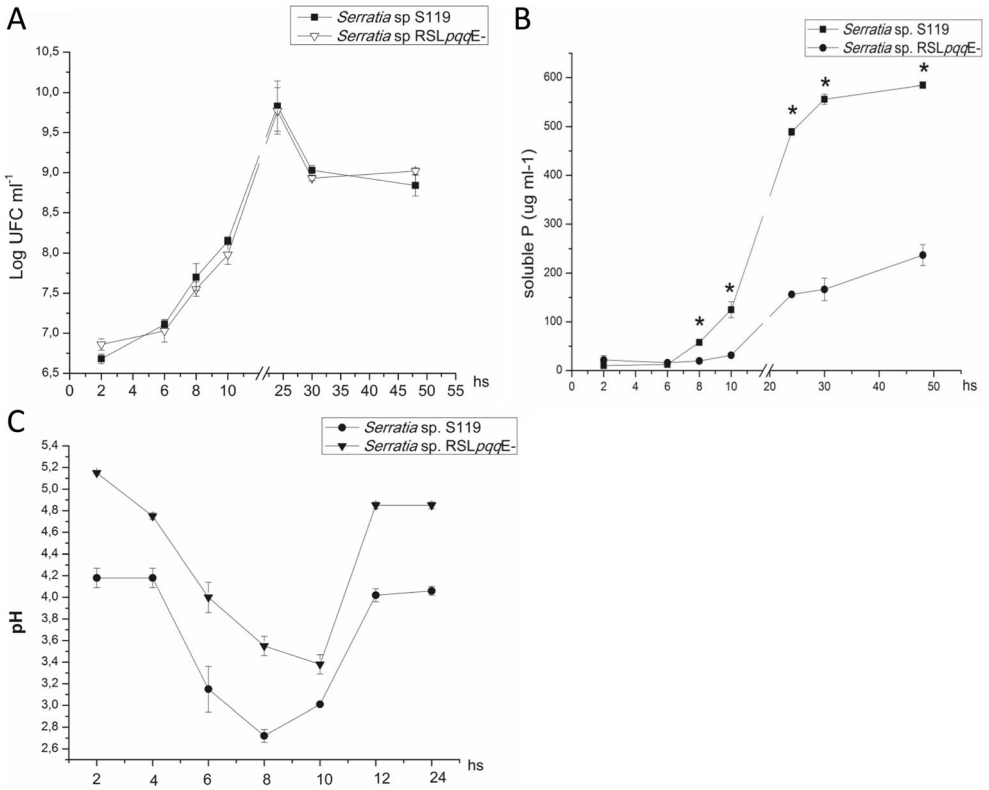


Fig. 1 Changes in the Log UFC ml^{-1} (**a**), soluble P (**b**) and pH (**c**) of culture supernatants of the wild type and RSL (*pqq*E-) mutant of *Serratia* sp. S119 when they were grown on NBRIP-BPB liquid medium at 30 °C. Each value is the mean ± S.E. of 3 independent replicates ($n = 5$) * Statistically different ($p < 0.05$)

orientations of the insert (Fig. 2a). The combination of pqqER/pknock primers allowed obtaining an 800-pb fragment confirming the pKNOCK540 insertion and its orientation (Fig. 2b).

The ability of RSL (*pqq*E-) to solubilize mineral phosphate was evaluated using tricalcium phosphate as sole P source in broth and agarized medium. After 2-days of incubation at 28 °C a clear zone around the wild-type *Serratia* sp. S119 was seen (Fig. 3a). Minimal traces of tricalcium phosphate were solubilized by the mutant strain as evidenced by lack of clear zones around the colonies. Quantification of soluble P released by RSL (*pqq*E-) in liquid NBRIP medium indicated a significant decrease compared to wild-type strain (Fig. 1b). Concurrently, pH values of mutant supernatant culture medium were higher than those from wild-type's supernatant (Fig. 1c).

PQQ was not identified in culture media of mutant RSL (*pqq*E-) indicating that this strain is unable to produce this cofactor (Fig. 4a). Wild type strain produced ~725 nmol of PQQ cofactor at 48 h of growth.

