

Evaluation of Glucose Dehydrogenase and Pyrroloquinoline Quinine (pqq) Mutagenesis that Renders Functional Inadequacies in Host Plants

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The rhizospheric zone abutting plant roots usually clutches a wealth of microbes. In the recent past, enormous genetic resources have been excavated with potential applications in host plant interaction and ancillary aspects. Two *Pseudomonas* strains were isolated and identified through 16S rRNA and *rpoD* sequence analyses as *P. fluorescens* QAU67 and *P. putida* QAU90. Initial biochemical characterization and their root-colonizing traits indicated their potential role in plant growth promotion. Such aerobic systems, involved in gluconic acid production and phosphate solubilization, essentially require the pyrroloquinoline quinine (PQQ)-dependent glucose dehydrogenase (GDH) in the genome. The PCR screening and amplification of GDH and PQQ and subsequent induction of mutagenesis characterized their possible role as antioxidants as well as in growth promotion, as probed *in vitro* in lettuce and *in vivo* in rice, bean, and tomato plants. The results showed significant differences ($p \leq 0.05$) in parameters of plant height, fresh weight, and dry weight, *etc.*, deciphering a clear and in fact complementary role of GDH and PQQ in plant growth promotion. Our study not only provides direct evidence of the *in vivo* role of GDH and PQQ in host plants but also reveals their functional inadequacy in the event of mutation at either of these loci.

Keywords: Glucose dehydrogenase, pyrroloquinoline quinine, rhizosphere, *Pseudomonas*, mutagenesis, phosphate solubilization, plant growth promotion

Introduction

Glucose dehydrogenase (GDH) is a quinoprotein enzyme that uses pyrroloquinoline quinine (PQQ) as a redox cofactor [13]. On the basis of localization within the cell, two types (GDH-A and GDH-B) have been reported so far. GDH-A has been reported in numerous bacterial species like *Acinetobacter calcoaceticus*, *Klebsiella aerogenes*, *Pseudomonas aeruginosa*, *Acinetobacter lwoffii*, *Gluconobacter suboxydans*, and *Escherichia coli*. It is a membrane-bound enzyme and also referred to as m-GDH, and has a similar primary structure to GDH-B but differs in substrate specificity. The N-terminal has five transmembrane segments, which anchor

the protein in the membrane, whereas the C-terminal domain has a large conserved PQQ-binding site with catalytic functions [38]. On the contrary, GDH-B is a soluble enzyme (s-GDH) reported only in *Acinetobacter calcoaceticus* [8]. The position of the GDH apoenzyme on the periplasmic side eases the link of PQQ to form a holoenzyme.

GDH plays an important role in phosphate solubilization by acidification in the periplasmic space of bacteria through direct oxidation of glucose into gluconic acid and then to 2-ketogluconic acid [3, 12]. These acids are the strongest naturally occurring moieties secreted into the extracellular medium by bacteria [11]. The production of acid is a direct consequence of the oxidation pathway. Although the GDH

enzyme is bound to the inner membrane, the original process takes place on the cell surface, whereas its catalytic activity occurs by the cytoplasmic membrane where the periplasmic space provides the substrate oxidation site [23, 24, 27]. The process of oxidation acidifies the medium through direct release into the extracellular space and consequently attains the phosphate solubilization [11]. Hence, GDH acts to dissolve the mineral phosphate multiplexes and fulfills the deficiency of phosphate [15] in soil. GDH also plays an important regulatory and bioenergetics role in bacterial systems where carbon is provided for intracellular metabolism [33].

PQQ serves as a redox cofactor for several bacterial dehydrogenases such as glucose and ethanol dehydrogenases [13]. The cofactor PQQ has multiple beneficial affects, yet only few studies are available on the functional role of PQQ in plants: such as the *in vitro* pollen germination in *Camellia*, *Tulipa*, and *Lillium* stimulated by PQQ [36, 37]. However, the mechanism as to how exactly this is done is not yet clear. PQQ may also be responsible for scavenging toxic free radicals, besides foraging superoxides, and is better than flavonoids, vitamin C, β -carotene and carotenoids, vitamin B, phenolic compounds, and conjugated linoleic acid [25]. PQQ is rarely found in plant and animal tissues. It even could not be produced by plants and animals themselves [21]; but a rhizobacterial source for PQQ has been reported [5]. Produced in plant-associated systems, PQQ aids in making the soil and the surrounding environment more acidic [32] and consequently plants get more phosphate. The PQQ synthesized by *P. fluorescens* B16 has been found to promote growth in tomato, cucumber, *Arabidopsis*, and hot pepper [5].

PQQ acts as a plant growth-promoting factor owing to its

antioxidant activity [5]. The functional role to scavenge reactive oxygen species (ROS) was demonstrated in *E. coli* [28] where the enhanced formation of ROS has resulted in the oxidative stress in cells. *E. coli* cells with the PQQ synthase gene were found to be UV resistant and oxidative stress tolerant [34] and demonstrated 4-fold higher protection of proteins against damage induced by γ -radiation. These results point to the fact that PQQ protects cells from oxidative stress [19]. The overall objective of this study was to interpret the *in vivo* role of GDH and PQQ found in different *Pseudomonas* species. The study was conducted by inducing mutagenesis at the GDH and *pqqC* loci. The mutants obtained for *P. putida* QAU90 and *P. fluorescens* QAU67 were characterized and compared for their performance in plant growth promotion and auxiliary activities.

Materials and Methods

The Isolates

Rhizospheric samples associated with cotton and wheat roots were taken from cultivated plots located in rural areas of Multan and Gujranwala, Pakistan, respectively. After washing, the roots were dried and placed in 50 ml of sterile 0.9% NaCl solution and stored overnight at 4°C. Each suspension was then centrifuged for 30 min at 350 rpm and inoculated in 1:9 ratios in King-B (KB) broth medium. The suspension was incubated overnight with slight agitation at 27°C. Serial dilutions were then prepared and spread onto KB agar plates. After incubation for 48 h, up to 50 colonies were selected per root sample for further analysis.

