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Regulation of Pyrroloquinoline Quinone-Dependent Glucose Dehydrogenase Activity in the Model Rhizosphere-Dwelling Bacterium *Pseudomonas putida* KT2440

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**ABSTRACT**

Soil-dwelling microbes solubilize mineral phosphates by secreting gluconic acid, which is produced from glucose by a periplasmic glucose dehydrogenase (GDH) that requires pyrroloquinoline quinone (PQQ) as a redox coenzyme. While GDH-dependent phosphate solubilization has been observed in numerous bacteria, little is known concerning the mechanism by which this process is regulated. Here we use the model rhizosphere-dwelling bacterium *Pseudomonas putida* KT2440 to explore GDH activity and PQQ synthesis, as well as gene expression of the GDH-encoding gene (*gcd*) and PQQ biosynthesis genes (*pqq operon*) while under different growth conditions. We also use reverse transcription-PCR to identify transcripts from the *pqq* operon to more accurately map the operon structure. GDH specific activity and PQQ levels vary according to growth condition, with the highest levels of both occurring when glucose is used as the sole carbon source and under conditions of low soluble phosphate. Under these conditions, however, PQQ levels limit in vitro phosphate solubilization. GDH specific activity data correlate well with *gcd* gene expression data, and the levels of expression of the *pqqF* and *pqqB* genes mirror the levels of PQQ synthesized, suggesting that one or both of these genes may serve to modulate PQQ levels according to the growth conditions. The *pqq* gene cluster (*pqqFABCDEG*) encodes at least two independent transcripts, and expression of the *pqqF* gene appears to be under the control of an independent promoter and terminator.

**IMPORTANCE**

Plant growth promotion can be enhanced by soil- and rhizosphere-dwelling bacteria by a number of different methods. One method is by promoting nutrient acquisition from soil. Phosphorus is an essential nutrient that plants obtain through soil, but in many cases it is locked up in forms that are not available for plant uptake. Bacteria such as the model bacterium *Pseudomonas putida* KT2440 can solubilize insoluble soil phosphates by secreting gluconic acid. This chemical is produced from glucose by the activity of the bacterial enzyme glucose dehydrogenase, which requires a coenzyme called PQQ. Here we have studied how the glucose dehydrogenase enzyme and the PQQ coenzyme are regulated according to differences in bacterial growth conditions. We determined that glucose dehydrogenase activity and PQQ production are optimal under conditions when the bacterium is grown with glucose as the sole carbon source and under conditions of low soluble phosphate.

Mineral phosphate solubilization is an essential activity of many rhizobacteria with the ability to promote plant growth, including a range of bacteria from genera such as *Pseudomonas*, *Bacillus*, *Rhizobium*, *Micrococcus*, *Acinetobacter*, *Flavobacterium*, *Achromobacter*, *Erwinia*, and *Agrobacterium* (1, 2). Most commonly, these bacteria release organic acids into the extracellular space to chelate divalent cations (e.g., Ca$^{2+}$) in poorly soluble mineral phosphate forms, such as hydroxyapatite or tricalcium phosphate, thus releasing phosphate in a form available for plant uptake (3). The best-characterized mechanism for microbial phosphate solubilization is through secretion of gluconic acid (4), which is produced from glucose through the activity of a glucose dehydrogenase (GDH) enzyme that requires the redox cofactor pyrroloquinoline quinone (PQQ).

Two types of PQQ-dependent GDH enzymes have been identified to date: an inner membrane-bound GDH and a soluble GDH (sGDH), both of which exhibit activity in the periplasm of Gram-negative bacteria. While membrane-bound GDH has been found in many Gram-negative bacteria, such as *Gluconobacter*, *Pseudomonas*, and *Acinetobacter* species, sGDH is less common and has been reported only from *Acinetobacter calcoaceticus* (5). Periplasmic gluconic acid can be imported into the cytoplasm, where it is further catabolized, or it can be exuded into the extracellular space, where it is proposed to play myriad roles, including reducing protist grazing, as an antifungal, and solubilizing mineral phosphate (6).

Soil-dwelling pseudomonads have become models for understanding GDH-mediated phosphorus solubilization (1, 4, 7). Miller et al. showed that this activity can be impaired by mutations of the GDH-encoding gene (*gcd*) or of certain genes in the PQQ biosynthesis pathway from *Pseudomonas fluorescens* F113 (8).
They and others have noted distinct differences in the number and genomic synteny of genes predicted to be involved in PQQ biosynthesis among pseudomonads (9, 10). In general, the pqqA, pqqB, pqqC, pqqD, and pqqE genes are conserved and arranged in that particular order in what is typically referred to as the pqq operon (pqqABCDE) (11). Other commonly found genes include pqqF and pqqG, which can be located either proximal or distal to the pqq operon (8, 12–14). While a fair amount is known about the genes necessary for PQQ biosynthesis, their specific roles and the mechanisms by which their expression is regulated are less clear (11, 15).

GDH enzyme activity, and, hence, phosphate solubilization, can be affected by the levels of both the GDH enzyme and the PQQ cofactor in the periplasm. Observational studies have suggested that substrates of PQQ-dependent enzymes as well as environmental factors, such as phosphorus availability and carbon source, can have an effect on the enzyme activity and levels of PQQ produced (13, 16–21). Previous work has suggested that the synthesis of PQQ and GDH is not coordinated (17, 22), but there is little information on the mechanisms by which either GDH activity or PQQ synthesis is regulated. Here we use the model rhizosphere-dwelling bacterium Pseudomonas putida KT2440 to explore how GDH enzyme activity, gcd gene expression, PQQ levels, and pqq gene expression are regulated according to variations in growth conditions. We also explore the structure of the PQQ biosynthetic operon to identify which gene(s) is limiting the levels of PQQ under conditions of low synthesis.