Complementation with synthetic PQQ restored the *pqq*E minus mutants' ability to solubilize phosphate (Fig. 3a and b). After 48 h growth, the amount of soluble phosphate determined in the culture media from wild-type strain and the complemented mutant was ~300 μg l^{-1} while it remained at ~100 μgl^{-1} in the *pqq*E- mutant culture medium. After 10, 24 and 48 h of growth in broth NBRIP medium, the pH values of both *Serratia* sp. S119 and the complemented mutant cultures dropped to ~3 (Fig. 3c), and this was correlated with increased levels of soluble phosphate. In contrast, the pH of the RSL (*pqq*E-) mutant culture medium remained at ~4–4.5.

In wild-type strains' culture growing with or without PQQ, the concentration of gluconate was 600 and 610 mg ml^{-1}, respectively. Complemented mutant restored gluconic acid production reaching levels similar to that of wt strain (580 mg ml^{-1}) while non-complemented mutant strain produced only 130 mg ml^{-1} (Fig. 4b).

3.2 Sequence analysis of *pqqE* gene of *Serratia* sp. S119

The nucleotide sequence of full-length (1137 pb) *pqqE* gene from *Serratia* sp. S119 was determined and compared to sequences available in data banks. Phylogenetic *pqq*E sequence analysis revealed that it was related to other *Serratia* strains, closely related to *S. marcescens* strains (Fig. 5). Analysis of phylogenetic tree obtained confirms that *pqqE* gene is highly conserved within genus taxonomic level (Wecksler et al. 2009).

Analysis of nucleotide sequences by BLAST-X indicated that *pqqE* codifies a 321 aminoacids peptide (data not shown) that shows an elevated identity (97–100 %) with other PqqE proteins of bacterial strains from *Serratia* genus and to other strains of *Enterobactereaceae* and *Pseudomonadaceae* family. Two highly conserved domains were identified in the aminoacidic sequence: SAM radicals and SPAMS superfamily.