Culturing Conditions and Characterization

Pseudomonas strains (Table 1) were cultured at 28°C in KB, except *E. coli* that was cultured in lactose broth (LB) at 37°C and *S. cerevisiae* on yeast extract peptone dextrose at 30°C. Gentamicin

Table 1. Strains, plasmids, and vectors used in this study.

Strains/plasmid	Description	References
<i>P. fluorescens</i> QAU67	Biocontrol, PGPR, GDH ⁺ , PQQ ⁺ , wild type (Pakistan)	[30]
<i>P. putida</i> QAU90	Biocontrol, PGPR, PHZ ⁺ , CLP, GDH ⁺ , PQQ ⁺ , wild type (Pakistan)	This study
<i>P. fluorescens</i> QAU67-14	PGPR ⁺ , GDH ⁺ , PQQ ⁺ , deletion mutant (<i>pqqC</i>), Pakistan	This study
<i>P. putida</i> QAU90-4	PGPR ⁺ , CLP ⁺ , GDH ⁺ , PQQ ⁺ , deletion mutant (<i>pqqC</i>), Pakistan	This study
<i>P. putida</i> QAU90-23	PGPR ⁺ , GDH ⁺ , PQQ ⁺ , Tn5 mutant (GDH), Pakistan	This study
<i>E. coli</i> WM3064	Donor strain for conjugation; carries the <i>pir</i> gene, which is necessary for plasmids with an <i>oriR6K</i> origin of replication	[9]
pfaj-1518 plasmid	mTn5 gfp-pgusA contains a promoterless <i>gfp</i> gene and a constitutively expressed <i>gusA</i> gene.	[6]
pMQ-30 Plasmid	Gene replacement vector for <i>Pseudomonas</i> sp.; sacB, <i>URA3</i> , Gm ^R	[35]
<i>S. cerevisiae</i> Inv Sc	Yeast strain for <i>in vivo</i> recombination (<i>ura3-52/ura3-52</i> mutation)	Invitrogen

The abbreviations: PGPR = plant growth-promoting rhizobacteria; PHZ⁺ = Phenazine producer; CLP⁺ = lipopeptides production; GDH = glucose dehydrogenase; and PQQ = Pyrroloquinoline quinone.

was added at a concentration of 25 µg/ml for *E. coli* strains and 25–50 µg/ml for *Pseudomonas* and mutant strains. The *E. coli* WM3064 vector with pfaj-1518 plasmid was cultured in 5 ml of LB broth with 10 ppm diammonium phosphate (DAP, 10 µl/ml) + kanamycin (Km, 50 ppm). All cultures were preserved at –80°C in LB medium supplemented with 40% glycerol. The *Pseudomonas* strains QAU67 and QAU90 [30] were selected for further study and characterized for indole acetic acid (IAA), catalase production, nitrogenase activity, quorum sensing, acyl homoserine lactone (AHL) production, secondary metabolite, antibiotics, antifungal activities, and phosphate solubilization activity on Pikovskaya medium [31].

Amplification, Sequencing, and Phylogenetic Analysis of 16S rRNA and RNA Polymerase Subunit D (RpoD) Genes

The genomic DNA of bacterium was extracted by the CTAB method with some modification as mentioned by Naveed *et al.* [30]. The 16S rRNA and *rpoD* loci were amplified using primers, as listed in Table 2. The Taq DNA polymerase and Q-solution were purchased from Qiagen (Venlo, The Netherlands) and polymerase chain reaction (PCR) was carried out with initial denaturation at 98°C for 5 min, followed by 29 cycles of denaturation at 98°C (30 sec), annealing at 54°C (30 sec), extension at 72°C (30 sec), and final extension at 72°C (10 min). The PCR products were resolved on a 2% agarose gel and visualized by staining with ethidium bromide. A nearly complete 16S rRNA gene was amplified as previously described [2]. The purified PCR product was sequenced at Macrogen, Korea (<http://dna.macrogen.com/eng>) using universal primers for the 16S rRNA gene, as described above. The sequences were assembled using the BioEdit software

to obtain a consensus sequence of the genes, which were then submitted to the NCBI servers. The strain was identified using the sequence of the 16S rRNA gene on the EzTaxon Server (<http://eztaxon-e.ezbiocloud.net>) and a BLAST search on the NCBI servers. The sequences of 16S rRNA gene for closely related validly published type strains were retrieved from the EzTaxon Server database, and phylogenetic trees were constructed as described previously [1] using neighbor-joining algorithm. The stability of the relationship was assessed using bootstrap analysis with 1,000 resamplings for the tree topology.

Amplification of *pqq* Operon

PCR amplification of the *pqq* gene was carried out with freshly designed oligonucleotides using sequence information of *Pseudomonas protegens* CHA0 (Accession No. CP003190.1) (Table 2). PCR was carried out in total volume of 50 µl in 1.5 ml microfuge tubes using GoTaq DNA polymerase (Promega, USA). The PCR mixture consisted of the following components: 33 µl H₂O; 10 µl Master Amp GoTaq 5× PCR Buffer; 1 µl dNTP mix; P1: 10 ppm; P2: 10 ppm; and 0.25 µl GoTaq polymerase. Colony PCR was carried out by initially denaturing DNA at 98°C for 5 min, followed by 30 cycles at 98°C for 30 sec, annealing temperatures (52°C to 60°C) for 30 sec, extension at 72°C for 30 sec, and a final extension at 72°C for 10 min. The amplified PCR products were sequenced commercially from LGC genomics (Germany) and compared with already published sequences in NCBI GenBank databases (<http://www.ncbi.nlm.nih.gov/blast/Blast.cgi>) for homology.

Induction of Mutation at GDH Locus and Characterization

E. coli WM3064 (Table 1) with pfaj-1518 [6] was cultured overnight

Table 2. Sequence information on the primers used in the study.

