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Original Article

Effects of P limitation and molecules from peanut root exudates on *pqq*E gene expression and *pqq* promoter activity in the phosphate-solubilizing strain *Serratia* sp. S119

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Abstract

The mineral phosphate-solubilizing phenotype in bacteria is attributed predominantly to secretion of gluconic acid produced by oxidation of glucose by the glucose dehydrogenase enzyme and its cofactor, pyrroloquinoline quinone. This study analyzes *pqq*E gene expression and *pqq* promoter activity in the native phosphate-solubilizing bacterium *Serratia* sp S119 growing under P-limitation, and in the presence of root exudates obtained from peanut plants, also growing under P-limitation. Results indicated that *Serratia* sp. S119 contains a *pqq* operon composed of six genes (*pqq*A,B,C,D,E,F) and two promoters, one upstream of *pqqA* and other between *pqqA* and *pqqB*. *Pqq*E gene expression and *pqq* promoter activity increased under P-limiting growth conditions and not under N-deficient conditions. In the plant−bacteria interaction assay, the activity of the bacterial *pqq* promoter region varied depending on the concentration and type of root exudates and on the bacterial growth phase. Root exudates from peanut plants growing under P-available and P-limiting conditions showed differences in their composition. It is concluded from this study that the response of *Serratia* sp. S119 to phosphorus limitation involves an increase in expression of *pqq* genes, and that molecules exuded by peanut roots modify expression of these phosphate-solubilizing bacterial genes during plant−bacteria interactions.

Keywords: Pyrroloquinoline quinone (PQQ) cofactor; *Serratia* sp.; Phosphorus; Peanut root exudates

1. Introduction

Phosphorus (P) is the second major macronutrient required for the growth and development of plants. Nevertheless, only 0.1% of P present in the soil is available to plants [1]. This problem can be overcome to some extent by P-solubilizing soil microbes which convert insoluble P compounds into accessible forms for plants [2]. In Gram-negative bacteria, this mineral phosphate-solubilization phenotype (MPS) is attributed predominantly to secretion of low molecular weight organic acids, with gluconate being the principal acid described. Glucose dehydrogenase (GDH) catalyzes oxidation of glucose to gluconic acid, requiring the pyrroloquinoline quinone (PQQ) cofactor for its activity [3]. It has been demonstrated that PQQ plays an essential role in phosphate-solubilizing ability in bacteria [4−6], shows antioxidant activity [7] and has been involved in antibiosis and antifungal properties [8]. In addition, PQQ-GDH was shown to be required by *Sinorhizobium meliloti* for optimal nodulation

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efficiency and competitiveness on alfalfa roots [9]. Genes associated with PQQ production have been identified in various bacteria, including *Enterobacter intermedium* 60-2G [10], *Gluconobacter oxydans* [11], *Methylobacterium extorquens* AM1 [12], *Pseudomonas fluorescens* B16 [4] and *Serratia* sp. S119 [6]. These genes are organized in an operon (*pqq*) whose gene number and organization vary within different bacterial genera and species. However, in all species studied so far, it has been reported that PQQ is encoded by at least 5 genes; *pqq*A, *pqq*B, *pqq*C, *pqq*D and *pqq*E [4,5]. Data from sequence alignment of *pqq*E gene from several bacterial genera, including strain *Serratia* sp. S119 used in this study, indicates that it is highly conserved and that it contains a cysteine motif that is also present in proteins known as radical SAM enzymes [6].

There are few studies regarding the transcriptional regulation of *pqq* operon in bacterial strains. In *M. extorquens*, it has been reported that the expression of this operon depends on a single promoter upstream of *pqq*A which codifies the PQQ cofactor precursor [13]. Other authors suggest the existence of a potential second downstream promoter inside this operon [14,15].

Serratia sp. S119 is a Gram-negative bacterium of the *Enterobacteriaceae* family isolated from peanut nodules [16]. This strain solubilizes high amounts of tricalcium phosphate and shows biocontrol activity against the peanut pathogen *Sclerotinia sclerotiorum* [16]. In addition, *Serratia* sp. S119 directly promotes the growth of peanut and maize plants in controlled conditions. This strain colonizes the rhizosphere as well as the surface and the interior tissues from both plants [6]. These previous studies indicated that *Serratia* sp. S119 is a valuable bacterial strain with potential for application as a biofertilizer in P-deficient soils.

The aim of this work was to evaluate the genetic basis of the P limitation response in this peanut native strain and to analyze the expression of specific bacterial genes related to the phosphate-solubilizing phenotype during plant–bacteria interactions. *Pqq*E gene expression and *pqq* promoter activity in *Serratia* sp. S119 growing under in P-limiting conditions were evaluated. In addition, we evaluated changes in *pqq*E gene expression and the activity of the *pqq* operon when bacteria are exposed to root exudates from peanut plants growing in P-limiting conditions. This is the first study to date that analyzes the effect of P-limiting growth conditions and plant root exudates on the expression of a specific bacterial *pqq* gene. It is expected that this study will broaden our understanding of the expression of genes associated with the phosphate-solubilizing phenotype in the P-limiting response. In addition, it is expected to provide knowledge of how molecules found in root exudates can influence bacterial gene responses in other beneficial associations different from the well-studied biological nitrogen fixation.

2. Material and methods

2.1. Bacterial strains and plasmids

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 nodules [16].

2.2. Media and bacterial growth conditions

Serratia sp. S119 and *Escherichia coli* MG1655 [17] were grown on Luria–Bertani (LB) agar medium or LB broth at 28 °C and 37 °C, respectively. When required, filter-sterilized antibiotics chloramphenicol (Cm), 30 μg ml^{-1}, gentamicin (Gm), 10 μg ml^{-1} and kanamycin (Km), 50 μg ml^{-1} were added.

2.3. Collection and analysis of peanut root exudates

Root exudates (REs) were collected from peanut plants (*Arachis hypogaea* L.) growing in P-limiting conditions (0.2%

Table 1
Bacterial strains and plasmids used in this study.

Strains/plasmids	Characteristics	Reference
Strains		
Serratia sp. S119	Isolated from peanut nodule, Cm^r	[18]
Serratia sp. S119 + pK18mobII-mCherry-470bp	*Serratia* sp. S119 carrying pK18mobII-mCherry-470bp (complete promoter region fragment)	This work
Escherichia coli MG1655	F^- λ^-. Derived from *E. coli* K12	[17]
E. coli MG1655 + pK18mobII	*Escherichia coli* MG1655 carrying the empty vector pK18mobII, Km^r	This work
E. coli MG1655 + pk18mobII-mCherry	*Escherichia coli* MG1655 carrying pK18mobII-mCherry vector, Km^r	This work
Escherichia coli MG1655 + pK18mobII-mCherry-380bp	*Escherichia coli* MG1655 carrying pK18mobII-mCherry-380bp (promoter region upstream *pqq*A gene), Km^r	This work
E. coli MG1655 + pK18mobII-mCherry-470bp	*Escherichia coli* MG1655 carrying pK18mobII-mCherry-470bp (complete promoter region fragment), Km^r	This work
Plasmids		
pK18mobII	Km^r, lacZ	[38]
pK18mobII-mCherry	Cloning vector Km^r, lacZ, + mCherry fluorescent protein	This work
pK18-mCherry-380bp	Cloning vector Km^r, lacZ, mCherry fluorescent protein +380 bp promoter region upstream *pqq*A gene from *Serratia* sp. S119 strain.	This work
pK18-mCherry-470bp	Cloning vector Km^r, lacZ, mCherry fluorescent protein +470 bp promoter region upstream *pqq*A gene and intergenic promoter region between *pqq*A and *pqq*B from *Serratia* sp. S119	This work