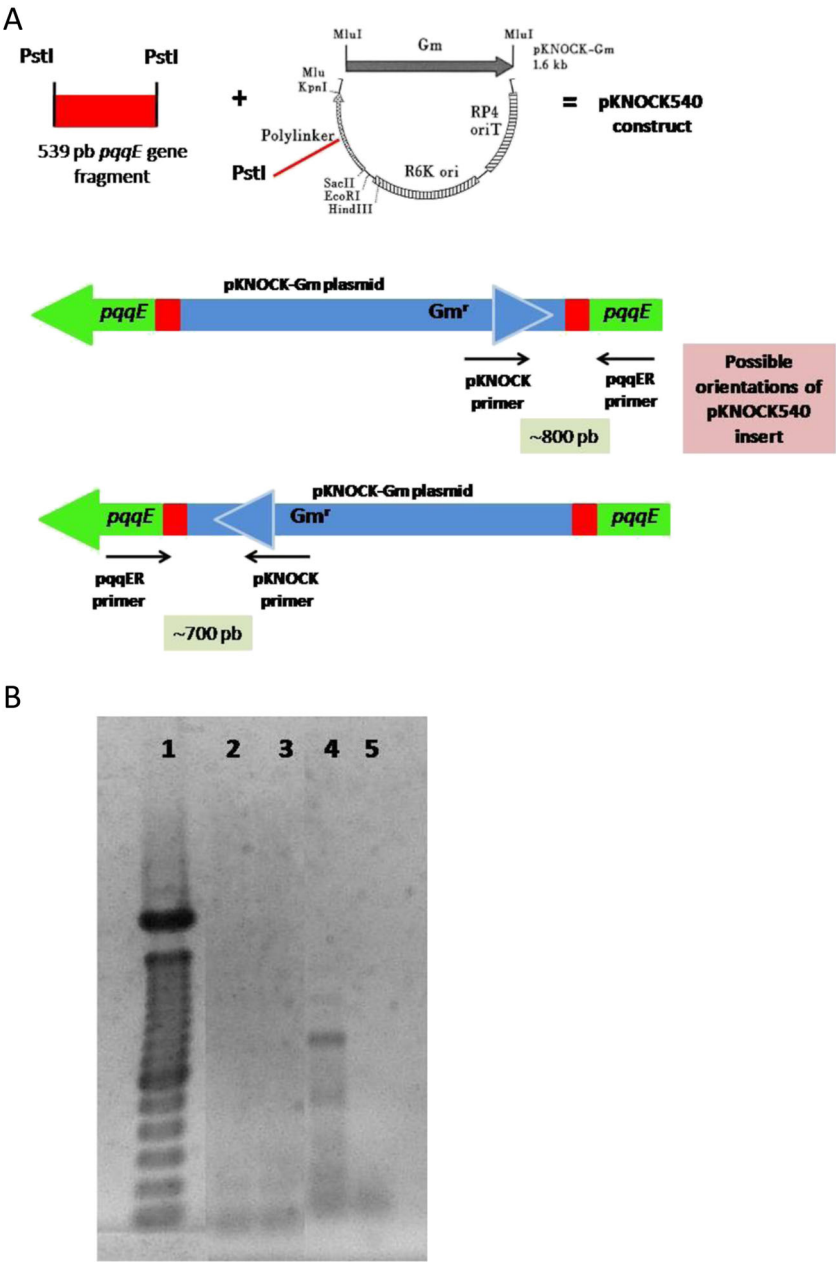


Fig. 2 Detection of pKNOCK540 insert. Scheme of pKNOCK540 construct and its possible orientations in the RSL (*pqq*E-) mutant (**a**). Gel electrophoresis of PCR products obtained using pknock/pqqEF and pqqER/pknock primers of DNA obtained from RSL (*pqq*E-) mutant. Lane 1: 100- pb molecular marker, 2–3: DNA from RSL (*pqq*E-) strain and negative control respectively using pknock/pqqEF primers, 4–5: DNA from RSL (*pqq*E-) strain and negative control respectively using pqqER/pknock primers (**b**)

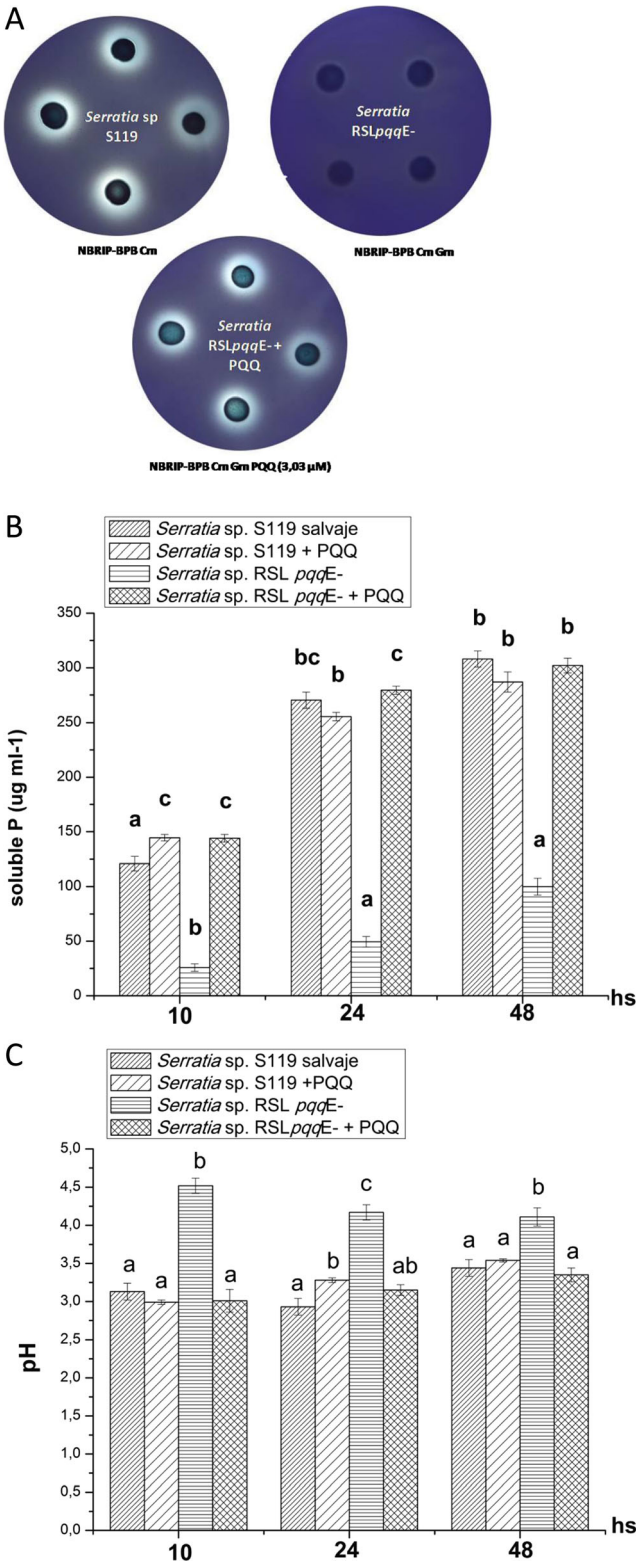
3.3 Sequence analysis of *rpo*B gene of Serratia sp. S119