Primer	Sequences (5' → 3')	Product size	References
pqqC-Up-F	GGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTGTTCAAGATGCTCAGCCACTG	1,000 bp	This study
pqqC-Up-R	CAGTTCATAGGCCATGCTCAATGGGGATGTTACCTGGTA		
pqqC-Down-F	TACCAGGTGAACATCCCCATTGAGCATGGCCTATGAACTG	1,000 bp	This study
pqqC-Down-R	CCAGGCAAATCTGTGTTTATCAGACCGCTTCTGCGTCTGATCGATCTTGTCGATGTTGTGC		
pqqBCD-F	TTCAAGATGCTCAGCCACTG	2,050 bp	This study
pqqBCD-R	CGATCTTGTCGATGTTGTGC		
16S rRNA P1	AGAGTTTGATCCTGGTCAGAACGAACGCT	1,500 bp	[30]
16S rRNA P6	TACGGCTACCTTGTTACGACTTCACCCC		
rpoD-F	CAGTTCATGGACCAGAACAAACCCGCT	508 bp	[14]
rpoD-R	CCCATCAACGCACGGTTGGCGTC		
PqqAB-F	TGTGGACCAAACCTGCATACACTG	900 bp	This study
PqqAB-R	GATGCTCATGCCATCGAA		
PqqE-F	GATCGTCCTCGCCTGAGTT	1,200 bp	This study
PqqE-R	GATGACACGGGAGTTTCGAT		
PqqF-F	CCAACTTACCCTCGCCAAT	2,300 bp	This study
PqqF-R	CAGCGTTGGCCAAACATAG		

and 1 ml of culture was centrifuged at 14,000 rpm for 5 min. After discarding the supernatant, 500 µl of overnight culture of wild strain was added. The conjugated culture of the two strains (*E. coli* WM3064 vector construct and QAU90) was mixed by pipetting and poured onto an LB+DAP agar plate and incubated overnight at 28°C. The overnight culture was then placed in 1 ml of LB. After mixing the culture, various (10^0 – 10^{-7}) dilutions of conjugated culture was made in LB or saline solution. Then 100 µl of each conjugated culture was plated on LB+Km (50 ppm) and incubated at 28°C. The growth of colonies was checked after incubation to the corresponding dilution factor, and afterwards the replica plating was done.

All colonies were checked for their ability to solubilize inorganic phosphate on Pikovskaya medium and further tested for their comparative role in kidney bean (*Phaseolus vulgaris*) growth promotion. The growth-promoting capacity of *P. putida* QAU90 (wild type) strain with *P. putida* QAU90-23 (GDH mutant) inoculated plants and water-treated control plants was compared using the parameters of plant height and fresh weight.

Induction of *pqqC* Mutagenesis

The PQQ operon was amplified using primers designed for *Pseudomonas* strain CMR12a (Table 2). To mutate the operon, the biosynthesis gene *pqqC* was targeted for site-directed mutation using a PCR-based knockout method. To construct a deletion plasmid for *pqqC*, an upstream fragment from *pqqB* consisting of a 0.8 kb region upstream of *pqqC* and an overlapping 100 bp part of *pqqC* (primers pqqC-UP-F and pqqC-UP-R), and a downstream fragment from *pqqD* consisting of an overlap of 100 bp with the end of *pqqC* and a 0.88 kb region downstream of *pqqC* (primers pqqC-DOWN-F and pqqC-DOWN-R) were amplified for the strains QAU67 and QAU90. The upstream fragment was cloned immediately in the suicide plasmid pMQ30 with the counter selectable *sacB* gene by means of an *in vivo* cloning technique with the yeast *Saccharomyces cerevisiae* InvScl. Deletion plasmids for both *P. fluorescens* QAU67 and *P. putida* QAU90 were constructed in the same way. After mobilization of the resulting deletion plasmid into *Pseudomonas* by *E. coli* WM3064, transconjugants that lost the suicide plasmid after a second crossover event were selected on LB with 10% sucrose. Deletion of the gene was confirmed by PCR. The *E. coli* strain with the plasmid was used as a positive control, and the wild type was used as a negative control.

pqqC Mutant Characterization

Comparative performance of wild type strains (*P. fluorescens* QAU67 and *P. putida* QAU90) and the *pqqC* mutants (*P. fluorescens* QAU67-14 and *P. putida* QAU90-4) was assessed for phosphate solubilization on Pikovskaya medium [31] with little modification. The halo zone formation around the bacterial colony was considered as an indicator of phosphate solubilization. The acidification of medium experiment was carried out in 100 ml of Pikovskaya broth medium. Before inoculation, the initial pH of the medium was determined. The wild-type (*P. fluorescens* QAU67 and *P. putida*

QAU90) and *pqqC* mutant (*P. fluorescens* QAU67-14 and *P. putida* QAU90-4) strains were then inoculated in sterilized Pikovskaya broth medium and incubated at 28°C on a shaker with continuous agitation for 5 days. The pH of the broth was then measured immediately after sampling, and the changes in pH caused by mutants and wild-type strains were determined.

DPPH Radical Scavenging Activity

The DPPH assay was carried out as previously described [16] with a few modifications. The stock solution was prepared by dissolving 24 mg of DPPH with 100 ml of methanol and a working solution was obtained by diluting DPPH with methanol to obtain an absorbance of about 0.980 (± 0.02) at 517 nm using a spectrophotometer. A 3 ml aliquot of this solution was mixed with 100 µl of the samples at varying concentrations (25–250 µg/ml). The solution in the test tubes were shaken well and incubated in the dark for 15 min at room temperature. Then the absorbance was taken at 517 nm. The scavenging activity was estimated based on the percentage of DPPH radical scavenged, per the following equation:

$$\text{Scavenging effect (\%)} = \frac{[(\text{control absorbance} - \text{sample absorbance}) / (\text{control absorbance})] \times 100.}$$

The EC₅₀ value was taken as an effective concentration to scavenge 50% of the DPPH radicals. Ascorbic acid and rutin were used as positive references. Each fraction was assayed in triplicate.

Reducing Power

The reducing power of the extracts was determined as described by Chung *et al.* [7] with some modification. First, 0.1 ml of each extract, ascorbic acid, and rutin or gallic acid (0.05–250 mg/ml) were mixed with an equal volume of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide and incubated at 50°C for 20 min. Then 0.25 ml of 1% trichloroacetic acid was added to the mixture to stop the reaction and the mixture was centrifuged at 2,790 ×g for 10 min. The supernatant (0.25 ml) was mixed with 0.25 ml of distilled water and 0.1% FeCl₃ (0.5 ml). The absorbance was measured at 700 nm.

