Disruption of Gene pqqA or pqqB Reduces Plant Growth Promotion Activity and Biocontrol of Crown Gall Disease by Rahnella aquatilis HX2

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Abstract

Rahnella aquatilis strain HX2 has the ability to promote maize growth and suppress sunflower crown gall disease caused by Agrobacterium vitis, A. tumefaciens, and A. rhizogenes. Pyrroloquinoline quinone (PQQ), a cofactor of aldose and alcohol dehydrogenases, is required for the synthesis of an antibacterial substance, gluconic acid, by HX2. Mutants of HX2 unable to produce PQQ were obtained by in-frame deletion of either the pqqA or pqqB gene. In this study, we report the independent functions of pqqA and pqqB genes in relation to PQQ synthesis. Interestingly, both the pqqA and pqqB mutants of R. aquatilis eliminated the ability of strain HX2 to produce antibacterial substance, which in turn, reduced the effectiveness of the strain for biological control of sunflower crown gall disease. The mutation also resulted in decreased mineral phosphate solubilization by HX2, which reduced the efficacy of this strain as a biological fertilizer. These functions were restored by complementation with the wild-type pqq gene cluster. Additionally, the phenotypes of HX2 derivatives, including colony morphology, growth dynamic, and pH change of culture medium were impacted to different extents. Our findings suggested that pqqA and pqqB genes individually play important functions in PQQ biosynthesis and are required for antibacterial activity and phosphorous solubilization. These traits are essential for R. aquatilis efficacy as a biological control and plant growth promoting strain. This study enhances our fundamental understanding of the biosynthesis of an environmentally significant cofactor produced by a promising biocontrol and biological fertilizer strain.
Introduction

The gram-negative bacterium *Rahnella aquatilis* is widely ubiquitous, thriving in soil, water, marshes, and on food, seeds and plant roots. Strains of *R. aquatilis* fix nitrogen in the rhizosphere, solubilize mineral phosphate, and have biocontrol capabilities \[1, 2, 3\]. Specifically, *R. aquatilis* HX2 has been shown to suppress sunflower crown gall disease caused by *Agrobacterium vitis*, *A. tumefaciens*, and *A. rhizogenes* \[4\]. Biocontrol activity by *R. aquatilis* is nonspecific. This species has demonstrated suppression of diseases caused by *Xanthomonas campestris*, *X. axonopodis*, *Penicillium expansum*, *Botrytis cinerea*, and *Erwinia amylovora* \[5, 6, 7\]. Competitive colonization of varied environments and the ability of HX2 to establish several beneficial interactions with plants make it an ideal candidate for a soil inoculant. A mechanistic understanding of plant disease suppression and mineral phosphate solubilization by a *R. aquatilis* was provided in previous research, which linked these activities to the glucose dehydrogenase cofactor, pyrroloquinoline quinone (PQQ) \[8, 9\].

In gram-negative bacteria, PQQ mainly functions as a non-covalently bound, redox cofactor of several membrane-associated sugar and alcohol dehydrogenases, including methanol dehydrogenase, ethanol dehydrogenase, and glucose dehydrogenase (GDH) \[10\]. Previous reports indicate that the GDH-PQQ holoenzyme is involved in the production of an antimicrobial substance by several genera of bacteria including *Rahnella* and notably, strain HX2 \[9, 11, 12\]. Organic acids (OA) such as gluconic acid (GA) are considered to be a main factor responsible for dissolution of insoluble phosphate through organometallic complex formation or through metal chelation processes \[13, 14\]. Bacterial mineral phosphate solubilization (MPS) activity most commonly occurs when bacteria produce and release OAs \[14, 15, 16\]. Furthermore, PQQ is a plant growth promotion factor, which in addition to GA production, has also been related to its antioxidant properties as well as unknown mechanisms \[17\].

Bacterial genes involved in PQQ biosynthesis have been identified in numerous species isolated from varying environments and are clustered in pqqABCDEF operons \[9, 18, 19, 20\]. The pqqA gene encodes a small peptide that contains tyrosine and glutamate and serves as the precursor and rate-determining step for PQQ biosynthesis \[21\]. This molecule remains attached to a precursor peptide and is cleaved off at a later step by other enzymes of the biosynthesis pathway. Often, pqqB is not directly required for PQQ biosynthesis. Its suggested role in *K. pneumoniae* is a carrier that facilitates the secretion of PQQ across the plasma-membrane into the periplasm \[22\]. Little information is available on PQQ biosynthesis in the *Rahnella* genus. Kim et al. \[8\] mobilized a cosmid library of *R. aquatilis* into *Escherichia coli* HB101 to isolate and clone the genes that confer the MPS trait from *R. aquatilis*. Consequently, it was revealed that the MPS locus of *R.
*R. aquatilis* contains the *pqq*D and *pqq*E genes. Mutants of *R. aquatilis* HX2 showed that a lack of antibacterial activity was due to a Tn5 insertion in the *pqq*E gene, which prevented synthesis of the PQQ [9]. The aim of our study is to investigate the individual roles of *pqq*A and *pqq*B genes in *R. aquatilis* HX2 PQQ biosynthesis. The relevance of these genes to growth and beneficial activities of HX2 are assessed with respect to synthesis of OAs, antibacterial activity, mineral phosphate solubilization, biological control of crown gall disease, and plant growth promotion.