The nucleotide sequence of partial (360 bp approximatelly) *rpo*B gene from *Serratia* sp S119 was determined and compared to sequences available in data banks. Phylogenetic *rpo*B gene sequence analysis revealed that it was 98 % identical to that of *Serratia marcescens* (data not shown).

3.4 Plant growth promotion ability and survival of P solubilizing deficient mutant

The ability of *Serratia* sp. RSL (*pqq*E-) to promote the growth of peanut and maize plants was analyzed in pot assays. Considering that peanut plant has its highest P requirement in the reproductive stages of growth (R1-R4) pot assays in which this plant was inoculated with RSL (*pqq*E-) mutant and wt strain were 40 day long. On the other hand, maize plants were harvested at 21 days postinoculation (V5) since this plant has its highest P requirement in vegetative growth stages. Results obtained indicated no differences between mutant and wt strain ability to promote plant growth (Fig. 6a and b). In the case of maize plants, both strains increased all plant growth parameters analyzed.

P content determined in maize plants and substrate where they were grown indicated no differences between treatments inoculated with mutant or with wt strains (Fig. 6c), being these values significantly higher than those obtained from substrate of uninoculated plants growing withouth P addition (Fig. 6d).



◀ **Fig. 3** Halo of clearance indicating P solubilizing activity of *Serratia* sp. S119 wild type strain, RSL (*pqq*E-) mutant strain and RSL (*pqq*E-) mutant strain supplemented with synthetic PQQ cofactor after 2 days of growth in NBRIP-BPB solid medium (**a**), Amounts of P solubilized (**b**) and pH values (**c**) on supernatants of wt and RSL (*pqq*E-) mutant strains growth cultures at 10, 24 and 48 hs of growth in NBRIP-BPB liquid medium supplemented with synthetic PQQ cofactor. Each value is the mean ± S.E. of 3 independent replicates ($n = 5$). * Statistically different, different letters indicate significant statistic differences ($p < 0.05$)

Both strains were isolated from the outside of peanut and maize root tissues (epiphytic) and plant growth substrate (Table 2). Number of bacteria recovered from maize plants was significantly higher than those obtained from peanut plants. The number of endophytic bacteria varied with plants and plant organs. Wild type strain was isolated from all tissues of both plants while RSL (*pqq*E-) mutant was not recovered from peanut root tissues nor from maize leaves tissues. Number of viable cells recovered of wild type strain was higher than that recovered of mutant strain.

4 Discussion

Strain *Serratia* sp. S119 was isolated from peanut nodules and it exhibits a strong ability to solubilize phosphate (Anzuay et al. 2013). This bacterium was also found to promote peanut growth (Taurian et al. 2010; Anzuay et al. 2013). We hypothesize that the phosphate solubilizing activity of *Serratia* sp. S119 is correlated with the production of gluconic acid, involving processes requiring PQQ as an essential cofactor for glucose dehydrogenase.