**MATERIALS AND METHODS**

Bacterial strains and growth conditions. *Escherichia coli* DH5α and *Pseudomonas putida* KT2440 were routinely grown at 37°C and 28°C, respectively, on LB agar plates. For preparation of the *E. coli* cell membrane fraction used in PQQ bioassays, a single colony of *E. coli* was picked and inoculated in 500 mL LB broth and shaken (220 rpm) overnight at 37°C. For measurements of *P. putida* KT2440 GDH enzyme activity, PQQ levels, and RNA extraction, three different medium types were used: LB medium, M9 minimal medium (23), and the National Botanical Research Institute’s phosphate (NBRIP) medium (24). M9 minimal medium was used for studies in which carbon sources were varied, and NBRIP medium was used for studies in which the level of soluble phosphate was varied. Glucose, glycerol, or citrate was added as the sole carbon source to M9 minimal medium to a final concentration of 22.2 mM. For growth in NBRIP medium, glucose was used as the sole carbon source at 22.2 mM. For studies varying the carbon source, a single colony of *P. putida* KT2440 was inoculated and grown overnight in 5 mL LB broth in a shaker at 28°C, and 1 mL of this starter culture was inoculated in a 1-liter flask containing either 250 mL of fresh M9 minimal medium with various carbon sources or LB medium. Each culture was performed in triplicate and grown at 28°C with shaking. Two replicates were for cell collection, and one was for monitoring bacterial growth by measuring the optical density at 600 nm (OD600) every 4 h using a Biomate 3 spectrophotometer (Thermo Scientific).

Preparation of cells and cell membrane fractions. Cells were grown to mid-exponential phase and harvested when the OD600 reached 0.5. The 500-mL cultures of *E. coli* DH5α or *P. putida* KT2440 were harvested by centrifugation at 2,320 × g for 15 min. Culture supernatants from each growth condition were collected for PQQ bioassays and stored at −80°C. A small amount of the same *P. putida* KT2440 culture was used for RNA isolation (described below). The cell pellets were washed twice with phosphate-buffered saline (PBS; pH 7.0), resuspended in PBS with 10% glycerol, and stored immediately at −80°C. This step and all the subsequent procedures were carried out at 4°C as described by Matsushita and Ameyama (25). The washed cell pellets were resuspended in PBS and disrupted with a sonicator dismembrator system (Fisher Scientific) for 10 cycles of 20 s each with a 2-min pause on ice between each cycle. The mixture was centrifuged at 1,800 × g for 10 min at 4°C to remove intact cells and cell debris, and the supernatant was centrifuged in an Optima L-XP ultracentrifuge (Beckman) at 68,000 × g for 60 min at 4°C to sediment membrane fractions. Pelleted membrane fractions were homogenized in ice-cold PBS. The total protein concentration was measured using the Bio-Rad protein assay and ranged from 1.5 to 2.4 mg mL−1.

**PQQ bioassay.** PQQ concentrations in the culture supernatant were determined using GDH assays with the extracted cell membrane fraction of *E. coli* DH5α. This approach is based on the fact that *E. coli* synthesizes apo-GDH but is unable to synthesize PQQ; therefore, the membrane fraction of *E. coli* shows GDH activity only after the addition of exogenous PQQ (26). A standard curve was generated from assays with the *E. coli* membrane fraction (as described above) with various concentrations of PQQ. The working range of the assay was determined based on the extent of the linear relationship between enzyme velocity and substrate concentration. The relationship was linear up to 1 μM PQQ (see Fig. S1 in the supplemental material). Filter-sterilized (0.22 μm; Sigma-Aldrich) super- natant was preincubated with the *E. coli* membrane fraction before measurement of the GDH activity, which was used to determine the PQQ concentration in each culture supernatant according to the standard curve. Several control assays were performed simultaneously to exclude the background DCIP reduction contributed by components in both the membrane fractions of *E. coli* DH5α and the supernatants of *P. putida* KT2440 cultures under different growth conditions.

Evaluation of phosphate-solubilizing efficiency. The phosphate-solubilizing efficiency of *P. putida* KT2440 was evaluated by cultivating the bacterium in liquid medium with insoluble tricalcium phosphate and measuring the content of soluble inorganic phosphate in culture filtrates over time. Fifty milliliters of NBRIP broth at pH 7.0 containing 22.2 mM glucose as the sole carbon source was added to 250-mL flasks. Insoluble tricalcium phosphate was added as the only phosphate source to the medium at a concentration of 5 g liter−1. M9 medium was not used here due to its high inorganic phosphate content. To determine whether the level of PQQ production was limiting bacterial phosphate-solubilizing efficiency, parallel experiments were conducted in which exogenous PQQ (Sigma-Aldrich, USA) was added to the medium at a concentration of 10 μM. A
200-μl volume of \textit{P. putida} KT2440 cells (at \(5 \times 10^8 \text{ CFU ml}^{-1}\)) grown in LB broth was inoculated to each flask. Each treatment was performed in triplicate, and uninoculated medium served as a negative growth control. Both inoculated and uninoculated flasks were shaken at 28°C and 220 rpm for 7 days. Inorganic phosphate concentration, cell density, and pH were monitored after removing 2 ml from each flask every 24 h. Of this 2-ml volume, 500 μl was used to determine the pH using a benchtop pH meter (Fisher Scientific), and 500 μl was used to harvest cells. Cells were washed twice with LB medium before final resuspension in 500 μl NBRIP broth. The OD_{600} was measured spectrophotometrically after adding the same volume of 3.7% HCl to dissolve the tricarboxylic phosphate and vortexing (8). The remaining 1 ml was centrifuged at 20,878 g for 20 min, and the supernatant was filter sterilized. The inorganic phosphate concentration in samples was determined by the construction of a standard curve using K₂HPO₄.