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w v^{-1} of $Ca_3(PO_4)_2$ as the sole source of P in the plant pot) named RE-TCP or, in P-available growth conditions (0.35% w v^{-1} of K_2HPO_4) named RE-SOLP. For this, seeds of *A. hypogaea* L cv. Granoleico were surface-disinfected and germinated at 28 °C until the radicle reached approximately 2 cm in length. Seedlings were transferred to sterilized plastic pots that contained a mixture of sterile vermiculite and sand (2:1) [18]. Plants were grown under controlled environmental conditions (light intensity of 200 μR m^{-2} s^{-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 [19] devoid of soluble P and diluted with water (1:4). In RE-TCP pots, $Ca_3(PO_4)_2$ (0.2% w v^{-1}) was supplemented as a unique source of P, while K_2HPO_4 (0.35% w v^{-1}) was included in Hoagland medium used for RE-SOLP pots. Plants were harvested at 40 days after emergence and immersed in a sterile flask containing 100 ml sterile water. Roots remained submerged in water for 24 h. After this period of time, they were removed and the water removed via lyophilization. RE were weighed, resuspended in a mix of methanol:water (1:1) and filtered using 0.22 μM pore size and stored at −80 °C.

For analysis of RE composition, a Waters HPLC pump equipped with an autosampler and UV detection was used. 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 different isocratic modes and UV optical densities. The mobile phases for the HPLC protocol were 30% methanol and 70% 0.06 M phosphoric acid, 50% methanol and 50% 0.06 M phosphoric acid, and 70% methanol and 30% 0.06 M phosphoric acid.

2.4. Genomic and plasmid DNA isolation

The genomic DNA of *Serratia* sp. S119 was isolated from an overnight bacterial culture by using the HiYield™ Genomic DNA Mini Kit (Real Genomics) or by the CTAB/NaCl method [20]. Plasmid DNA was isolated by using NucleoSpin Plasmid kit (Macherey Nagel).

2.5. Primer design

All primers used are listed in Table 2. The primers designed in this study were analyzed with four different software packages; Web Primer, Oligo Analyzer 1.0.2, primer3 v.0.4.0 and AmplifX 1.5.4 considering similar melting temperatures, size (18–25 bp), G–C% content (40–60) and product size. The specificity of the primers was scanned in silico with known sequences available in the GenBank database using BlastN [21]. PCRs were made using genomic DNA of *Serratia* sp. S119 as the template.

2.6. Characterization and nucleotide sequence of the pqq *operon of* Serratia *sp. S119*

The complete sequence of the *pqq* operon of *Serratia* sp. S119 was obtained by amplification of eight partially overlapped fragments (Fig. 1A) from *Serratia* sp. S119 genome using 16 specific primers (Table 2). These primers were designed using the *pqq* genes sequence of the *Serratia marcescens* SM39 strain (AP013063.1) as template. In addition, a 382 bp DNA fragment upstream *pqq*A gene was obtained using primers FwpromS119 and RvpromS119 (Table 2). This

Table 2
Primers used in this study.

Primers	Gene/region amplified	Sequence	Reference
rpoBFserratia	*rpo*B -*Serratia* sp. S119	5′TCGGGTGTACGTCTCGAACT3′	This work
rpoBRserratia	*rpo*B -*Serratia* sp. S119	5′TCTCTGGGCGATCTGGATAC3′	This work
FwpromsS119	Promoter región	5′CTAAACTTGGTGAAGATCGCGT3′	This work
RvpromAs119	*pqq*-Promoter región	5′GTTGGAAATGTACAGCGTCACT3′	This work
FwApromS119-HindIII	*pqq*-Promoter región	5′CACAAGCTTCTAAACTTGGTGAAGATCGCGT3′	This work
RvApromS119-XbaI	*pqq*-Promoter región	5′CACTCTAGAGTTGGAAATGTACAGCGTCACT3′	This work
RvBpromS119-XbaI	*pqq*-Promoter región	5′CACTCTAGAGCCGAGAACTTTTATCTGCATG3′	This work
Primers used for the amplification of *pqq* genes from *pqq* operon of *Serratia* sp. S119			
FwpqqF1-29	*pqq*F	5′TACGTTTATATTGCGTCGGGT3′	This work
RvpqqR1-29		5′CATCGGGAAGGACATTTAAGCT3′	
FwpqqF2-29	*pqq*F	5′ATGGCGATGTCGTTTGCT3′	This work
RvpqqR2-29		5′CTGCTGAGTTTTACCTTTACCGTC3′	
FwpqqF3-29	*pqq*F	5′TCGTAATGCGCAAGCTGG3′	This work
RvpqqR3-29		5′TTTTCGCAATCAGGCCAACT3′	
Fw1pqqFserratiaS119	*pqq*F/*pqq*E	5′TCTGCGCTTACTTCGAAGAAGA3′	This work
Rv1pqqEserratiaS119		5′ACCTGCCAGTTCTACGGCT3′	
Fw2pqqEserratiaS119	*pqq*E/*pqq*D	5′ACTGTGACAGGGTAACGCCAT3′	This work
Rv2pqqDserratiaS119		5′ATCATCGCCCAGTTGAACG3′	
FwpqqD-29	*pqq*D/*pqq*C	5′TTGGAGCAATACGGACACTG3′	This work
RvpqqC-29		5′AGACCAGCATTCCGATCAAA3′	
FwpqqC-29	*pqq*C/*pqq*B	5′AGCACCAATTCTTCAGAGAGCA3′	This work
RvpqqB-29		5′TGCGCTTTACCGCCATTC3′	
FwpqqB-29	*pqq*B/*pqq*A	5′ATGTCCTTGCCGGTATTGC3′	This work
RvpqqA-29		5′TGGGTTCGACTTATTACGATGC3′	

Underlined nucleotide sequence corresponds to recognition site of endonuclease used.