In order to examine whether the cofactor PQQ is involved in the ability of *Serratia* sp. S119 to solubilize inorganic phosphate we obtained a *pqq*E gene mutant. As expected, this strain showed significant decrease in its phosphate solubilizing ability. In addition, the fact that a less acidification and a diminution of the gluconic acid content in the culture media of the mutant strain was also observed indicates that *pqq*E gene of *Serratia* sp. S119 is involved in the solubilization of insoluble phosphate. The biochemical functions of many proteins coded by the *pqq* genes have been elucidated, and it has also been reported that some of them (such PQQB and PQQF) are not essential to PQQ biosynthetic pathway (Puehringer et al. 2008; Shen et al. 2012; Velterop et al. 1995). In this work the absence of PQQ in the supernatants of RSL (*pqq*E-) bacteria indicates that PQQE is a key protein in *Serratia* sp. S119 PQQ's biosynthetic pathway. Phylogenetic analysis of *pqq*E gene of *Serratia* sp. S119 indicated high identity with nucleotide sequences of this gene from *Serratia marcescens* strains. This confirms its close phylogeny together with the analysis of partial *rpo*B gene sequence obtained and with 16S sequence of this bacterium that showed high identity with several *Serratia marcescens* strains (Anzuay et al. 2013). It was interesting to note that *pqq*E gene sequence grouped with sequences of *Serratia marcescens* strains isolated form clinical samples. This could be explained since few sequences of soil/rhizospheric strains have been deposited in GenBank. In addition, in the