40 cycles of denaturation at 95°C for 30 s and annealing and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The resulting PCR products were identified on a 1.5% agarose gel. Band intensity was analyzed using ImageJ software.

Relative intensity (RI) was assessed by the following formula, where \(B_I\) represents band intensity:

\[
RI = \frac{B_{\text{target}}}{B_{\text{Reference}}/\text{control}}
\]

Following verification of the appropriate sizes, band intensity was normalized to the 16S rRNA gene level.

Analysis of \(pqq\) operon structure. A two-step RT-PCR was performed to validate the computationally predicted \(pqq\) operon in \textit{P. putida} KT2440. One microgram of total RNA, extracted as described above from cells grown in LB medium or M9 minimal medium with glucose, glycerol, or citrate as the sole carbon source, was used to synthesize cDNA in a 20-μl reaction mixture containing 10 μM random hexamer primers, 0.5 mM deoxynucleoside triphosphates (dNTPs), and 4 U Omniscript reverse transcriptase (Qiagen). The same amount of RNA was added with nucleic-acid-free water up to 20 μl, to serve as the RT-negative (RT (−)) control. Reverse transcription reaction mixtures were incubated at 50°C for 30 min and inactivated by incubating them at 85°C for 3 min. The cDNA products were subsequently amplified by PCR using the RT-PCR primers listed in Table 1. These primers were designed to amplify intergenic regions (where possible) of 250 to 400 bp that span adjacent \(pqq\) genes, such that the forward primer was located at the 3′ end of one \(pqq\) gene and the reverse primer was located at the 5′ end of the \(pqq\) gene immediately downstream (Fig. 1A). PCR was carried out in a 25-μl reaction mixture consisting of 1 μl freshly synthesized cDNA, 250 nM each primer, and 1× DreamTaq master mix (2 mM MgCl₂, 2 mM dNTPs, and 0.625 U DreamTaq DNA polymerase [Thermo Scientific]). Amplification was performed with an initial denaturation of 95°C for 3 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 65°C for 30 s and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. The resulting PCR products were identified on a 1.5% agarose gel.

Following verification of the appropriate sizes, band intensity was analyzed using ImageJ software. Relative intensity (RI) was assessed by the following formula, where BI represents band intensity:

\[
RI = \frac{BI_{\text{target}}}{BI_{\text{Reference}}/\text{control}}
\]

### Table 1 qRT-PCR and RT-PCR primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
<th>RT-PCR</th>
<th>qRT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>(pqqF-pqqA)</td>
<td>FA-F</td>
<td>gcd-F</td>
</tr>
<tr>
<td>(pqqA-pqqB)</td>
<td>AB-F</td>
<td>gdd-R</td>
</tr>
<tr>
<td>(pqqB-pqqC)</td>
<td>BC-F</td>
<td>(pqq)F</td>
</tr>
<tr>
<td>(pqqC-pqqD)</td>
<td>CD-F</td>
<td>(pqq)R</td>
</tr>
<tr>
<td>(pqqD-pqqE)</td>
<td>DE-F</td>
<td>(pqq)G</td>
</tr>
<tr>
<td>(pqqE-pqqG)</td>
<td>EG-F</td>
<td>(pqq)E</td>
</tr>
<tr>
<td>(pqqG)</td>
<td>EG-R</td>
<td>(pqq)G</td>
</tr>
<tr>
<td>(pqqA)</td>
<td>FA-F</td>
<td>(pqq)A</td>
</tr>
<tr>
<td>(pqqB)</td>
<td>AB-F</td>
<td>(pqq)B</td>
</tr>
<tr>
<td>(pqqC)</td>
<td>BC-F</td>
<td>(pqq)C</td>
</tr>
<tr>
<td>(pqqD)</td>
<td>CD-F</td>
<td>(pqq)D</td>
</tr>
<tr>
<td>(pqqE)</td>
<td>DE-F</td>
<td>(pqq)E</td>
</tr>
<tr>
<td>(pqqG)</td>
<td>EG-F</td>
<td>(pqq)G</td>
</tr>
</tbody>
</table>
The 16S rRNA gene was used as the reference gene, and the bands for the intergenic regions between pqqF and pqqA (designated FA), pqqA and pqqB (AB), pqqB and pqqC (BC), pqqC and pqqD (CD), pqqD and pqqE (DE), and pqqE and pqqG (EG) were normalized to the reference gene band from the same growth condition (Fig. 1A). The following controls were included for each PCR: (i) a positive control with genomic DNA as the template, (ii) a negative control with the RT(-) reaction as the template, and (iii) a no-template control (NTC) without any nucleic acid added as the template.