Plant Growth Promotion Activity

Experiments on lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*), rice (*Oryza sativa* C-039), and bean (*Phaseolus vulgaris* var. prelude) were carried out to assess the performance of wild type and *pqqC* mutants. *In vitro* experiment was done on lettuce seeds, pre-germinated in MS media for 3 days at 22°C before bacterial inoculation. The optical density (OD₆₂₀) of overnight LB culture with strains was taken in three replicates. The dilutions at 10⁶ CFU of all strains in saline solution were made and the treated pre-germinated seeds (10 each) were placed in the MS agar plates in three replicates (*i.e.*, those treated with the wild type; those treated with the mutants, and the control) and incubated in a growth chamber for one week. The data on root length were taken for all plants after one week.

The *in vivo* experiments were carried out on plants as described above. Seeds were surface sterilized in 1% sodium hypochlorite solution for 5–10 min and rinsed three times in sterile distilled water, and after air drying, 25 seeds of each species were sown in a petri dish and incubated at 28°C. The seeds were pre-germinated before sowing to reduce variability in emergence.

Inoculum Preparation and Experimental Setup

Suspensions of the bacterial strains (QAU67 and QAU90) were grown on KB plates for 48 h at 28°C and collected from plates by adding sterile saline solution. The OD of these bacterial suspensions was determined at 620 nm and the suspensions were diluted using saline solution to obtain $2,800 \text{ g} \times 10^6 \text{ CFU}$ per 200 ml, but for rice the bacterial suspensions were $5 \times 10^7 \text{ CFU/g}$ soil. These dilutions served as bacterial inoculum for plant experiments. After 3 days, the germinated seeds were sown in perforated plastic trays ($22 \times 15 \times 6 \text{ cm}$) filled with 700 g of a soil mixture composed of 50% sand and 50% non-sterile potting soil (w/w) (Structural; Snebbout, Kaprijke, Belgium). Then 200 ml of diluted bacterial suspension was mixed thoroughly with 2.8 kg of soil for 2 min to get a final concentration of 10^6 CFU/kg soils, with four replicates for each treatment. Ten pre-germinated bean (*Phaseolus vulgaris*) seeds were sown in each box and the plants were incubated in a growth chamber (28°C, relative humidity of 70%, and 16 h photoperiod). A completely randomized design was employed with four replications per treatment. FeSO_4 and $(\text{NH}_4)_2\text{SO}_4$ (2:1 g/l) were used as nutrients to fertilize (250 ml solution/box) the rice plants, and soil mixed with 200 ml of saline solution served as a positive control.

Data Analysis

Different growth parameters (root length, shoot length and weight, leaf area index, dry weight, and total number of leaves) were collected and recorded as an average value of 10 plants in three replicates. The data were assessed using SPSS 15.0 (SPSS Inc., USA). The data revealed non-normal distribution and therefore nonparametric analyses such as Kruskal-Wallis and Mann-Whitney comparisons ($\alpha = 0.05$) for the mean and medians were made.

Results

Bacterial Characterization

The phenotypic characterization of strains QAU67 and QAU90 revealed both to be gram-negative cocci. The strains were positive for IAA, catalase, and AHL production; both the strains were however found negative for the nitrogenase activity. QAU90 also produced secondary metabolites and antibiotics, and showed antifungal activity, thus revealing its biocontrol traits. The rhizospheric strains QAU67 and QAU90 showed halo-zone formation around the colony on Pikovskaya agar medium. QAU90 showed a

solubility index (SI) of 3.9 mm, whereas QAU67 showed the lower value of 3.2 mm. In Pikovskaya broth, a decrease in pH was observed for QAU90, from an initial pH value 7.0 to 4.14, and for QAU67 the pH value was 4.50, depicting their phosphate solubilization capacity.

Strain Identification

Homology based on the 16S rRNA full-length sequence revealed that QAU90 belonged to *P. putida* (Fig. 1A). The sequence similarity of this strain was 100% with that of *P. mosselii*, a subgroup of *P. putida*, whereas strain QAU67 gained 91% bootstrap value and belonged to *P. mohnii*, a subgroup of *P. fluorescens*. The *rpoD*-based analysis reconfirmed the QAU67 homology with *P. mohnii* and it was therefore identified as *P. fluorescens*, whereas QAU90 was identified as *P. putida* (Fig. 1B).

GDH Mutation and Impact on Plant Growth Promotion

P. putida strain QAU90 was mutated through Tn5 insertion mutation with *E. coli* WM3064 and pfaj-1518 plasmid. A total of 340 colonies were screened for mutation by measuring their ability to solubilize the inorganic phosphate on Pikovskaya medium, and only two GDH mutants (QAU90-23 and QAU90-40) were detected. Intriguingly, the mutant QAU90-23 still showed some capacity of phosphate solubilization; however, it did not develop any halo-zone on Pikovskaya agar media.

The inoculum treatment of *P. putida* mutant QAU90-23 to bean plants showed no impact on the growth promotion originally revealed by the wild-type inoculation (Fig. 2A). Comparing the two treatments, the difference in performance was obvious in plant height (23 cm), fresh weight (6.3 cm), and leaf area (23.2 cm). These data clearly demonstrated differences among wild, mutant, and control treated plants (Fig. 3). This experiment suggested that glucose dehydrogenase has a positive impact on phosphate solubilization and consequently on plant growth promotion.

PQQ Mutation and Impact on Plant Growth Promotion

Both the strains and their *pqqC* mutants were assessed *in vitro* with lettuce as well as *in vivo* with bean, tomato, and rice models.