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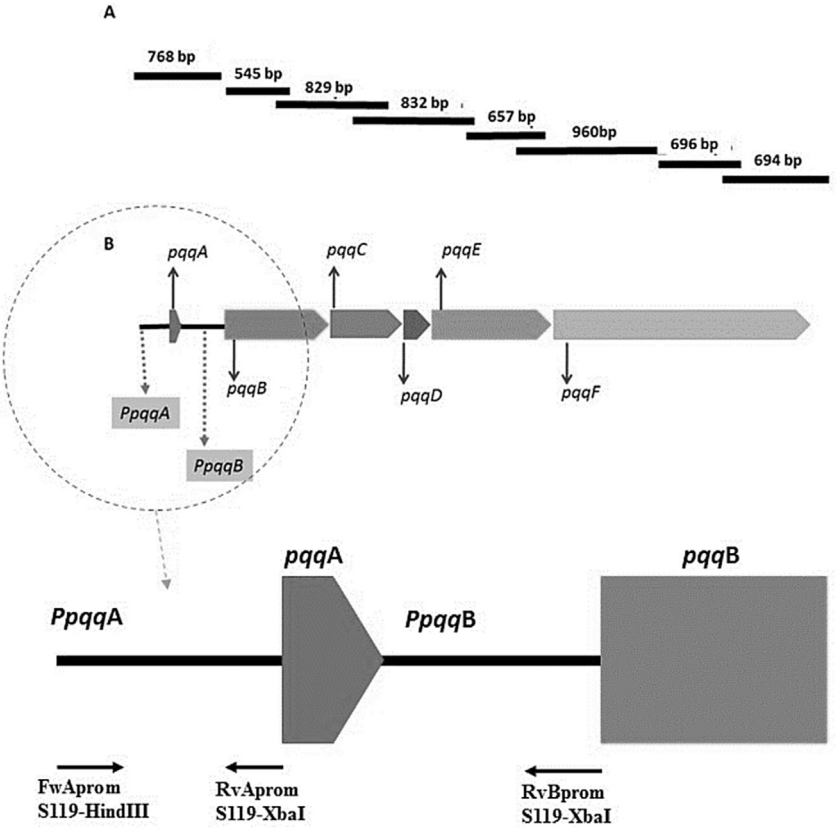

Fig. 1. **A**. Fragments of *pqq* genes obtained by PCR for construction of full length nucleotide sequence of the *pqq* operon. **B**. Scheme of the *pqq* operon and promoter region organization of the *Serratia* sp. S119 strain.

fragment was sequenced by Operon Eurofins (Germany) and the sequence obtained was analyzed along with the complete sequence of the *pqq* operon. Putative promoter regions were evaluated using BPROM online software [22].

2.7. Pqq*E* gene expression analysis by qRT-PCR

2.7.1. Bacterial growth conditions

The *Serratia* sp. S119 strain was grown under P-limiting conditions in liquid NBRIP medium [23] containing 16 mM $Ca_3(PO_4)_2$ as the sole source of insoluble P (designated NBRIP-TCP), or under P-available conditions in liquid NBRIP medium containing 2 mM K_2HPO_4 as a soluble P source (designated NBRIP-SOLP-2mM). Cultures were incubated on a shaker at 28 °C in 250 ml Erlenmeyer flask with continuous agitation (150 rev min^{-1}). Bacterial growth in each medium was determined at 2, 6, 8, 10, 12 and 24 h by counting bacterial CFU ml^{-1} in LB medium using the drop plate method [24].

The effects of peanut root exudates (REs) on bacterial *pqq*E gene expression was analyzed by growing *Serratia* sp. S119 in liquid NBRIP medium (with available P) supplemented with 10 μg ml^{-1} of RE-TCP or RE-SOLP. All growth experiments were performed in triplicate with a starting population of 10^8 cells ml^{-1}.

2.7.2. RNA isolation and cDNA synthesis

Total RNA was isolated from cell cultures with ~10^8 cells ml^{-1} at different growth phases (6, 8, 10 and 24 h) using two distinct extraction methodologies: (i) the total RNA NucleoSpin RNA II kit (Macherey Nagel) or (ii) the TRIZOL reagent (Invitrogen) method, according to the manufacturer's instructions. When necessary, genomic DNA was eliminated by extra RNase-free DNase I digestion (Epicentre). Subsequently, RNA was purified by the RNA Clean up kit (Macherey Nagel). The integrity of total RNA was checked on agarose gels (0.8%), and quantity and purity of RNA obtained was determined by fluorescence using the Qubit RNA BR assay kit and Qubit DNA BR assay kit (Life Technologies). cDNA was synthesized using the MMLV reverse transcriptase 1° Strand cDNA synthesis kit (Life Technologies) according to the manufacturer's instructions. The concentration of cDNA of each sample was measured using the fluorescence quantification Qubit ssDNA Assay kit (Life Technologies).

2.7.3. Quantitative real-time PCR (qRT-PCR)

qRT-PCR was performed using 2 μl (1/10 dilution) of cDNA as template. cDNAs were amplified using primers F1PQQEserratia and R1PQQEserratia for *pqq*E target gene and primers rpoBFserratia and rpoBRserratia for the *rpo*B housekeeping gene (Table 2). Different concentrations of primers that ranged from 50 to 900 nM were evaluated. Standard curves were created by plotting the Ct (cycle threshold) values of the qRT-PCR performed on different cDNA dilutions of strain *Serratia* sp. S119 growing in P-limiting and P-available conditions. The coefficient of efficiency (E) was calculated as: efficiency $= 10^{(-1/\text{slope})} - 1$ [25].

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Gene expression of each sample (6 and 24 h of growth) was quantified in a Stratagene Mx3005P Real-Time PCR System programmed with the following temperature profile: an initial cycle at 95 °C for 3 min, followed by 40 cycles at 95 °C for 20 s, at 60 °C for 20 s and a final step of 72 °C for 1 min. Fluorescence data acquisition was done during the exponential phase of amplification (60 °C), before reagents were consumed, accumulation of inhibitors or inactivation of polymerase had started to have an effect on the efficiency of amplification. A final melting curve was performed to check for product specificity. Reactions were performed using Brilliant III ultra-Fast SYBR Green QPCR Master Mix (Agilent Technologies) in a 20 µl final volume containing 6.8 µl of sterile distilled water, 10 µl of SYBR Green mix, 0.8 µl of each primer (400 nM) and 0.3 µl of ROX reference dye. Primer dimers and other artifacts were detected by analyzing the dissociation curve. Data were analyzed with the software MxPro QPCR (Stratagene). Relative *pqq*E expression along *Serratia* sp. S119 growth in NBRIP-TCP or NBRIP-SOLP media was calculated against expression of the housekeeping gene. The resulting Ct was analyzed using the comparative critical threshold ($^{\Delta\Delta}$Ct) method in which the amount of target RNA is adjusted to a housekeeping gene [26]. All samples were analyzed in triplicate and the expression values were averaged by software analysis (Stratagene) using the ANOVA test and LSD statistical analysis.

2.8. Analysis of pqq operon promoter activity by fusion with fluorescent protein m-Cherry

The analysis of the activity of the *pqq* operon promoter was done by fusion of this promoter region with the fluorescent protein m-Cherry integrated into the pK18mobII plasmid. Two fragments of this promoter were cloned: a fragment of 382 bp that includes only the promoter region upstream of the first gene *pqq*A and a second larger fragment that includes the 382 bp sequence plus 84 bp downstream between *pqq*A and *pqq*B genes.