**Bioinformatic analysis of the putative pqq operon in P. putida KT2440.** The DNA sequence and the gene arrangement of the putative pqq gene cluster in P. putida KT2440 were obtained from the Pseudomonas Genome Database (29). The seven genes potentially involved in PQQ biosynthesis are in the region from bp 454815 to 462463, annotated as pqqA, pqqB, pqqC, pqqD, pqqE, and pqqG (PP_0375). In this study, we refer to PP_0375 as pqqG in P. putida KT2440 because the gene locus and sequence in this strain are highly similar to those of the pqqG gene in *P. fluorescens* F113 (8). Figure 1A shows a schematic of the putative PQQ biosynthesis genes in *P. putida* KT2440. An additional predicted paralog of the pqqD gene (PP_2681) is found distal to the above-mentioned genes and is referred to here as pqqD2 (not shown in the schematic) (21). The presence of three promoters, which are upstream of pqqF, pqqA, and pqqC, and two rho-independent terminators between pqqA and pqqB, as well as between pqqB and pqqC, was predicted using the promoter prediction tool Virtual Footprint (30) and the terminator prediction tool Web-GeSTer (31). The structures of the putative promoters and terminators are described in Fig. S2 and S3 in the supplemental material.

**Assessment of the effect of soluble phosphate on GDH enzyme activity and PQQ production.** To determine whether GDH activity and PQQ production were affected by the presence of insoluble phosphate and/or the levels of soluble phosphate, additional experimentation was done using NBRIP growth medium. A single colony of *P. putida* KT2440 was inoculated and grown in 500 ml LB broth and shaken (220 rpm) overnight at 28°C. Cells were harvested by centrifuging the bacterial culture at 4,000 rpm for 15 min, washed twice with sterile normal saline (0.85% NaCl), and resuspended in 30 μl of the same saline. This starter culture was inoculated in 250-ml flasks containing 50 ml of NBRIP medium without any soluble phosphate (no phosphate) or amended with K2HPO4 as a soluble phosphate source at two concentrations: 1 mM (low phosphate) and 50 mM (high phosphate). The cell density at the time of inoculation for each flask reached at least 5 x 10^6 cells ml^-1 to ensure that sufficient cells would be available for membrane extraction. Medium without bacterial inoculation served as a negative control. Each culture was performed in quadruplicate and grown at 28°C with shaking, and pH and OD_{600} were monitored every 4 h. Culture supernatants were collected at the same time for a PQQ bioassay by cen-
trifugation at 20,878 × g for 30 min, followed by sterilization through a 0.22-μm filter. GDH enzyme assays, PQQ bioassays, RNA isolation, and qPCR determination of gene expression levels were conducted as described above for each growth condition.

Statistical tests. Enzyme kinetic analysis, GDH specific enzyme activity, PQQ production, and gene expression levels are presented as a mean value for three replicates. One-way analysis of variance (ANOVA) was performed, and multiple comparisons were made by Dunnett’s tests at a significance level of 0.05.

RESULTS

Kinetic analysis of GDH enzyme activity. The apparent \( K_m \) and \( V_{\text{max}} \) values for GDH enzymes from both \( P. putida \) KT2440 and \( E. coli \) DH5α were measured from their respective membrane fractions (Table 2). Both GDH isoforms showed apparent \( K_m \) values in the low millimolar range for glucose and in the low micromolar to submicromolar range for PQQ. The apparent \( K_m \) for PQQ of the \( P. putida \) KT2440 GDH was too low to accurately measure using this assay. It is likely that the binding between this GDH enzyme and PQQ is strong enough to tolerate the extraction process of the membrane fraction without notable separation, as significant GDH enzyme activity was detected when no exogenous PQQ was added to the \( P. putida \) KT2440 membrane fraction. \( V_{\text{max}}/K_m \) values for glucose indicated a very similar overall enzyme efficiency between GDH enzymes of \( E. coli \) DH5α and \( P. putida \) KT2440. The kinetic values determined here were used to verify that concentrations of both PQQ and glucose were not limiting in the specific activity assays described below.

GDH enzyme activity of \( P. putida \) KT2440 grown on different carbon sources. GDH specific activity assays were conducted to determine which growth conditions enabled the highest enzyme activity. Exogenous PQQ (10 μM) was added to ensure that PQQ was not limiting for specific activity assays. The GDH activity of cells grown in glucose was significantly higher than that in other conditions (\( P < 0.05 \)) (Table 3). No significant difference in specific enzyme activity was observed between the cells grown in LB medium and those grown in M9 minimal medium, with the exception of those with glucose as the sole carbon source, and the GDH activity in glucose was 1.2- and 1.4-fold higher than that in glycerol and citrate, respectively. Incidentally, the GDH activities of membrane fractions not supplemented with exogenous PQQ showed a trend similar to those supplemented with PQQ (data not shown), consistent with our suggestion that a certain amount of PQQ remains with the GDH enzyme during membrane preparation.

PQQ production of \( P. putida \) KT2440 grown on different carbon sources. The PQQ standard curve established that the relationship between enzyme activity and PQQ was linear up to approximately 1 μM (see Fig. S1 in the supplemental material). It should be noted here that this assay only provides measurements of PQQ exuded from the cell and does not account for PQQ that remains in the cells. PQQ concentrations under each growth condition fell within the detectable limit and varied significantly with the growth conditions (\( P < 0.05 \)) (Table 3). The PQQ levels in all minimal medium growth conditions were considerably higher than the PQQ levels in LB medium, and carbon sources in M9 minimal medium had a prominent impact on PQQ production, as the PQQ concentration in glucose was 1.4- and 3.8-fold higher than that in glycerol and citrate, respectively.