The *in vitro* effect. Both wild-type strains (*P. fluorescens* QAU67 and *P. putida* QAU90) demonstrated positive impact on *in vitro* elongation of lettuce roots (Fig. 2D). However, the impact of all mutants of the *pqqC* locus did not show any significant difference from the control (no treatment). Hence, mutation at this locus has resulted in dysfunction for the strain causing proliferated root growth. When

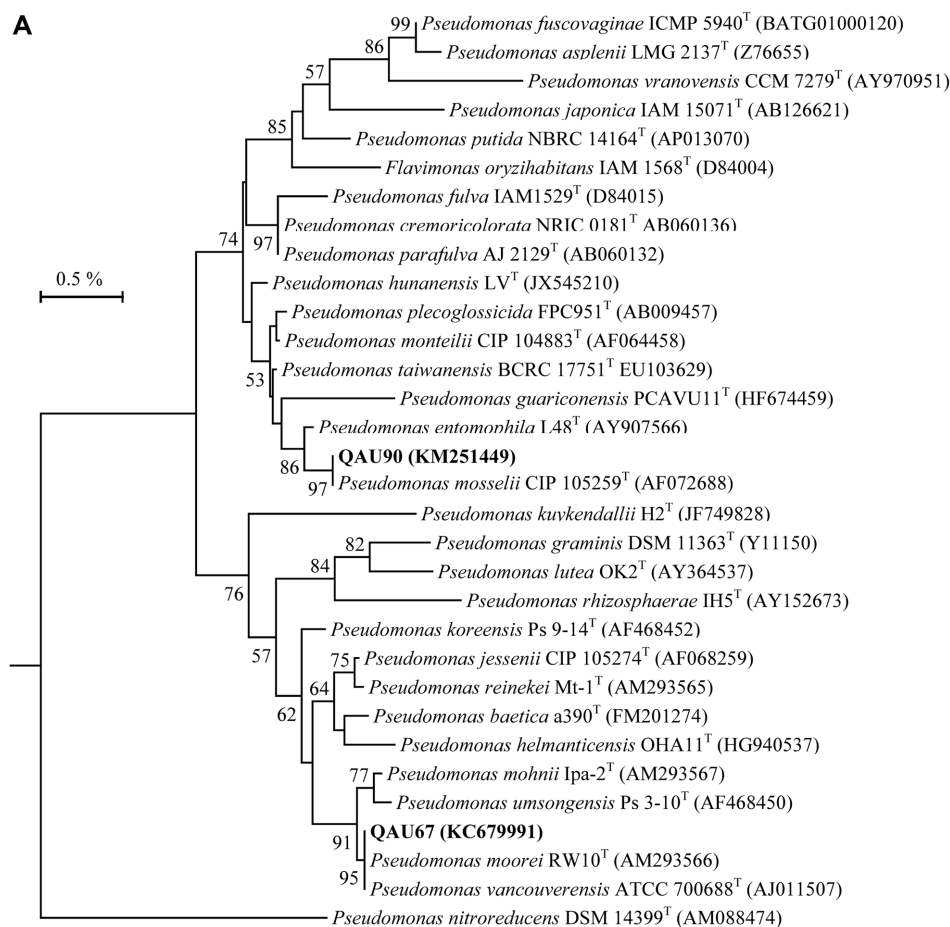


Fig. 1. Neighbor-joining phylogenetic tree showing inter-relationship of strains QAU67 and QAU90 with the closely related validly published type species inferred from sequences of the 16S rRNA gene.

Pseudomonas nitroreducens DSM 14399T (AM088474) was used as the outgroup. The bootstrap values are expressed as a percentage of 1,000 replications analysis and are given on respective branch points. (B) Neighbor-joining phylogenetic tree showing inter-relationship of strains QAU67 and QAU90 with the closely related validly published type species inferred from sequences of the *rpoD* gene. *Pseudomonas fluorescens* LMG 18387^T (FN554465) was used as the outgroup. The bootstrap values (>50) are expressed as a percentage of 1,000 replications analysis and are given on respective branch points.

compared for their performance, both species demonstrated almost similar proliferation in the roots, and any possible impact of species difference was ruled out. The differences revealed here corresponded to the impact of *pqqC* mutation and not to the genetic background by any chance.

The *in vivo* effect.

Tomato. The performance of *P. fluorescens* QAU67 and *P. putida* QAU90 did not show clear difference for parameters like fresh weight and dry weight; however, for plant height, the experimental performance was clear (Fig. 2B). However, the null hypothesis (that there is no difference between the wild and mutated strains) could not be supported for the three parameters assessed. A comparison of median values also concluded in the same

manner. The *p* values for fresh weight and dry weight in the case of QAU67 were $0.05 > p > 0.001$, and the probability was $0.05 > p > 0.001$ for fresh weight in the case of QAU90 treatment; however, the other two parameters did not show any evidence (Fig. 4A).

Rice. The treatment of wild-type strains (both *P. fluorescens* and *P. putida*) on rice plants demonstrated plant growth promotion. This was evident by comparing the performance of wild-type treatment by either strains QAU67 and QAU90 with the respective mutants and also the control (no treatment) treatment (Fig. 2C). For instance, the plant height in rice revealed a difference of up to 25%, whereas the fresh weight demonstrated up to 24% difference in performance for the wild type strains to that of their