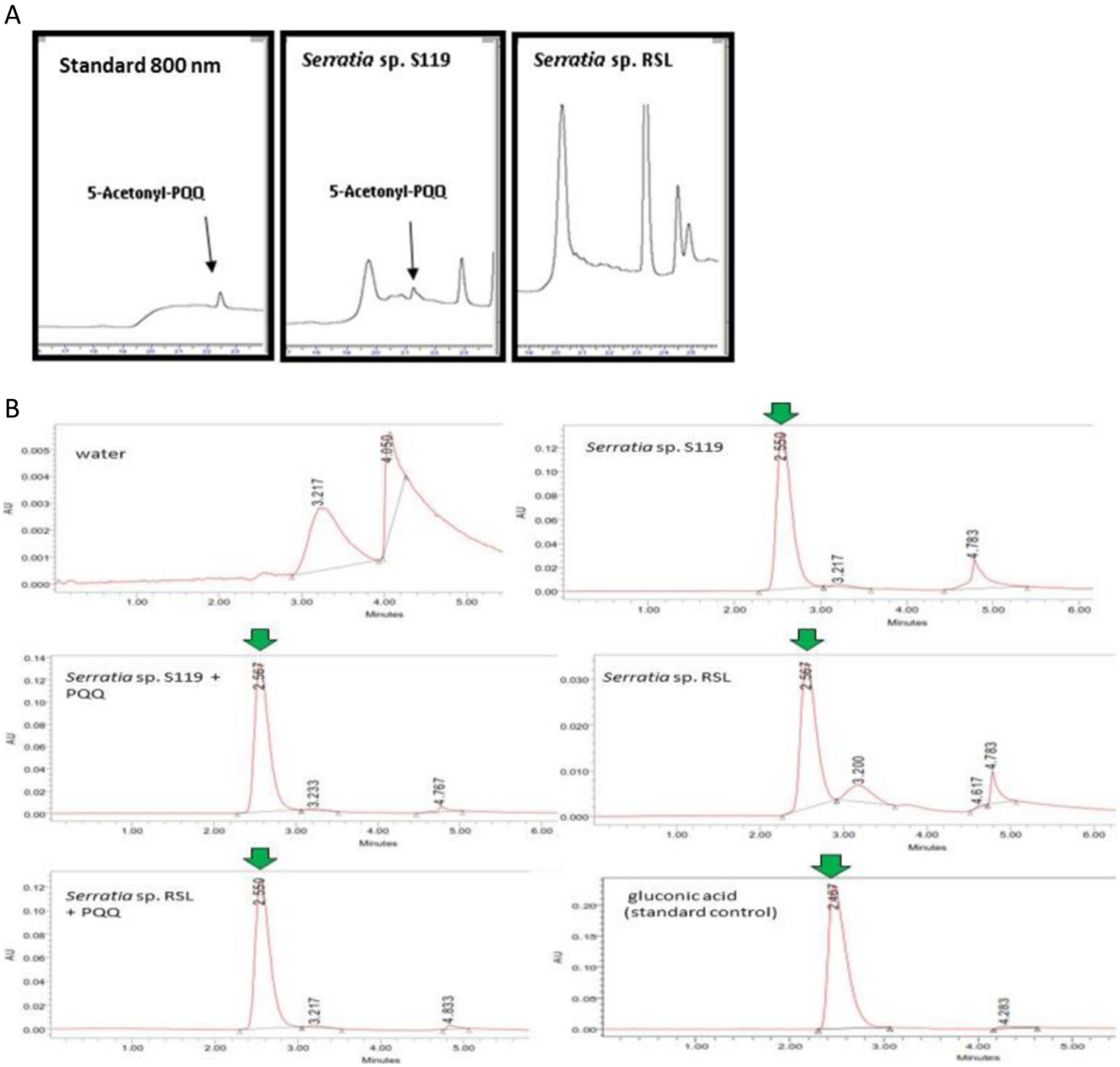


Fig. 4 Quantities of PQQ synthesized by wild-type strain *Serratia* sp. S119 and mutant RSL (*pqq*E-). HPLC detection of 5-acetonyl-PQQ in S119 and RSL (*pqq*E-) strains, arrows indicate 5-acetonyl-PQQ at 48 h of growth (**a**). Quantities of gluconic acid synthesized by wild-type strain *Serratia* sp. S119 and mutant RSL (*pqq*E-) growing with or without PQQ. HPLC detection at 48 h of growth (**b**)

aminoacidic sequence of PqqE, SAM radicals and SPAMS superfamily domains were identified. It has been described that gene products that contain SAM radicals domains presents a CxxxCxxC conserved motif and catalyzes cellular metabolic reactions such as ADN repair and biosynthesis of vitamins, coenzymes and several antibiotics. The SPASM domain of the C-end is characteristic of proteins that contain SAM radical domain and is found in those that activate other proteins (Springer et al. 1996; Wang and Perry 2007; Wecksler et al. 2009; Haft and Basu 2011; Latham et al. 2015).

In general the ability of both strains to promote plant growth was higher in maize than in peanut plants. Maize crop is used in rotation practices in peanut crop producing area of Córdoba, and it has been reported that this cereal has an elevated demand of phosphorus. Our data indicate that this bacterium *Serratia* sp. S119 and its isogenic mutant RSL (*pqq*E-) promote maize at the same extent. In contrast, mutation in the gene *pqq*H from *Pseudomonas fluorescens* caused the loss of its phosphate solubilizing phenotype and its plant growth promotion ability on tomato plants (Choi et al. 2008).