2.8.1. Cloning of pqq promoter regions of Serratia sp. S119

DNA manipulations for cloning and subcloning of *pqq* promoter regions were carried out as described by Sambrook et al. [27]. The 382 bp fragment flanked by restriction enzyme sites (*Hind*III and *Xba*I) was amplified using primers FwApromS119-HindIII/RvApromS119-XbaI (Table 2). The 466 bp fragment flanked by the same restriction enzymes was obtained using FwApromS119-HindIII/RvBpromS119-XbaI. Both fragments were digested with the corresponding restriction enzymes and cloned into the pK18mobII-mCherry plasmid to give pK18mobII-mCherry-380bp and pK18mobII-mCherry-470bp (Table 1). These plasmids were then introduced separately into chemically competent *E. coli* MG1655 cells [28] and into *Serratia* sp. S119. Transformation of these cells was done following TSS protocol [29].

2.8.2. Bacterial growth conditions

The *E. coli* MG1655 strain carrying the corresponding plasmids was grown at 37 °C in MOPS medium [30] with 0.1 mM K_2HPO_4 as P-limiting growth conditions or 1 mM K_2HPO_4 as the P-available growth condition. *Serratia* sp. S119 carrying the corresponding plasmids was grown at 28 °C in NBRIP-SOLP-0.1mM (0.1 mM K_2HPO_4) as P-limiting growth conditions or in NBRIP-PSOL-1.2mM (1.2 mM K_2HPO_4) as the P-available growth condition.

To evaluate the effects of REs on *pqq* operon promoter activity, *Serratia* sp. S119 carrying the pK18mobII-mCherry-470bp construct was grown in liquid NBRIP-PSOL-1.2mM (K_2HPO_4) medium supplemented with 10, 20, 50 and 100 µg ml^{-1} of REs (RE-PSOL and RE-PTC).

For *pqq* operon promoter activity analysis under carbon (C) and nitrogen (N) starvation growth conditions, *Serratia* sp. S119 carrying pK18mobII-mCherry-470bp was grown in liquid modified NBRIP medium containing 0.1% glucose (10-fold less glucose than the original composition) or 0.001% $(NH_4)_2SO_4$ (10-fold less nitrogen than the original composition). All growth experiments were performed in triplicate with a starting population of 10^8 cells ml^{-1}.

2.8.3. Expression analysis of pqq promoter regions

Expression analysis were first performed in *E. coli* MG1655 strain carrying the corresponding plasmids (*E. coli* + pK18mobII EV, *E. coli* + pK18mobIIm-Cherry, *E. coli* + pK18mobm-Cherry-380bp, *E. coli* + pK18mobm-Cherry-470bp) and secondly, in *Serratia* sp. S119 carrying the corresponding plasmids (*S.* sp. S119 + pK18mobII EV, *S.* sp. S119 + pK18mobIIm-Cherry, *S.* sp. S119 + pK18mobm-Cherry-470bp, *S.* sp. S119 + pK18mobm-Cherry-380bp).

The fluorescence of the red protein m-Cherry (e_x: 587 and e_m: 610 nm) was measured in each bacterial culture every 30 min for 24 h for the *E. coli* assay (in MOPS-0.1mM K_2HPO_4 or MOPS-1mM K_2HPO_4 media) and every 2 h for 24 h in the *Serratia* sp. S119 assay (in NBRIP-PSOL-0.1mM or NBRIP-PSOL-1.2mM media). Bacterial growth was analyzed simultaneously by determining optical density at 620 nm.

2.9. Statistical analyses

Statistical analyses were performed using Infostat software by ANOVA, and differences among treatments were detected by the LSD or Tukey test ($p < 0.05$).

3. Results

3.1. Analysis of the pqq operon sequence of Serratia sp. S119

In order to confirm the presence of the complete *pqq* operon sequence in *Serratia* sp. S119, as well as its intrinsic variability compared to *pqq* operons from other *Serratia* strains, we amplified from its genome eight DNA fragments (partially

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overlapped) that potentially correspond to this operon (Fig. 1A). The sequences of these fragments were aligned showing high identity (97–99%) with *pqq* gene sequences of *Serratia* strains available in Gene Bank NCBI (For *pqq*A gene: *Serratia* sp. FS14 (CP005927.1), *S. marcescens* WW4 (CP003959.1), *pqq*B gene: *S. marcescens* CTM50650 (HE820729.1), *S. marcescens* SmUNAM836 (CP012685.1), *S. marcescens* SM39 (AP013063.1), *pqq*C gene: *S. marcescens* SmUNAM836 (CP012685.1), *S. marcescens* SM39 (AP013063.1), *S. marcescens* CAV1492 (CP011642.1), *pqq*D gene: *S. marcescens* SM39 (AP013063.1), *S. marcescens* SmUNAM836 (CP012685.1), *pqq*E gene: *S. marcescens* SmUNAM836 (CP012685.1), *S. marcescens* SM39 (AP013063.1), *S. marcescens* CAV1492 (CP011642.1), *pqq*F gene: *S. marcescens* SmUNAM836 (CP012685.1) and *S. marcescens* SM39 (AP013063.1)). The *pqq* operon from *Serratia* sp. S119 is 5538 bp length and consists of genes *pqq*A, *pqq*B, *pqq*C, *pqq*D, *pqq*E and *pqq*F (Fig. 1B). A promoter region upstream *pqq*A (PpqqA) and a potential promoter region downstream, between *pqq*A and *pqq*B (PpqqB), were identified.

3.2. Pqq*E gene expression is increased under P-limiting growth conditions*

The expression of *pqq*E gene was analyzed in *Serratia* sp. S119 at the exponential growth phase (6, 8 and 10 h) in NBRIP minimal medium supplemented with TCP (insoluble source of P) or 2 mM K_2HPO_4 (soluble P) (Fig. 2A). It was found that *pqq*E gene expression increased over time when *Serratia* sp. S119 was grown in P-limiting conditions (NBRIP-TCP) (Fig. 2B). This increase was correlated with a higher level of solubilized P by this strain in the NBRIP-TCP medium (Fig. 2C). On the other hand, *pqq*E gene expression in bacteria growing under P-available growth conditions remained constant during this period.

3.3. *The activity of the promoter of the* pqq *operon is increased under P-limiting growth conditions*

To analyze changes in the expression of *pqq* genes at different growth stages of bacterial culture under P-limiting conditions, a second approach was used. The promoter regions of the *pqq* operon were fused with the gene that codes for the m-Cherry fluorescence protein. To analyze whether both PpqqA and PpqqB promoter regions (Fig. 1B) are involved in *pqq* operon transcription under P-limiting conditions, they were cloned into the pK18mobII-mCherry vector.

These constructs (pK18mobIIm-Cherry-380 (382 bp PpqqA) and pK18mobIIm-Cherry-470 (466 bp PpqqA + PpqqB)) were initially introduced into *E. coli* MG1655-competent cells and grown under P-available or P-limiting conditions. The highest fluorescent signal was observed in the *E. coli* strain containing the 466 bp fragment (PpqqA + PpqqB) under both growth conditions assayed (Fig. 3A), suggesting that either most of the transcription activity is due to the downstream promoter region, or that both promoter regions are necessary for full transcription activity. Further studies are necessary to determine whether the upper promoter region contributes to the transcription of *pqq* operon.