Evaluation of phosphate-solubilizing efficiency. The initial soluble inorganic phosphate concentration in NBRIP medium supplemented with glucose was around 5.0 mg liter\(^{-1}\) and dramatically increased with the \( P. putida \) KT2440 growth time, reaching 419.0 mg liter\(^{-1}\) after 144 h (Fig. 2). In the absence of added PQQ, the highest phosphate-solubilizing rate, 4.4 mg liter\(^{-1}\) h\(^{-1}\), was observed in the second 24 h of growth, at which time the cells were in the late exponential phase. The addition of exogenous PQQ (10 μM) had a positive impact on the phosphate-solubilizing efficiency of \( P. putida \) KT2440 in glucose. The positive effect was significant (\( P < 0.01 \)) and most noteworthy in the second 24 h of growth, at which time the rate of phosphate solubilization increased from 4.4 mg liter\(^{-1}\) h\(^{-1}\) to 5.4 mg liter\(^{-1}\) h\(^{-1}\) in the presence of exogenous PQQ. However, no significant difference in cell density was observed between the growth conditions with or without PQQ added during the same period (Fig. 2, inset). The pH decline with exogenous PQQ was significantly faster than that without PQQ, particularly in the first 24 h of growth (\( P < 0.05 \)). Parallel experiments were not conducted with the other carbon sources because either the level of soluble phosphorus was already high in that medium (i.e., LB) or the carbon source in minimal medium could solubilize insoluble phosphorus under the assay conditions (i.e., citrate).

Expression levels of the \( gcd \) and \( pqq \) genes of \( P. putida \) KT2440 grown on different carbon sources. The expression level of the \( gcd \) gene, encoding GDH, varied significantly with the carbon source (\( P < 0.05 \)) (Fig. 3), showing the trend of expression in glucose > glycerol > LB > citrate, which was consistent with the GDH enzyme specific activity. Overall, the expression levels of the \( pqqF \), \( pqqA \), and \( pqqB \) genes were highest in glucose (although \( pqqA \) is not significant), while others were highest in LB medium. Expression of \( pqqF \), the first gene of the \( pqq \) gene cluster, was

### TABLE 2 Kinetic analysis of \( E. coli \) DH5α and \( P. putida \) KT2440 glucose dehydrogenase

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Bacterial strain</th>
<th>( K_m ) (μM)</th>
<th>( V_{\text{max}}/K_m ) (min(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>( E. coli ) DH5α</td>
<td>2.71 ± 0.14 mM</td>
<td>42.14 ± 0.98</td>
</tr>
<tr>
<td></td>
<td>( P. putida ) KT2440</td>
<td>4.91 ± 0.83 mM</td>
<td>67.64 ± 1.92</td>
</tr>
</tbody>
</table>

### TABLE 3 GDH enzyme activity and PQQ production of \( P. putida \) KT2440 grown on different carbon sources

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>GDH activity (U/mg of protein)</th>
<th>PQQ production (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LB medium</td>
<td>857.58 ± 63.85 AB</td>
<td>0.083 ± 0.012 A</td>
</tr>
<tr>
<td>Glucose</td>
<td>1,100.00 ± 15.75 C</td>
<td>0.532 ± 0.017 B</td>
</tr>
<tr>
<td>Glycerol</td>
<td>890.91 ± 18.18 B</td>
<td>0.385 ± 0.012 C</td>
</tr>
<tr>
<td>Citrate</td>
<td>787.88 ± 54.80 A</td>
<td>0.140 ± 0.012 D</td>
</tr>
</tbody>
</table>

*Data are the average result of three replicates ± standard deviation.
approximately 2-fold higher in glucose than in other growth conditions ($P < 0.05$), yet no significant difference was observed between LB medium and glycerol or citrate. Interestingly, LB medium and glucose conditions exhibited very similar $pqqA$ gene expression levels, and both were significantly higher than those exhibited in glycerol and citrate conditions ($P < 0.05$). The expression level of the $pqqB$ gene was significantly higher in minimal medium than in LB medium ($P < 0.05$). In the minimal medium growth conditions, the $pqqB$ gene expression level was highest in glucose and lowest in citrate, showing the same pattern as the PQQ production observed under these conditions. The $pqqC$, $pqqD$, $pqqE$, and $pqqG$ genes all showed the highest expression in LB medium, with lower expression in minimal medium conditions, and showed no obvious relatedness to the expression of other $pqq$ genes. Overall, the gene expression data indicate that changes in expression of the $pqqF$, $pqqA$, and $pqqB$ genes correlated with the changes in PQQ levels measured above. Further, the differences in relative abundance suggest that the genes are likely regulated differently at the transcription level.

**Effect of soluble phosphate availability on GDH enzyme activity and PQQ production of* P. putida KT2440.** GDH specific activity and PQQ production of cells grown in NBRIP medium with glucose as the sole carbon source. Data are the average result of three replicates with standard deviations. Control conditions (ctrl) are uninoculated cultures. The inset plot shows cell density (OD$_{600}$) of the same cultures with growth time. An asterisk indicates a significant difference in inorganic phosphate concentration between conditions with and without PQQ addition ($P < 0.05$); a dagger indicates a significant difference in pH between conditions with and without PQQ addition ($P < 0.05$).