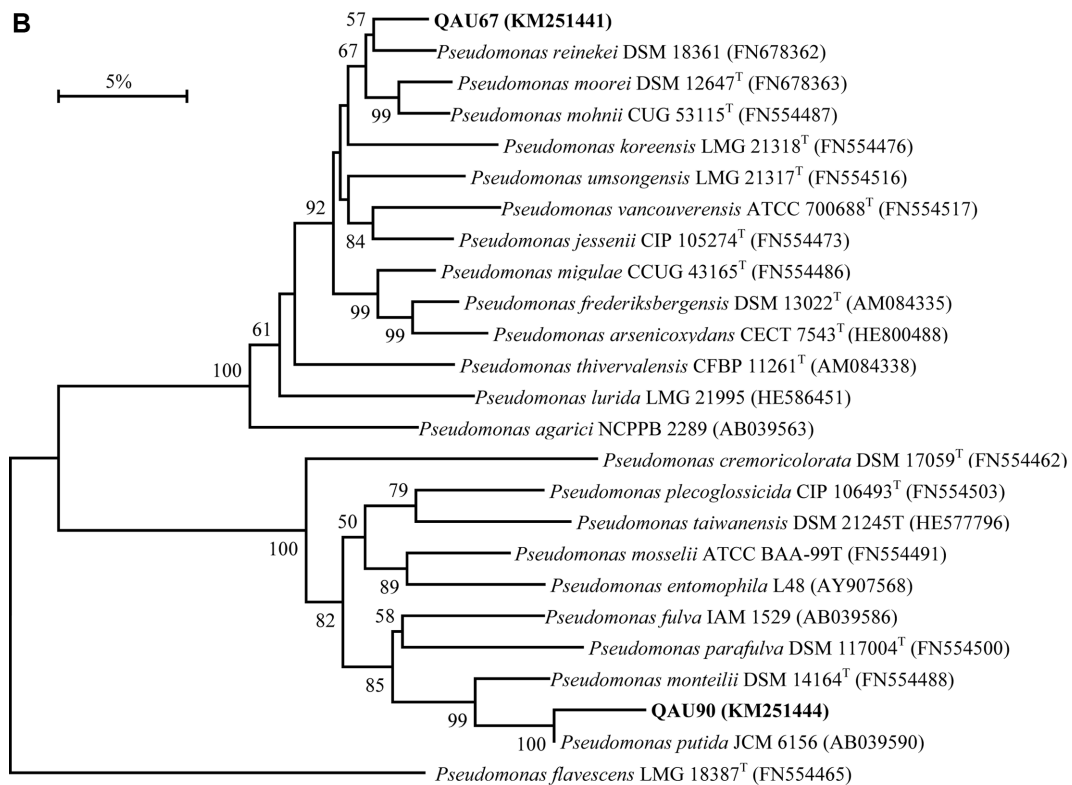


Fig. 1. Continued.

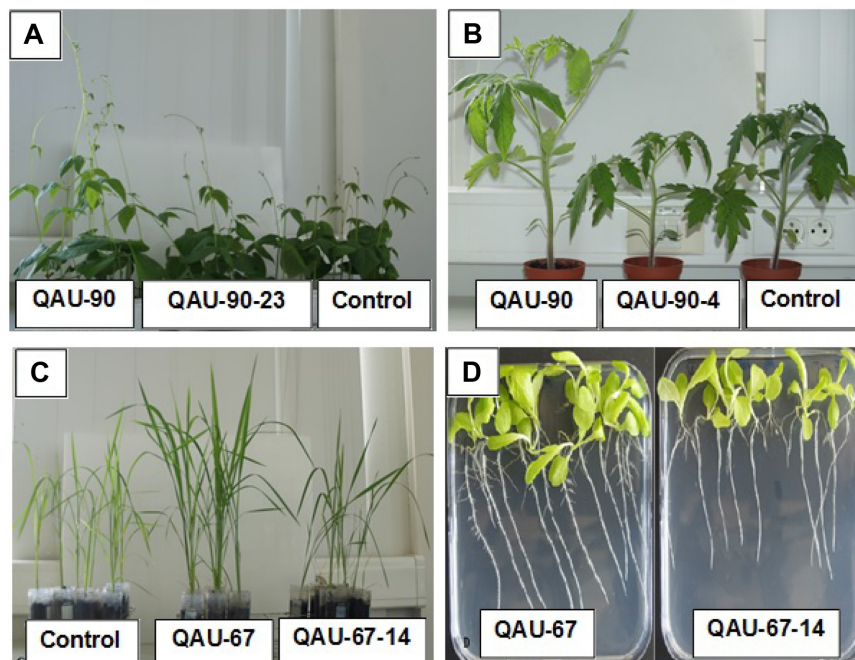


Fig. 2. Plant growth promotion activities of wild-type and mutant strains in lettuce, bean, tomato, and rice. (A) *P. putida* QAU90 and its GDH mutant (*P. putida* QAU90-23) with water-treated control bean plants, (B) *P. putida* QAU90 and its *pqqC* mutant (*P. putida* QAU90-4) with water-treated control tomato plants, (C) *P. fluorescens* QAU67 and its *pqqC* mutant (*P. fluorescens* QAU67-14) with control-treated rice plants, and (D) *P. fluorescens* QAU67 and its *pqqC* mutant (*P. fluorescens* QAU67-14) with control-treated lettuce plants.

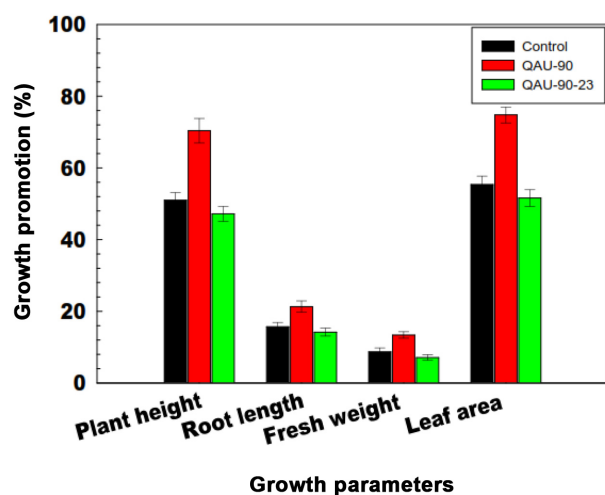


Fig. 3. Growth promotion activities of bean (*Phaseolus vulgaris*) by *P. putida* QAU90 (wild type) and Tn5-based insertional GDH mutant *P. putida* QAU90-23 inoculated plant with water-treated control plants.

Plant parameters are plant height, root length, fresh weight (shoot $\times$ root), and leaf area (length $\times$ width), with an average of 10 plants from three replicates.

mutants, respectively. A comparative performance analysis for the species revealed no significant difference between them and therefore species type has been considered as not an influencing factor in these experimental results (Fig. 4B). The statistical analysis further confirmed the performance for parameters such as plant height and fresh weight. Hence, we obtained the p value = $0.00 < 0.05 = \alpha$. Therefore, we rejected our null hypothesis, meaning that our experimental data revealed differences among treatments based on the test scores. Furthermore, the data were tested for the median scores, again revealing the $p = 0.00 < 0.05 = \alpha$, thus providing reasons to reject the null hypothesis.