In addition, expression of the *pqq* promoter regions was analyzed in *Serratia* sp. S119. This strain was transformed with the same constructs and grown in P-limiting conditions or with an available source of P. The data obtained confirmed that, in *Serratia* sp. S119 (as in *E. coli*), the longer construct carrying both promoter regions (PpqqA + PpqqB) was the most transcriptionally active (Fig. 3B). Fluorescence from the larger construct, pK18mobIIm-Cherry-470, indicated that at 6, 10 and 24 h (exponential and stationary growth phase), the *pqq* promoter region increases its activity in P-limiting conditions over time, reaching values significantly higher during the stationary growth phase (24 h) compared to the exponential growth phase (Fig. 3C and D). Under P-available growth conditions, *pqq* promoter activity also increased over time (from 6 to 24 h of growth). During the exponential phase (6 and 10 h), it was considerably lower than that obtained from bacteria growing under P-limiting conditions, suggesting that expression is enhanced by P-limitation (data not shown). Nevertheless, at stationary phase (24 h), promoter activity was enhanced despite an excess of phosphate, suggesting that limitation of growth via depletion of nutrients other than phosphate can also enhance the promoter expression. Also notable was that fluorescence levels detected in *Serratia* sp. S119 were around 1000-fold stronger than those detected in *E. coli* MG1655, suggesting either the presence of a specific activator of expression in the native host or some incompatibility between the *Serratia* promoter and the *E. coli* RNA polymerase.

3.4. *The activity of the* pqq *promoter is not affected by carbon and nitrogen deficiency*

In agreement with the analysis of bacterial growth in each medium, *Serratia* sp. S119 had a comparable growth behavior in C-deficient medium and in complete medium, while in media deficient in P or N nutrients, it was poorer (Fig. 4A). After 24 h of growth, the highest *pqq* promoter activity was observed under normal growth conditions (minimal medium with all the nutrients), followed by that obtained under P-deficient growth conditions. At this time point, fluorescence levels indicated lower *pqq* promoter activity under C and N limitation than under P-deficient growth conditions (Fig. 4B). The *pqq* promoter activity was about 2-fold higher in bacteria growing under P-deficient than those growing under N-deficient conditions, suggesting that promoter activity responds to specific nutritional conditions.

3.5. *REs from peanut plants growing in P-limiting conditions modify* pqq*E gene expression and promoter activity of the* pqq *operon*

The quantification of *Serratia* sp. S119 *pqq*E gene transcripts showed no differences between bacteria cultured in th

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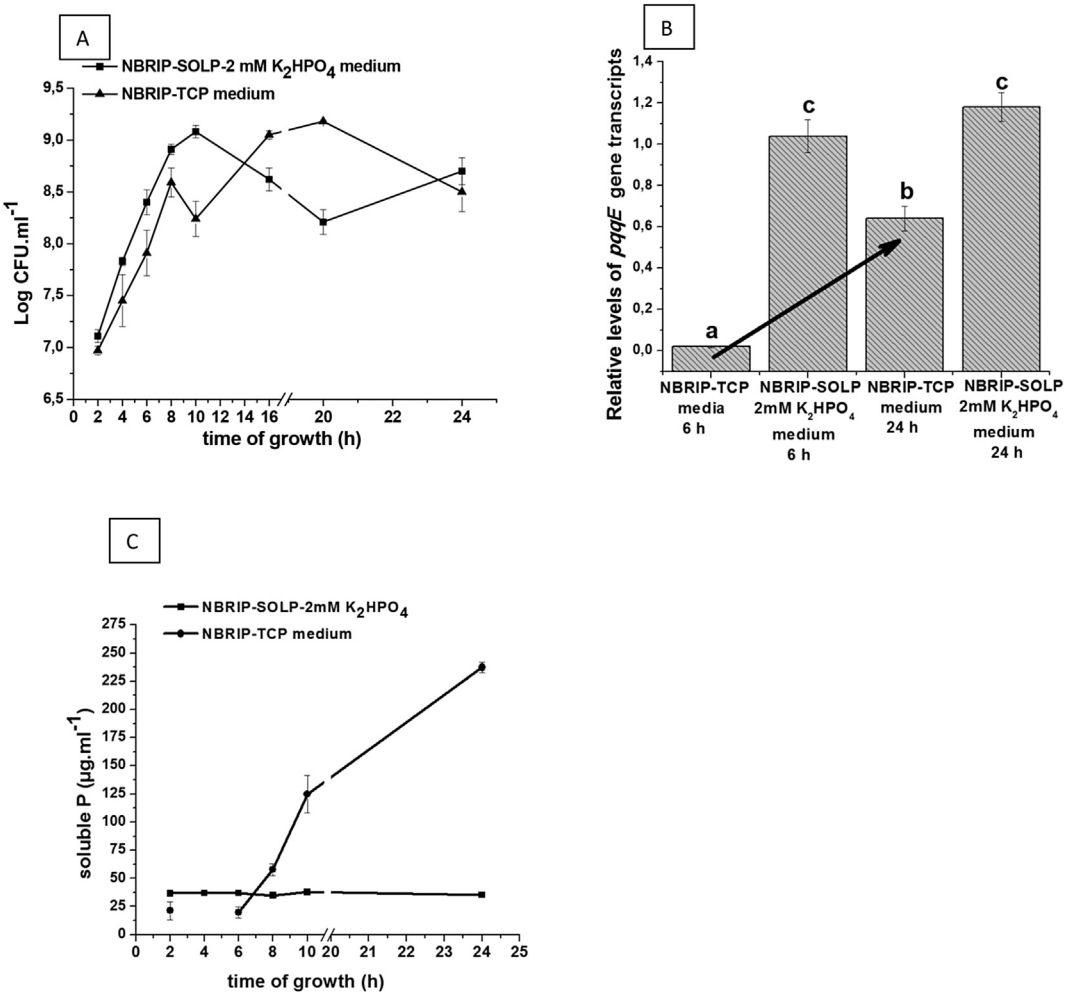

Fig. 2. **A**. *Serratia* sp. S119 growing in NBRIP medium supplemented with soluble P (2 mM K_2HPO_4) or with tricalcium phosphate (insoluble source of P) (Log CFU ml^{-1}). Data are means ± SE of 3 replicates (n = 3), $p < 0.05$ according to the LSD test ($p < 0.05$). **B**. Relative expression levels of *pqq*E gene from *Serratia* sp. S119 growing under P-starvation and P-available at 6 and 24 h of growth relativized to *pqq*E gene expression at 6 h of growth. Data are means ± SE of 3 replicates (n = 3), $p < 0.05$ according to the LSD test ($P < 0.05$). **C**. Levels of soluble P released by strain *Serratia* sp. S119 growing in NBRIP medium supplemented with soluble P (2 mM K_2HPO_4) or with tricalcium phosphate (insoluble source of P). Data are means ± SE of 3 replicates (n = 3).

presence of RE-TCP (plants grown with an insoluble source of P) and RE-SOLP (plants grown with available P) (10 µg ml^{-1}) at 6 h of growth (Fig. 5). At 10 (data not shown) and 24 h of growth, lower levels of *pqq*E transcripts in bacteria growing in the presence of RE-TCP compared to those observed in the presence of RE-SOLP were detected.