**FIG 2** Monitoring inorganic phosphate (PO$_4^{3-}$) concentration (mg/liter), pH, and cell density of *P. putida* KT2440 cultured in NBRIP medium with glucose as the sole carbon source. Data are the average result of three replicates with standard deviations. Control conditions (ctrl) are uninoculated cultures. The inset plot shows cell density (OD$_{600}$) of the same cultures with growth time. An asterisk indicates a significant difference in inorganic phosphate concentration between conditions with and without PQQ addition ($P < 0.05$); a dagger indicates a significant difference in pH between conditions with and without PQQ addition ($P < 0.05$).

**FIG 3** Expression levels of the $gcd$ and $pqq$ gene cluster of *P. putida* KT2440 grown in LB medium or M9 minimal medium with glucose, glycerol, or citrate as the sole carbon source. Data are the average result of three replicates with standard deviations. Different letters above the columns indicate a significant difference between the expression levels of a gene under different growth conditions ($P < 0.05$). Fold change is set relative to the LB medium growth condition for each gene.
amended with 50 mM K₂HPO₄ (high phosphate) were comparable to those of M9 medium with glucose as the carbon source (Tables 3 and 4). Both GDH specific activity and PQQ levels were significantly induced in NBRIP medium with 0 and 1 mM (low) soluble phosphate, with the highest observed with no soluble phosphate added (P < 0.05). The expression levels of gcd and the pqq genes exhibited the same pattern as the GDH enzyme activity and PQQ production under these conditions: they were significantly increased by zero and low soluble phosphate in NBRIP and PQQ production under these conditions: they were significantly increased by zero and low soluble phosphate in NBRIP medium compared to those under the high-phosphate conditions (Fig. 4). The gcd and pqq gene expression levels were approximately 1.5- to 3-fold higher in the zero-soluble-phosphate condition than in high-soluble-phosphate condition.

**Analysis of pqq operon structure.** A semiquantitative RT-PCR was used to determine whether adjacent genes from the predicted pqq operon were part of the same transcript and if the relative abundance of these transcripts changed according to growth conditions. The PCR primers used for this are listed in Table 1, and their approximate locations are indicated in Fig. 1A. A strong PCR band observed from amplification using a particular primer set from cDNA would indicate that the two genes exist, at least partially, on the same transcript. If a band cannot be detected, the same cannot be said of these two genes, although this does not prove that they are not on the same transcript. Figure 1B shows the bands observed in their respective agarose gels, and ImageJ quantification of the bands is given in Table 5. RT(−) controls and no-template controls (NTC) excluded the possibility of genomic DNA contamination and primer contamination, respectively. Bands were normalized to the 16S rRNA gene band from the same growth condition. No PCR band was observed for the FA region (the intergenic region between the pqqF and pqqA genes) under any of these conditions, suggesting that the pqqF gene is transcribed independently, at least under the conditions in our study. In contrast, CD, DE, and EG bands were observed under all conditions, with the highest expression in LB medium and the lowest in glycerol, indicating that the pqqC-pqqD-pqqE-pqqG region exists on one transcript. In contrast, there is some variation in the AB and BC regions. Bands of differing intensities can be seen for the AB region, with the most intense band in the glucose condition and no apparent band in the glycerol condition. Similarly, a very faint band is noted in the BC region in the LB medium and glucose conditions but not in the remaining conditions. Taken together, the data suggest an independent promoter and terminator for pqqF and at least one additional promoter driving the expression of the remaining genes. The fact that AB bands and/or BC bands are not uniformly found but the CDEG region is consistently transcribed suggests the presence of a terminator or terminators downstream of pqqA and/or pqqB as well as a promoter upstream of pqqC. A BC band is not observed without also seeing an AB band, suggesting that there is not a separate promoter between the pqqA and pqqB genes.

**DISCUSSION**

Kinetic analysis of GDH from *E. coli* DH5α and *P. putida* KT2440 revealed apparent *Kₘ* values with glucose (2.7 mM and 4.9 mM, respectively) that were comparable to those from other related organisms. Purified GDH enzymes from other Gram-negative bacteria (including *E. coli*, *Enterobacter asburiae*, *Erwinia* sp. 34-1, *Acinetobacter calcoaceticus*) gave *Kₘ* values with glucose in the range of 1.1 mM to 4.0 mM (32, 33). While less information is available about *Kₘ* values with PQQ for quinoproteins in general, the measured value of 1.1 μM for PQQ with GDH from *E. coli* DH5α falls within the range of values measured with other *E. coli* strains, including gcd mutants, at 0.05 to 21 μM (34–36), and is in

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>GDH sp act (U/mg of protein)</th>
<th>PQQ production (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No P</td>
<td>1,809.01 ± 7.42 A</td>
<td>0.861 ± 0.007 A</td>
</tr>
<tr>
<td>Low P</td>
<td>1,569.70 ± 22.68 B</td>
<td>0.633 ± 0.013 B</td>
</tr>
<tr>
<td>High P</td>
<td>1,375.76 ± 8.57 C</td>
<td>0.488 ± 0.014 C</td>
</tr>
</tbody>
</table>

*Data are the average result of three replicates ± standard deviation. Values given for each determination were calculated for cultures with an OD₆₀₀ of 0.5. Values followed by different letters are significantly different under the different phosphate conditions (P < 0.05).*