Antioxidant Activity of PQQ

The α -diphenyl- β -picrylhydrazyl antioxidant activity of PQQ was estimated through comparing extracts obtained from the wild type as well as the mutants at different concentrations (20, 40, and 80 $\mu\text{g/ml}$). The results suggested that the scavenging activity of extracts was concentration dependent, with maximum activity for both the wild type (*P. fluorescens* QAU67 and *P. putida* QAU90) treatment and their *pqqC* mutants (*P. fluorescens* QAU67-14 and *P. putida* QAU90-4) observed at 80 $\mu\text{g/ml}$. Comparing the species performance, the activity pattern was higher when treatment involved the wild-type *P. putida* as compared with that of

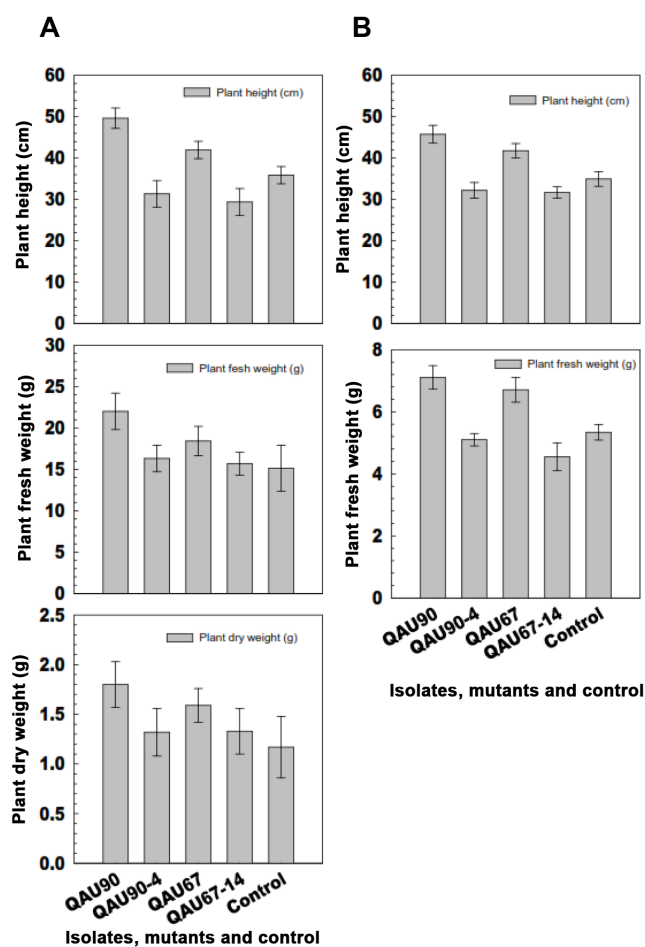


Fig. 4. Comparison in performance of wild-types (*P. fluorescens* QAU67 and *P. putida* QAU90), their derived *pqqC* mutant (*P. fluorescens* QAU67-14 and *P. putida* QAU90-4) strains, and control treatments as assessed in (A) tomato and (B) rice.

P. fluorescens. These results revealed novel traits for PQQ, demonstrating the proton-donating ability while inhibiting the free radicals (Fig. 5A).

Furthermore, the reducing capacity of PQQ (*i.e.*, the transition of Fe^{3+} /ferricyanide complex to Fe^{2+}) also exhibited a dosage dependency when studied at various concentrations (0, 10, 20, 40, and 80 $\mu\text{g/ml}$). At 80 $\mu\text{g/ml}$, the *P. putida* QAU90 strain showed maximum reducing potential with a value of 0.98, whereas *P. fluorescens* QAU67 exhibited a reducing value of 0.75 (Fig. 5B). With the same dosage, the mutant *P. putida* QAU90-4 showed a reducing value of 0.71 and *P. fluorescens* QAU67-14 revealed a value of 0.54. These results suggested that PQQ was an electron-donating moiety and has free radical scavenging capacity. Its reducing capacity showed its potential antioxidant activity.

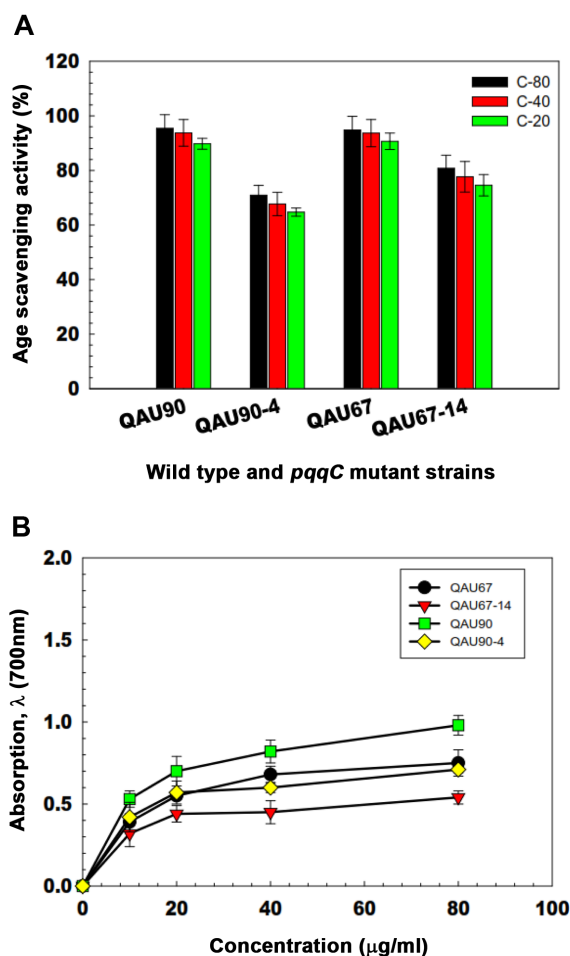


Fig. 5. Antioxidant activities of PQQ.

(A) DPPH radical showed a concentration (20, 40 and 80 mg/ml) dependent percent scavenging antioxidant activity of PQQ extract from wild-type (*P. fluorescens* QAU67 and *P. putida* QAU90) strains and their *pqqC* mutants (*P. fluorescens* QAU67-14 and *P. putida* QAU90-4) at absorbance of 517 nm. (B) Antioxidative activities of the wild-type (*P. fluorescens* QAU67 and *P. putida* QAU90) strains and their PQQ mutants (*P. fluorescens* QAU67-14 and *P. putida* QAU90-4) with different concentrations (0, 10, 20, 40, and 80 mg/ml) were measured by the reducing power method. Each absorbance value represented the average of triplicates of different samples analyzed. Increase in the absorbance at 700 nm indicates the reducing power.