Pqq promoter activity was evaluated using the *Serratia* sp. S119 strain transformed with the pK18mobIIm-Cherry-470 construct grown in the presence of different concentrations of RE-TCP and RE-SOLP (10, 20, 50 and 100 µg ml^{-1}). In all treatments, *pqq* promoter activity was enhanced by addition of root exudates (Fig. 6). In the presence of 10 and 100 µg ml^{-1} of RE-TCP, fluorescence detected was significantly higher than that observed in RE-SOLP treatment. When *Serratia* sp. S119 was exposed to 20 and 50 µg ml^{-1} of RE, fluorescence from RE-TCP and RE-SOLP treatments were similar.

HPLC analysis of RE-SOLP and RE-TCP samples indicated differences in their composition. Three defined peaks in RE-SOLP sample after 15 min of run and only one peak (with a different retention time) in the RE-TCP sample were detected (data not shown).

4. Discussion

Soil P-solubilizing Gram-negative bacteria can transform insoluble phosphorus compounds into soluble forms for plants mainly through secretion of gluconic acid (GA). Glucose dehydrogenase enzyme (GDH) catalyzes oxidation of glucose to GA and requires pyrroloquinoline quinone (PQQ) cofactor for its activity [3]. *Pqq* genes are organized in an operon that differs in the number and organization of genes within different bacterial genera and species [4,5].

In this study, we identified in the genome of native peanut strain *Serratia* sp. S119 the most studied and well known *pqq* genes (*pqq*ABCDE) and the *pqq*F gene. Also, a promoter region upstream from *pqq*A and a promoter region downstream, between *pqq*A and *pqq*B, were identified. Expression analysis of *pqq* promoter regions with m-Cherry protein in *E.*

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coli (heterologous) and in the native *Serratia* sp. S119 strain indicated that either both *pqq* operon promoter regions are necessary for expression of *pqq* genes, or that almost all transcription activity is due to the downstream promoter region. The comparison of common boxes of both promoter regions with the sequences of other bacteria indicated a similar promoter region of PpqqA in *E. coli* that corresponds to the −35 box and a −10 box consensus sequences from the *trp* promoter, one of the most well-studied promoters in the *E. coli* genome which is constitutively active. In light of this result, it

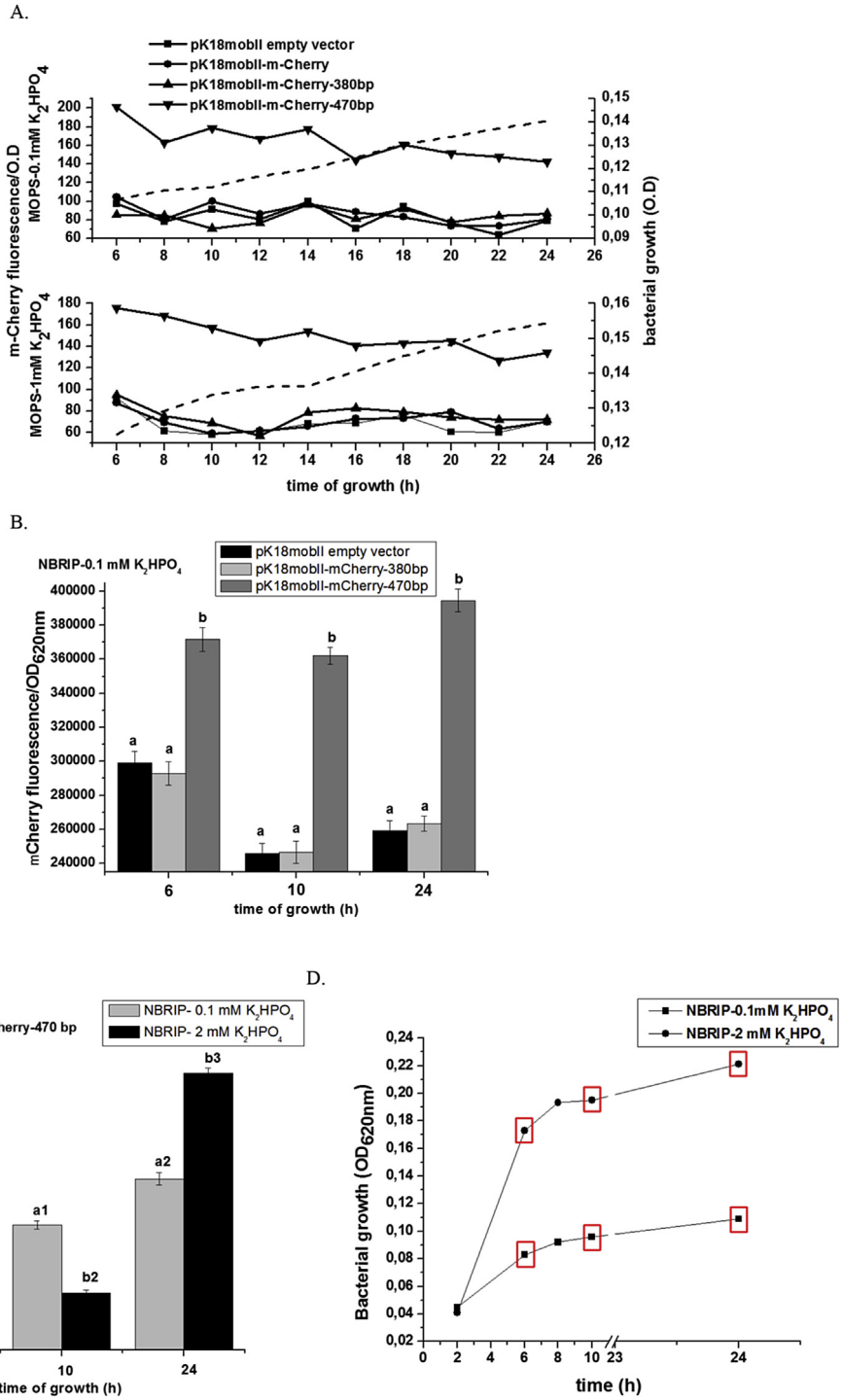

Fig. 3. **A**. *Pqq* promoter heterologous expression in *E. coli* growing in MOPS medium supplemented with 0.1 mM and 1 mM of K_2HPO_4 and bacterial growth determination (by optical density 600 nm). Data are means ± S.E. **B**. *Pqq* promoter expression in *Serratia* sp. S119 growing in NBRIP medium supplemented with 0.1 mM of K_2HPO_4. Data are means ± SE of 3 replicates (n = 3), $p < 0.05$ according to LSD test ($p < 0.05$). **C**. *Pqq* promoter region activity (466 bp fragment) in native *Serratia* sp. S119 growing in NBRIP medium supplemented with 0.1 mM and 1 mM of P at 6, 10 and 24 h of growth. Data are means ± SE. **D**. *Serratia* sp. S119 growth behavior in NBRIP medium supplemented with 0.1 and 2 mM of K2HPO4 over time. Data are means ± SE of 3 replicates (n = 3), $p < 0.05$ according to LSD test ($p < 0.05$).