**Materials and Methods**

**Bacterial Strains, Plasmids, and Culture Conditions**

The bacterial strains, plasmids, and primers used in this study are listed in Table 1. *R. aquatilis* strains were cultured at 28°C on potato dextrose agar (PDA) medium or with shaking (170 rpm) in potato dextrose broth (PDB) [3, 9]. The *E. coli* strains DH5α [23] and DH5α (λ-pir) were grown at 37°C on Luria-Bertani (LB) medium. To test PQQ production, cultures were grown in AB minimal medium (containing, per liter: K₂HPO₄, 3 g; NaH₂PO₄, 1 g; NH₄Cl, 1 g; MgSO₄.7H₂O, 0.3 g; KCl, 0.15 g; CaCl₂, 0.01 g; FeSO₄.7H₂O, 2.5 mg; glucose, 0.5%) [24]. *Agrobacterium vitis* strain K308 [25] was grown either on yeast extract broth (YEB) or yeast extract agar (YEA) at 28°C [26]. When required, media supporting the growth of *R. aquatilis* and *E. coli* were supplemented with filter-sterilized antibiotics (kanamycin, 50 μg ml⁻¹; ampicillin, 50 μg ml⁻¹), isopropyl-β-D-thiogalactopyranoside (IPTG) at 1 mM, and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 40 μg ml⁻¹. All of the studies involving *R. aquatilis* HX2 inoculation were carried out in a closed and protected greenhouse at the China Agricultural University. This study did not involve endangered or protected species.

**General Genetic Techniques**

Isolation of genomic DNA from strain HX2 and plasmid DNA from *E. coli* were performed according to standard procedures [27]. Restriction enzyme digestions were performed as recommended by the suppliers (TaKaRa, Japan) and ligations were carried out using T4 DNA ligase (TaKaRa, Japan). Gel electrophoresis was performed in 0.8–1.0% agarose gels. For cloning purposes, Ex® Taq DNA polymerase (TaKaRa, Japan) was used to PCR amplify inserts and Taq DNA polymerase (TaKaRa, Japan) was used for PCR amplification in test reactions (e.g., colony PCR). DNA sequencing was performed by Invitrogen Life Technologies (Beijing, China) and analyzed by using the National Center for Biotechnology Information BLAST server (http://www.ncbi.nlm.nih.gov/BLAST). The partial genome sequence of *R. aquatilis* HX2 (accession number CP003403-6) was used for primer design [28]. Primers used in this study are listed in Table 1.
Construction of \( pqqA \) and \( pqqB \) In-frame Deletion Mutants