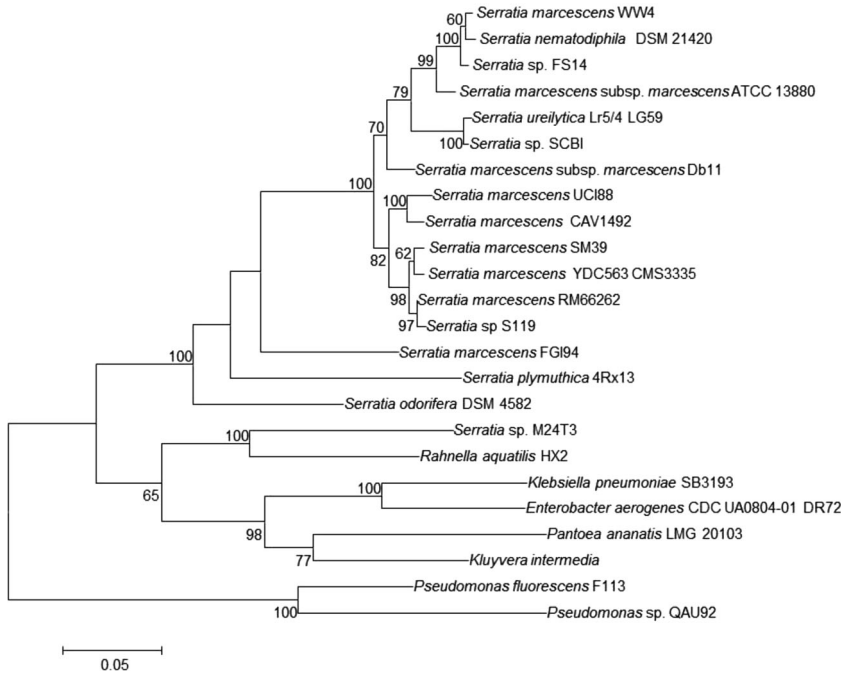


Fig. 5 Neighbor-joining phylogenetic tree based on 1137 bp *pqqE* gene sequence of *Serratia* sp. S119 strain. The tree was inferred under the "General Time Reversible" substitution model. Bootstraps values (over 50 %) for 100 replicates are shown (Nei and Kumar 2000). Símbols indicate source of isolation of bacteria ♦: different environmental sources (insects, water, etc.) • clinical samples. Δ: rizospheric or plant tissues samples