**FIG 4** Expression levels of the gcd gene and pqq gene cluster of *P. putida* KT2440 grown in NBRIP medium without any soluble phosphate source (No P) or amended with K₂HPO₄ as a soluble phosphate source at two concentrations, 1 mM (Low P) and 50 mM (High P). Data are the average result of three replicates with standard deviations. Different letters above the columns indicate a significant difference between the expression levels of a gene under different phosphate levels. Fold change is set relative to the no-phosphate growth condition for each gene.
line with $K_{m}$ values for the quinoprotein alcohol dehydrogenase from *Glucobacter suboxydans* IFO 12528 at 11 μM (37). The high affinity for PQQ of the *P. putida* KT2440 GDH prevented an accurate measurement of the apparent $K_{m}$ as the membrane fractionation process did not completely release PQQ from the GDH enzyme. With an estimate of an apparent arrangement process did not completely release PQQ from the GDH enzyme activity was induced when the strain had glucose catabolism and may be specifically repressed under other conditions in which the substrate is readily available (Table 4; Fig. 4), which is consistent with work with *P. putida* KT2440 is grown in glucose, yet the rate of phosphate solubilization is significantly increased when exogenous PQQ is added to the culture. This implies that the level of PQQ available to GDH is limiting the enzyme activity and therefore limiting phosphate solubilization under conditions that may be considered optimal for GDH activity (i.e., when glucose is the carbon source, with low levels of soluble phosphorus). Earlier studies that found that increasing the copy number of certain *P. putida* KT2440 GDH prevented an accordingly increased GDH activity, with lower levels of soluble phosphate showing that low soluble phosphate significantly increased and could therefore easily serve to enhance or limit the amount of carbon source, with low levels of soluble phosphorus). Earlier studies found that increasing the copy number of certain PQQ biosynthetic genes in *Pseudomonas fluorescens* F113, *Burkholderia cepacia*, and a *Pseudomonas* sp. increases the gluconic acid production and mineral phosphate solubilization further support the idea that the level of PQQ limits GDH activity (8, 38). Moreover, our data indicate that PQQ levels fluctuate according to carbon source (Table 3), indicating that PQQ synthesis is not constitutive and is activated in the presence of glucose in comparison with other carbon sources. Our studies on GDH activity and PQQ production according to carbon source were done in M9 minimal medium, which ensures that soluble phosphate is not limiting. Additional experimentation in NBRIP medium with various levels of soluble phosphate showed that low soluble phosphate significantly induced GDH enzyme activity and PQQ production (Table 4; Fig. 4), which is consistent with work with *Pantoea eu- calypsi* where GDH enzyme activity was induced when the strain was grown without K$_2$HPO$_4$ in comparison to that with 50 mM K$_2$HPO$_4$ (3).

In addition to PQQ levels, phosphorus solubilization can be limited by the level of active GDH enzyme. GDH specific activity of *P. putida* KT2440 varies significantly with growth condition, and the variations are consistent with the levels of *gcd* gene expression (Tables 3 and 4; Fig. 3 and 4), indicating that *gcd* is not constitutively expressed and that active GDH protein is most abundant under conditions in which the substrate is readily available and when soluble phosphate is low. Further studies will be necessary to establish the mechanism by which this occurs, but carbon catabolite repression may be at play here, as GDH is involved in glucose catabolism and may be specifically repressed under other conditions. Variation in the enzyme activity of PQQ-saturated (100 μM) GDH according to environmental conditions has been noted in other bacteria, such as *Sinorhizobium meliloti* RCR2011, specifically under phosphate-limiting conditions (20).

The PQQ coenzyme is synthesized exclusively in microbes, yet the precise mechanism is not fully understood (15, 39). The genes required for its synthesis comprise a combination of the following: *pqqA*, *pqqB*, *pqqC*, *pqqD*, *pqqE*, and *pqqG*. Not all of these genes are present in all PQQ-producing organisms, and their arrangement varies considerably (9, 11, 15). PqqA is a small, ribosomally produced peptide (23 or 24 amino acids) that serves as the precursor of the PQQ molecule, which is synthesized from conserved tyrosine and glutamate residues within the peptide (40). The remaining genes in the *pqq* operon are predicted to carry out functions such as hydroxylation of the PqqA Tyr residue (*pqqB*), enzymatically linking the Tyr and Glu residues (*pqqF*), excising the cross-linked dipeptide (*pqqF*), and cyclizing and oxidizing the dipeptide (*pqqC*) (11, 15, 39). Functions for the remaining genes have not been delineated, although bioinformatic analysis offers some clues as to what they may do. For example, *pqqG* (PP_0375) in *P. putida* KT2440 is uncharacterized but predicted to encode prolyl oligopeptidase (41), and orthologs are referred to as *pqqG* in *P. fluorescens* F113, *pqqM* in *Pseudomonas protegens* Pf-5 and *P. fluorescens* B16, and *pqqH* in *Pseudomonas aeruginosa* PA01 (8–10, 29).

Considering the levels of PQQ produced versus *pqq* gene expression, the expression patterns of *pqqF* and *pqqB* most closely mirror the levels of PQQ produced under their respective growth conditions, meaning that *pqqF* and *pqqB* are expressed highest under conditions in which the *pqq* levels are highest. This is consistent with the result that *Klebsiella pneumonia* mutants lacking the PqqB or PqqF protein synthesize only small amounts of PQQ compared to that produced by the wild type (42). Whether either of the putative reactions catalyzed by PqqB or PqqF (tyrosine hydroxylation or Tyr-Glu excision) is rate limiting in PQQ biosynthesis remains to be seen, but the RT-PCR results offer suggestions as to how gene expression may be enhanced under certain growth conditions. The *pqqF* gene appears to be under the control of an independent promoter and terminator, and no evidence exists to suggest that it is coexpressed with any other *pqq* genes under these conditions. As such, the levels of active PqqF can theoretically be altered without regard for the remaining genes and could therefore easily serve to enhance or limit the amount of PQQ available. The *pqqC-pqqD-pqqE-pqqG* region appears to be entirely on one transcript, making it unlikely that any of these genes independently limits PQQ synthesis. Collectively, expression of these genes could limit PQQ synthesis, but each of these