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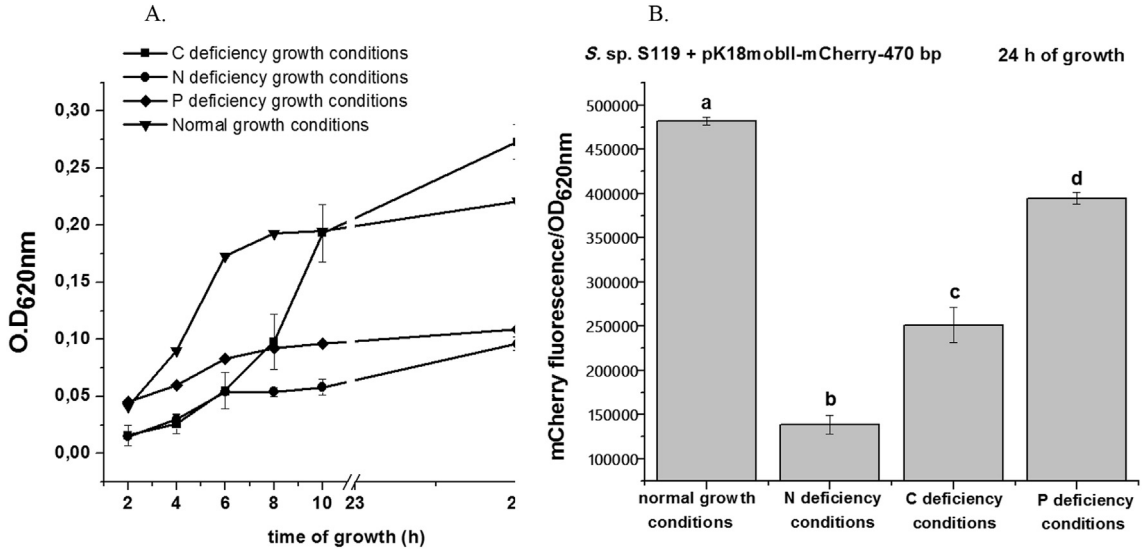

Fig. 4. **A**. *Serratia* sp. S119 growth behavior under carbon, nitrogen and phosphorus deficiency over time. **B**. *Pqq* promoter region activity (470 bp fragment) in native strain *Serratia* sp. S119 in NBRIP under carbon, nitrogen and phosphorus deficiency during stationary growth phase (24 h). Data are means ± SE.

is possible to speculate that he PpqqA promoter is responsible for constitutive expression of PQQ and that promoter PpqqAB is upregulated under P deficiency. Further studies are necessary to deepen the understanding of upper promoter activity in transcription of the *pqq* operon under P-limiting growth condition. Contrary to *Serratia* sp. S119, Holscher and Gorisch [11] reported that, in *G. oxydans*, the *pqq*AB genes are co-transcribed and that the *pqq*A–*pqq*B intergenic sequence may not have promoter activity. In addition, Ramamoorthi and Lidstrom [15] and Velterop et al. [14] observed increased expression of the *pqq*A gene with respect to the other *pqq* genes in *M. extorquens* and *Klebsiella pneumonia*, respectively, although both studies described a unique promoter region upstream from *pqq*A. They suggested that transcription differences observed between *pqq*A and the other *pqq* genes could be related to a potential termination signal located downstream from the *pqq*A gene. In those studies, enzymatic fusions were used. Therefore, this is one of the first studies using *pqq* fusion with a fluorescent reporter protein.

One of the aims of this study was to evaluate the genetic basis for the P limitation response in the peanut native strain *Serratia* sp. S119. The P starvation response has been studied in Gram-negative bacteria like *E. coli* [31] and *S. meliloti* [32,33]. Nevertheless, there is little literature concerning this topic in phosphate-solubilizing bacteria belonging to the *Serratia* genus. Gene expression is one of the first responses to environmental changes, typically activated by environmentally sensitive transcription factors, and is the main control for phenotypic changes in bacteria. We analyzed *pqq*E gene expression and the activity of the *pqq* promoter during growth under P-limitation using two different approaches; for *pqq*E expression analysis, P limitation was obtained by adding insoluble tricalcium phosphate to the medium, while for *pqq* promoter activity, a low soluble P concentration was used.

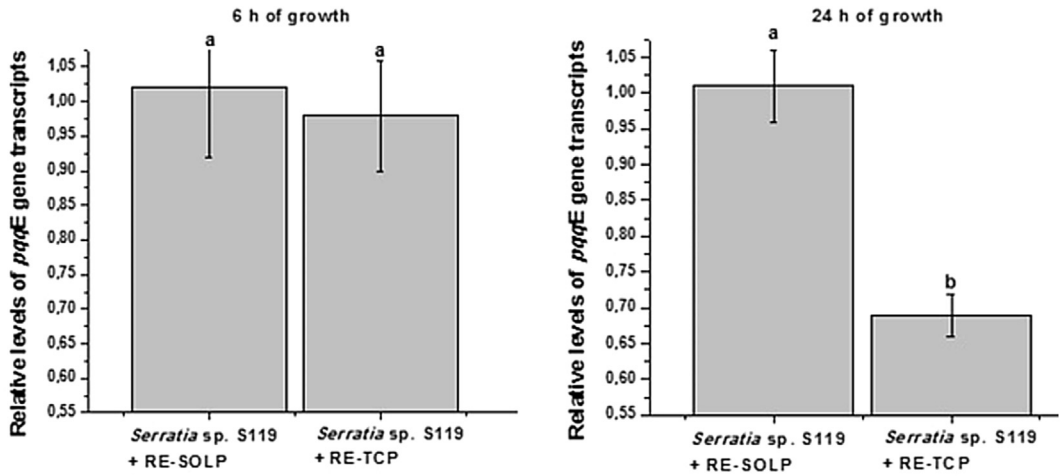

Fig. 5. Relative expression levels of *pqq*E gene from *Serratia* sp. S119 growing with 10 μg ml^{-1} REs (RE-TCP and RE-SOLP) at 6 and 24 h of growth. Data are means ± SE of 3 replicates (n = 3), $p < 0.05$ according to the LSD test ($P < 0.05$).