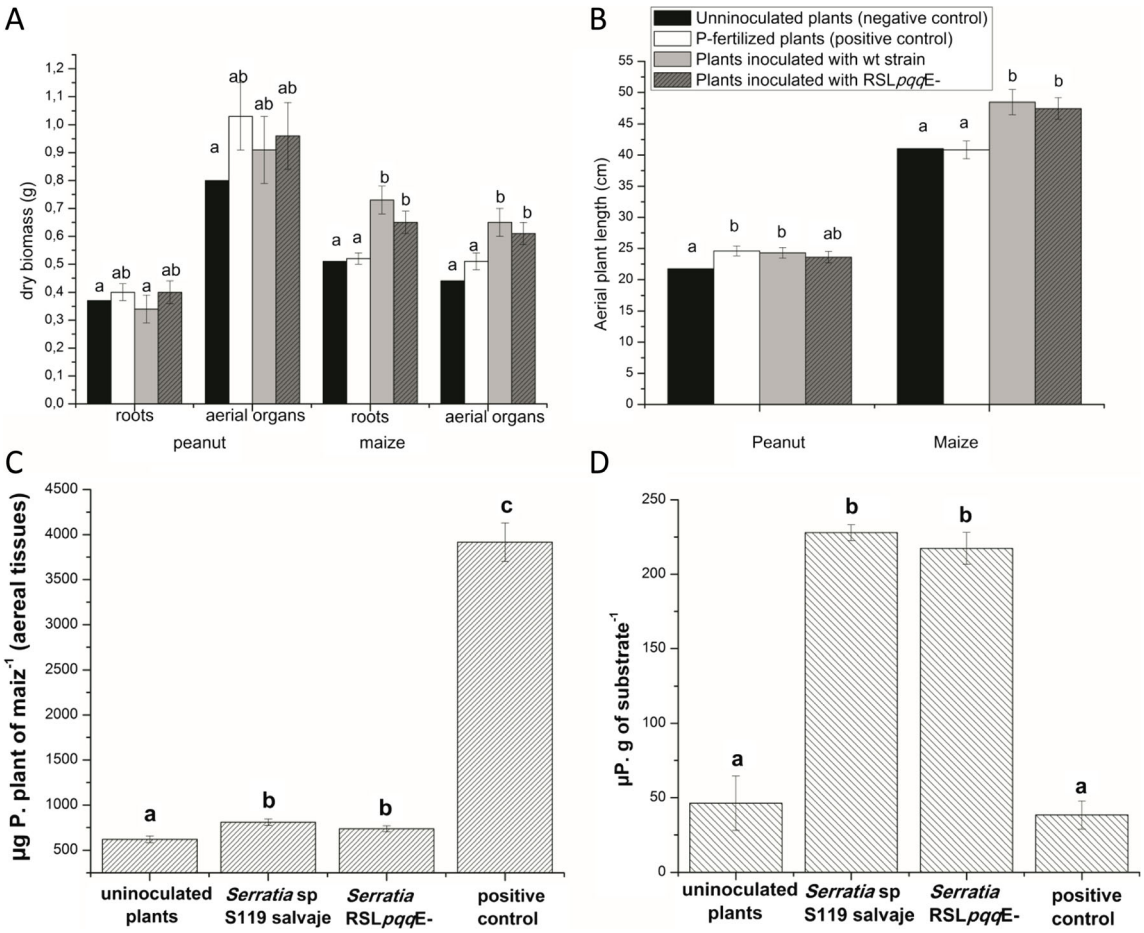


Fig. 6 Root and Aerial dry biomass (**a**) and Aerial length (**b**) of peanut and maize plants inoculated with wt and RSL (*pqq*E-) strains at 40 and 21 days post-bacterial inoculation, respectively. P content in aerial organs of maize plants inoculated with wild-type strain and RSL (*pqq*E-) mutant (**c**). P content in substrate used for growth of maize plant inoculated with wild-type strain and RSL (*pqq*E-) mutant (**d**). Each value is the mean ± S.E. of 2 independent replicates ($n = 5$) * Statistically different, different letters indicate significant statistic differences ($p < 0.05$)

Table 2 Survival of *Serratia* sp. S119 and *Serratia* sp. RSL (*pqq*E-) strains in peanut and maize plants and growth substrate

Log ufc g^{-1} susbtrate/plant tissue	Peanut		Maize	
	Serratia sp. S119	*Serratia* sp. RSL (*pqq*E-)	*Serratia* sp. S119	*Serratia* sp. RSL (*pqq*E-)
Bacteria isolated from plant growth substrate	5.16 ± 0.05^{a1}	5.16 ± 0.07^{a}	6.46 ± 0.07^{a2}	5.61 ± 0.14^{b}
Root epiphytic bacteria	4.4 ± 0.13^{1}	4.26 ± 0.18^{a}	5.32 ± 0.02^{2}	5.46 ± 0.10^{a}
Endophytic bacteria				
Leaves	ND	ND	1.72 ± 0.00	ND
Shoots	3.62 ± 0.23^{b1}	2.5 ± 0.16^{a}	1.97 ± 0.08^{b2}	1.59 ± 0.04^{a}
Roots	2.27 ± 0.06^{1}	ND	3.14 ± 0.01^{b2}	2.94 ± 0.06^{a}

Each value is a mean ± S.E. $p < 0.05$. Different letters indicates statistically significant difference between strains (wt and RSL mutant) in both treatments and different numbers indicates statistically significant difference between maize and peanut plants inoculated with the wt strain

ND not detected

The higher number of epyphitic and endophytic bacteria observed on the plant tissues of maize would indicate a better colonizing ability of the strains in this plant that could suggest the better promoting effect observed with respect to peanut plants. On the other hand a lesser number of viable cells of mutant strain was recovered from both plants suggesting a role of cofactor PQQ in endophytic colonization or survival.

High values of P content were found in maize plants inoculated with RSL (*pqq*E-) mutant suggesting that PQQ deficient mutant is likely providing P to the plant by other mechanism different to gluconic acid production such as the synthesis of inorganic acids, proton extrution, etc. P content of the substrate of bacterial inoculated plants was higher than P-fertilized plants suggesting that P supplied externally seems to be uptaken by the plant more efficiently. Nevertheless, it is possible to speculate that soil bacteria would solubilize insoluble phosphate from soil substrate more gradually maintaining a desirable level of this nutrient during all crop development.

The present study provides evidences that the phosphate solubilizing activity of the native peanut bacterium *Serratia* sp. S119 is correlated with the production of gluconic acid, involving processes requiring PQQ as an essential cofactor for glucose dehydrogenase and that, in the experimental conditions used, it is not required for growth promotion in peanut and maize.

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