<table>
<thead>
<tr>
<th>Growth condition</th>
<th>BI</th>
<th>RI</th>
<th>BI</th>
<th>RI</th>
<th>BI</th>
<th>RI</th>
<th>BI</th>
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<tbody>
<tr>
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<td>3,545.64</td>
<td>1.00</td>
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<td>1.00</td>
<td>4,618.40</td>
<td>1.00</td>
<td>5,853.08</td>
<td>1.00</td>
<td>5,321.52</td>
<td>1.00</td>
<td>11,781.90</td>
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<tr>
<td>Glucose</td>
<td>0.00</td>
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<td>3,257.93</td>
<td>0.90</td>
<td>3,527.74</td>
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<td>2,933.79</td>
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<td>1,057.36</td>
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<tr>
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<td>773.11</td>
<td>0.16</td>
<td>535.28</td>
<td>0.09</td>
<td>1,953.48</td>
<td>0.35</td>
<td>12,237.37</td>
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<td>1,217.55</td>
<td>0.35</td>
<td>0.00</td>
<td>0.00</td>
<td>1,577.18</td>
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<td>1,044.11</td>
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<td>11,494.00</td>
<td>0.98</td>
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</table>

*Indicates that *pqqF* and *pqqA*, *pqqA* and *pqqB*, *pqqB* and *pqqC*, *pqqC* and *pqqD*, *pqqD* and *pqqE*, and *pqqE* and *pqqG* are represented as FA, AB, BC, CD, DE, and EG, respectively. Relative intensity (RI) is calculated from the normalizing band intensity (BI) of the RT-PCR (target) to the PCR of the 16S rRNA gene (reference), with the LB medium growth condition serving as the control treatment.
genes is expressed at its highest level under the growth conditions (LB medium) that gave the lowest levels of PQQ.

While the PqqA peptide is the molecule from which PQQ is ultimately derived, variations in its expression are minor compared to those in the expression of both pqqF and pqqB and it seems unlikely that its expression is the limiting factor in PQQ synthesis under these conditions. Nonetheless, each of the three minimal medium conditions showed the expression pattern of glucose > glycerol > citrate for both pqqA and pqqB, suggesting that the promoter driving the expression of a transcript harboring both PqqA and PqqB may be responsive to glucose. Under LB medium growth conditions, pqqA expression is high but pqqB expression is low relative to their expression under the other growth conditions. Bioinformatic analysis of the putative pqq gene cluster (Fig. 1A; see Fig. S2 and S3 in the supplemental material) predicts the presence of three promoters, which are upstream of pqqF, pqqA, and pqqC, which is consistent with our RT-PCR data. Rho-independent terminators are predicted between pqqA and pqqB, as well as between pqqB and pqqC, which is also consistent with our RT-PCR data. Taken together with RT-PCR data showing various levels of AB- and BC-containing transcripts, these findings suggest that intrinsic termination may occur under certain conditions to terminate transcription from the Pₐ promoter. Our experimental evidence also predicts a terminator between pqqF and pqqA, yet no obvious evidence is found for intrinsic termination, which suggests a rho-dependent termination event.

Other closely related pseudomonads have different operon structures (8), with one notable difference among PQQ synthetizers being the presence and location of the pqqF gene. In some instances (e.g., Acinetobacter calcoaceticus and Gluconobacter oxydans ATCC 9937), the genome encodes no obvious pqqF homolog (11, 14, 43), and in other instances (e.g., Methylobacterium extorquens AM1 and P. aeruginosa PA01), the pqqF gene is located distal to the remainder of the genes (10, 13). Gene knockout studies in K. pneumoniae have suggested that the pqqF gene is not essential in PQQ biosynthesis in some organisms, with the expectation that other peptides may fulfill this role (42). The fact that this gene typically exists either distal from the remaining pqq genes or, as is the case here, on a separate transcript, suggests that this gene has undergone evolution independent of the remaining genes (11, 30). The organization of the pqq gene cluster (pqqFABCDEG) in P. putida KT2440 is identical to that seen in several Pseudomonas fluorescens strains, such as Pf0-1, F113, and B16, as well as Pseudomonas protegens PF-5 (8, 9, 44, 45). It is perhaps noteworthy that, among orthologous pqq genes from P. putida KT2440 and P. protegens PF-5, the pqqF homologs show by far the lowest percent identity despite the conservation of genomic synteny: pqqF, 42%; pqqA, 96%; pqqB, 98%; pqqC, 96%; pqqD, 86%; pqqE, 86%; and pqqG, 67%.

Previous studies demonstrate noncoordinated synthesis between GDH and PQQ in Acinetobacter and Pseudomonas species, yet little is known about the regulation of PQQ biosynthesis and its role in phosphorus solubilization via GDH activity (22). Our results show that PQQ limits the phosphate solubilization rate under optimal conditions and that PQQ levels varied significantly according to growth condition. Gene expression analysis under optimal PQQ production conditions suggest that PQQ levels appear to be most affected by the levels of pqqF and pqqB expression. While the structure of the pqq gene cluster in P. putida KT2440 offered some information as to how this regulation is achieved, future work will be required to further address the rate-limiting biochemical step in PQQ biosynthesis.

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