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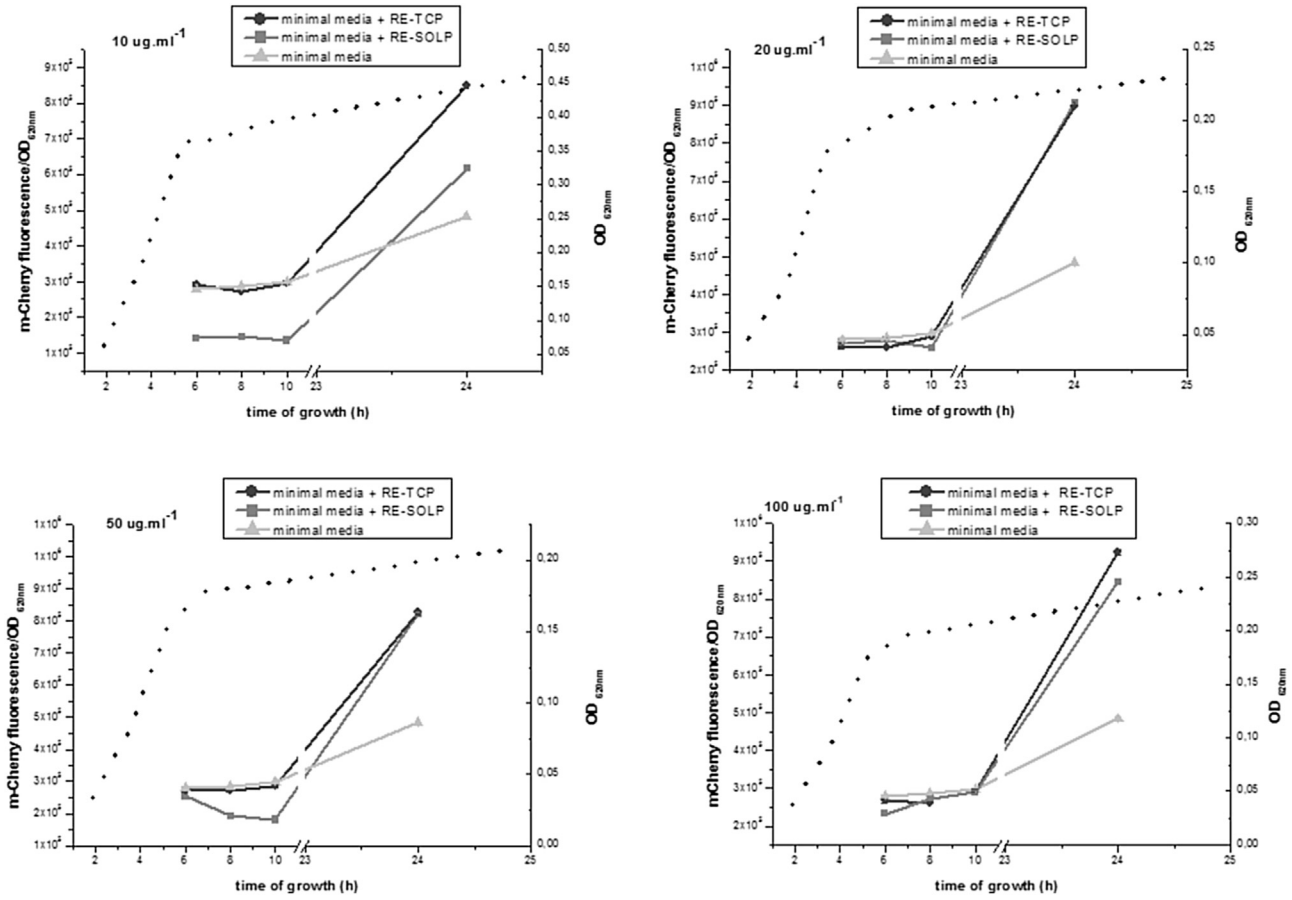

Fig. 6. *Pqq* promoter region activity in native strain *Serratia* sp. S119 in minimal medium supplemented with different concentrations of REs (RE-TCP and RE-SOLP) (10, 20, 50 and 100 μg/ml). Data are means ± SE of 3 replicates (n = 3), $p < 0.05$ according to the LSD test ($P < 0.05$).

Quantitative analysis suggested that expression of the *pqq*E gene is constant when the bacterium grows with available P, but increases over time under P-limiting growth conditions. In the presence of available P, the high levels of *pqq*E gene transcripts could be due to the other cellular functions attributed to the PQQ cofactor, e.g. as a reactive oxygen species scavenger or as a cofactor of enzymes involved in antimicrobial metabolite synthesis, etc [34]. *Pqq* promoter activity in *Serratia* sp. S119 under P limitation during the exponential phase was also higher than that observed under P-available growth conditions, suggesting that whole *pqq* operon activity increases as a response to P limitation. Using these two approaches, it is possible to infer that increased expression of *pqq* genes at 24 h of growth may be part of the P-limiting response in *Serratia* sp. S119.

The fact that *pqq* promoter activity in *Serratia* sp. S119 was around 1000-fold stronger than in *E. coli* MG1655, and that there was no increase in *pqq* promoter activity in *E. coli* when grown in P-limiting conditions, suggests two possibilities that are not necessarily mutually exclusive. One possibility is that promoter activity is dependent upon the presence of specific activators. These activators could regulate *pqq* operon transcription and presumably other genes related to nutrient limitation responses. The other possibility is that the RNA polymerase of *E. coli* was not able to recognize these promoter regions.

An interesting question was whether the promoter activity of the *pqq* operon increases in stationary phase during bacterial growth under N deficiency, since it is the primary macronutrient required for growth. Although some promoter activity was observed under N deficiency, it was much lower than that observed under P deficiency conditions. According to these results, it is possible to suggest that the increase in expression of the *pqq* operon is due to the P limitation response of *Serratia* sp. S119.

Molecular signaling between microbes and plants plays a fundamental role both in pathogenesis and in beneficial interactions. It has been reported that, under P starvation conditions, plants change the profile of the root exudates and increase the synthesis of important signal molecules involved in plant–microorganism interactions, e. g. strigolactones [35]. P deficiency during plant growth also stimulates production of specific molecules like carboxylates (organic acids and amino acids) and reduces H^+ release and uptake of some ions like Ca^{2+}, K^+, Mg^{2+} and Cl^- [36]. In fact, in a previous work, we reported that peanut plants grown with an insoluble source of P (tricalcium phosphate) significantly acidified the plant's semi-solid medium compared to plants grown with an available source of P [37]. Thereby, a second aim of this study was to analyze expression of the *pqq*E gene and the activity of the *pqq* promoter during plant–bacteria interaction under P-

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limiting conditions. It was interesting to note that the *pqq* promoter is mainly active during stationary phase (24 h), and that this activity is enhanced by addition of the root extracts. It is important to note that this is the first work that compares the effect on expression of a specific bacterial gene using REs obtained from plants growing in P-limiting conditions. Although, using HPLC analysis, some differences in REs composition (RE-TCP and RE-SOLP) were detected, further experiments are necessary to confirm the compositional differences observed.

On the other hand, the decrease in expression levels of the *pqq*E gene when *Serratia* sp. S119 grew in the presence of RE from peanut plants grown in P-limiting conditions led us to speculate that there is additional transcriptional regulation concerning *pqq*E, further studies are needed to reveal which root molecules are involved during this plant−bacterium interaction.

In conclusion, in the bacterium *Serratia* sp. S119, the P limitation response involves an increase in expression of *pqq* genes, consistent with the role of PQQ in the bacterial phosphate-solubilizing phenotype. Changes in expression of these genes when this strain is exposed to different concentrations of peanut REs indicate that some molecules released could act as signals during this plant−bacteria interaction under P-limiting environmental conditions.

Conflict of interest

None of the authors have present or potential conflicts of interest, nor financial, personal or other relationships with other persons or organizations that might inappropriately influence or be perceived to influence their work.

Acknowledgments

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