In-frame nonpolar deletions of \( pqqA \) and \( pqqB \) were constructed utilizing a two-step homologous recombination strategy as described previously \cite{29}. Primers
were designed based on sequences upstream and downstream of either \textit{pqqA} or \textit{pqqB} and were used to PCR amplify fragments from the genome of HX2 (Table 1). Briefly, primers \textit{\Delta pqqA-L1} and \textit{\Delta pqqA-L2} with sites for \textit{SalI} and \textit{BamHI}, were used to amplify a 1132 bp region upstream of the \textit{pqqA} open reading frame (ORF). A 1553 bp fragment, created by primers \textit{\Delta pqqA-R1} and \textit{\Delta pqqA-R2} containing \textit{BamHI} and \textit{EcoRI} sites, is downstream of the \textit{pqqA} ORF (Table 1). The standard PCR reactions involved 15 min at 94°C, then 35 cycles of 45 s at 94°C, 40 s at 66°C and 1 min at 72°C, and final extension at 72°C for 10 min. Similarly fragments flanking \textit{pqqB} were amplified. These included a 982 bp upstream fragment that included the first 193 codons of the \textit{pqqB} ORF, generated from primers \textit{\Delta pqqB-L1} and \textit{\Delta pqqB-L2} with sites \textit{EcoRI} and \textit{BamHI}, and a 958 bp downstream fragment including the last 171 codons of the \textit{pqqB} ORF from primers \textit{\Delta pqqB-R1} and \textit{\Delta pqqB-R2} with \textit{BamHI} and \textit{SalI} sites. These PCR reactions were carried out at 94°C for 15 min, followed by 35 cycles of 94°C for 45 s, 71°C for 2 min, and a final extension at 72°C for 10 min. After being digested with appropriate restriction enzymes, the \textit{\Delta pqqA-L/R} and \textit{\Delta pqqB-L/R} fragments were ligated into pBSNot6 [30] creating pBSNot6\textit{\Delta pqqA} and pBSNot6\textit{\Delta pqqB}. Then, the approximately 2.7 kb Not I fragment from pBSNot6\textit{\Delta pqqA} and the approximately 2.0 kb Not I fragment from pBSNot6\textit{\Delta pqqB}, including a \textit{pqqA} gene with 32 bp deletion and a \textit{pqqB} gene with 564 bp deletion, were lifted and ligated into pSR47S [31] to obtain pSR47S\textit{\Delta pqqA} and pSR47S\textit{\Delta pqqB}. The two suicide plasmids were transformed into \textit{E. coli} DH5\textalpha (\textit{\lambda-pir}) by heat shock and were mobilized from DH5\textalpha (\textit{\lambda-pir}) into the wild-type \textit{R. aquatilis} strain HX2 by triparental mating with helper strain DH5\textalpha carrying plasmid pRK600 [32]. Exconjugants were selected on AB minimal agar plates containing kanamycin and second recombination events were selected according to the methods previously described [9]. The mutants of HX2\textalphaA and HX2\textalphaB were each screened from approximately 1500 first recombination clones for the absence of kanamycin resistance. A fragment 33 bp from the \textit{pqqA} 72 bp gene was deleted in mutant HX2\textalphaA and 564 bp of the 912 bp \textit{pqqB} gene was deleted in HX2\textalphaB. The disruption of each gene and the absence of the vector within the genome of these mutants were confirmed by PCR with primers \textit{\Delta pqqA-L1/\Delta pqqA-R2} and \textit{\Delta pqqB-L1/\Delta pqqB-R2} and sequencing analysis.

**Genetic Complementation of the \textit{pqqA} and \textit{pqqB} Mutants**

To complement the \textit{pqq} mutants an 8.0 kb \textit{BamHI} I fragment containing the entire \textit{pqqABCDEF} operon of HX2 was cloned into the broad host range vector pRK415G [33], resulting in the complementation plasmid pCH15 [9]. The plasmid pCH15 was mobilized into the HX2\textalphaA or HX2\textalphaB strain by triparental mating, and the complement strains CHX2\textalphaA and CHX2\textalphaB were created.
Antibiosis Test in Vitro and Biocontrol of Crown Gall Disease in Greenhouse

The antagonist HX2 and its derivative strains were tested for in vitro antibiosis against pathogenic strain A. vitis K308 via a modified Stonier’s method described by Chen et al [3]. Biocontrol activity assays were performed on sunflower (Helianthus annuus L.) stems with two true leaves grown in a greenhouse according to a previously described method [3]. Briefly, a suspension of pathogenic agrobacterial strain K308 (ca. 2 x 10^8 CFU ml^-1) was mixed with an equal volume of HX2 or its derivative strains suspension (ca. 2 x 10^8 CFU ml^-1). A 10 μl drop of this mixture was injected into a 1.0 cm longitudinal incision in sunflower stem. The inoculation site was wrapped with Parafilm. Gall formation was observed, and the gall was excised and weighed 15 days after inoculation. Sterile buffered saline (SBS, 0.85% NaCl) was applied as a negative control and K308 mixed with SBS served as a positive control. The effectiveness index (EI) was calculated using the following formula: EI (%) = [(C-T)/C] x 100, where C is the mean fresh weight of the crown gall tumor of the positive control group and T is the mean fresh weight of the crown gall tumor in the treated group. The assay was performed with 4 replicates and 10 plants were used per treatment.

Determination of Mineral Phosphate Solubilization and pH

HX2 and derivative strains were inoculated and shaken in PDB at 28°C, 170 rpm for 48 h. Ten microliters of bacteria culture were dropped on a sterile filter paper (diameter, 5 mm), placed in the middle of agar plates containing the differential medium, national botanical research institute’s phosphate (NBRIP). The NBRIP growth medium contains (per liter): glucose, 10 g; Ca_3(PO_4)_2, 5 g; MgCl_2.6H_2O, 5 g; MgSO_4.7H_2O, 0.25 g; KCl, 0.2 g; (NH_4)_2SO_4, 0.1g [34] with 18 g agar. The phosphate-solubilizing halo diameter was measured after the plates were incubated at 28°C for 7 days.