Discussion

With increasing attention to various sides of plant-microbe interaction, enormous potentials of microbes have been focused on developing biofertilizers [17], nitrogen fixation [22], bioprocessing, and biofilming [4]. The phosphate solubilizing potential of microbes and their functional role in plant growth promotion [29] have found applications in

developing biofertilizers. Likewise, other aspects with predispositions have led to discovery of the biological and genetic diversity, still being exhumed. The present study focused on understanding the role of genetic factors *gdh* and *pqq* in plant growth promotion, as demonstrated in *Pseudomonas* species: *P. fluorescens* QAU67 and *P. putida* QAU90. The study provided *in vivo* experimental evidence on the functional role of these loci. Experimental details on the hosts kidney bean, tomato, and rice demonstrated the complementary role of PQQ in phosphate solubilization and regulating the pH of the medium, besides its antioxidant activity, all contributing to plant growth promotion. Previous studies demonstrated this phenomenon [5] with little evidence based on *in vitro* analysis. This study extends the current understanding for the host-associated microbial role in plant growth promotion.

Pseudomonas, known for plant growth promotion, also shows phosphate solubilization (PS) capability [26]. Hence, PS in turn predicts the growth promotion ability of strains under study. We used these indicators to select our strains for the genetic characterization. A similar slant was used for the catalase activity in bacteria [18, 20] to depict resistance to environmental, mechanical, and chemical stress mitigation and the role in crop yield improvement and plant protection. Previously, both of our selected strains were also found positive for catalase activity, pointing to a similar potential. Furthermore, our strains were also found positive for AHL production. AHL has been reported to increase salicylic acid production [10], a factor well known for enhanced systemic resistance.

GDH: Role in Plant Growth Promotion

So far, little is known about the role of GDH and its cofactor PQQ. Some recent studies provide *in vitro* evidence on their possible role in phosphate solubilization [5]. The paucity of GDH-based studies makes this aspect poorly understood and therefore presents a case to meticulously decipher its role in host plants. Sashidhar and Podile [33] reported the expression of GDH in *E. coli* from *Azotobacter*, exploiting its enhanced biofertilizer potential and plant growth promotion. For this purpose, *P. putida* QAU90 was mutated at the GDH locus and studied on bean plants. The treatment of wild-type strains on beans clearly showed enhanced performance in plant height as compared with the mutant, and therefore outperformed the mutated strain, thus providing *in vivo* evidence of GDH's role in plant growth improvement. Therefore, it was concluded that GDH together with its cofactor PQQ is a major genetic factor in phosphate solubilization.

The paucity of studies reporting GDH's role in plant growth promotion indicate two possibilities. First, there is experimental difficulty in probing and mutating the GDH locus and, therefore, barring its characterization. Our experimental design was based on site-directed mutagenesis, which remained successful by chance or that it targeted the right locus at the right position. An alternative opinion is that GDH has only recently gained attention and therefore still remains uncharacterized. However, studying the genetic basis of phosphate solubilization requires attention to both GDH and PQQ loci. Recent studies have paid more attention to PQQ [36, 37], which suggests the importance of this locus in this scenario and, therefore, the inclusion of both loci in the present study was inevitable.

The PQQ Role

PQQ, described as redox cofactor, has an important assistive role in GDH activities [26]. It is rare in nature to see the independent functioning of GDH [8]. The data from the present study showed that PQQ mutation has an antagonistic impact on phosphate solubilizing activity both in *P. fluorescens* QAU67-14 and *P. putida* QAU90-4. This not only depicted that our system was a PQQ-dependent GDH but also that PQQ has a complementary role to play in GDH activity for phosphate solubilization and consequently growth promotion in the host plants. If we look at the mechanism, the phosphate solubilization essentially involves both GDH and PQQ. Biochemically, this process involves conversion of glucose into gluconic acid, thus making the medium acidic and consequently causing phosphate solubilization. The process of phosphate solubilization was assessed on Pikovskaya medium, as seen in both the strains. It is pertinent to mention that the acidification capacity of *P. putida* QAU90 was relatively higher than that of *P. fluorescens* QAU67. Nonetheless, the fundamental role of GDH and PQQ was clearly observed in both the strains. Hence, the acidification of medium may be used as a quick confirmation to indicate ancillary traits in the strains.

Besides deciphering PQQ's role in phosphate solubilization and complementing GDH, this study also highlighted PQQ's role as an antioxidant, further endorsing its growth promotion capacity. The confirmation of its antioxidant trait was even clearer when the wild-type strains for PQQ locus were compared with their *pqqC* mutants. Both *in vivo* and *in vitro* experiments depicted reduced potential following mutagenesis, and therefore depicting the importance of PQQ in host plants. Previously, it was reported that PQQ has a role in phosphate uptake and *in vitro* pollen germination in tulips, *Lilium* and *Camellia* [36, 37]. The present study

describes an extended role of PQQ; that is, the reducing capacity and antioxidant properties, besides promoting host plant growth. This information promotes the hypothesis that PQQ is in fact involved in many ancillary processes, all supplementing the process of plant growth promotion many fold, where previously this was evident only through *in vitro* studies [5].

The results showed *in vivo* evidence for the functional roles of GDH and PQQ in phosphate solubilization and consequently in plant growth promotion in bean, rice, and tomato plants. PQQ has an indispensable role above and beyond GDH, as mutagenesis in either of the two renders functional inadequacy in host plants. Studying two different species that differ slightly in their performance depicted not only the possible widespread occurrence of the phenomenon but also points to the fact that there are procedural variations in the process of mutagenesis and that the interpretations should be made with caution.

Sequence Submission Numbers

The EMBL GenBank accession numbers for the 16S rRNA gene sequence of strains QAU67 and QAU90 are KC679991 and KM251449, respectively. The *rpoD* gene sequences of the strains QAU67 and QAU90 are KM251441 and KM251444, respectively. The PQQ operon of QAU67 are *pqqB* (KM251422), *pqqC* (KM251423), *pqqD* (KM251424), and *pqqE* (KM251425), and for QAU90 are as *pqqA* (KM251426), *pqqB* (KM251427), *pqqC* (KM251428), *pqqD* (KM251429), *pqqE* (KM251430), and *pqqF* (KM251431).

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