HX2 and derivative strains were cultured in liquid NBRIP at 28°C for 7 days to detect the soluble phosphorus (P) in the medium. Two-milliliters of bacterial supernatants were collected by centrifuge at 12,000 g for 5 min every other day. Soluble phosphate in the culture supernatants was detected using the Molybdenum-blue method [35]. Simultaneously, pH of the corresponding NBRIP medium was detected using a pH monitor (Mettler Toledo FE20, Shanghai, China). This assay was performed in triplicate with 3 replicates per treatment. Results from the third experiment are reported here.

Greenhouse Experiments for Plant Growth Promotion

Soil and coarse sand (0.35–0.5 mm) (1:1, w/w) were air-dried, passed through a sieve (2 mm), and sterilized by autoclaving at 121°C for 2 h before filling the pots. The soil texture is sandy loam (70.8% sand, 26.9% silt, and 2.3% clay). The soil type is a calcareous cambisol according to the FAO/UNESCO soil map of the world. Maize caryopses (Zea may L., Zhengdan 958, Henan Academy of Agricultural
Sciences, China) were surface sterilized with 70% ethanol for 30 sec, washed with sterilized distilled water for three times, and germinated in a sterilized Petri dish (270 mm) for 2 days. The bacteria strains were cultured with PDB at 28˚C for 2 days and diluted to OD$_{600}$ of 0.6 (ca. $2 \times 10^8$ CFU ml$^{-1}$). Maize seeds were coated with the diluted bacteria suspension or control (PDB) for 3 h before sowing. Coated maize caryopses were cultivated individually in pots (diameter 180 mm, length 160 mm) containing 750 g of soil ($P_2O_5$, 6.53 mg kg$^{-1}$; soil organic matter, 18.74 g kg$^{-1}$; pH, 7.49) amended with rock phosphate (1%, w/w), and 20 ml Hoagland’s nutrition liquid without phosphorus (containing per liter) $[Ca(NO_3)_2, 945$ mg; $KNO_3, 607$ mg; $MgSO_4, 493$ mg and 2.5 ml Ferrum salt solution (per liter) $FeSO_4 7H_2O, 5.56$ g; EDTA, 7.46 g, pH 5.5]). The pots were maintained in a greenhouse and irrigated with 50 ml sterile water every other day. After 42 days the plants were harvested and lengths and fresh weights of the plants were determined. The dry weights of shoots and roots were measured following drying at 65˚C for 48 hours. Total P of plants and soluble P of soils were detected according to the methods of Murphy and Riley [35]. The experiments were repeated 3 times and 20 plants were used in each treatment.

Detection of PQQ
The presence of PQQ in culture supernatants was determined as described previously [9]. In brief, bacterial strains were grown at 28˚C in AB minimal medium for 48 h, cell cultures were then mixed with methanol at a 1:9 ratio (v/v). Precipitated material was removed by centrifugation (12,000 g, 15 min) and the methanol was evaporated with rotary evaporator. The sample was acidified with HCl to pH 2.0 and loaded onto a Sep-Pak C$_{18}$ cartridge (Agilent Technologies, USA). The cartridge was washed with 20 ml of 2 mM HCl and then PQQ was eluted with 70% methanol. To identify the PQQ peak, 200 µl of the sample was mixed with 100 µl of 0.2 M Na$_2$B$_4$O$_7$ buffer, adjusted to pH 8.0 with HCl, and mixed with 90 µl 0.5% acetone, then incubated for 30 min at room temperature. Reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out using a Shimadzu LC-6A HPLC system with a fluorescence detector on ZORBAX SB-C$_{18}$ columns (4.6 × 250 mm, 5 µm; Agilent Technologies, USA) eluted with 27% methanol and 0.4% H$_3$PO$_4$ at a flow rate of 0.8 ml min$^{-1}$. The excitation and detection wavelengths of the fluorescence detector were set at 360 nm and 480 nm.

Identification of Organic Acid
Organic acids (OA) produced by bacterial strains were detected as described previously with some modifications [36]. HX2 and derivative strains were cultivated in NBRIP medium at 28˚C for 7 days. The culture was centrifuged at 12,000 g for 5 min and filtrated through a 0.22 µm filter (Pall Corporation, USA). Twenty microliters of filtrates were injected to high-performance liquid chromatography (HPLC) (Waters 2998, USA) equipped with ZORBAX SB-C$_{18}$
columns (4.6 × 250 mm, 5 μm; Agilent Technologies, USA). The chromatogram class \( (NH_4)_2HPO_4 \) (0.5%, w/v) with a pH of 2.81 was used as mobile phase at flow rate of 0.4 ml min \(^{-1}\). UV absorption was routinely monitored at a wavelength of 214 nm. Five types of OA served as standards for all samples; gluconic acid (GA), lactic acid (LA), citric acid (CA), succinic acid (SA) and propionic acid (PA).

Statistical analysis
In this study, mean values among treatments were compared by Duncan’s Multiple Range test at \( P<0.05 \). Analysis of variance (ANOVA) was performed on the data using SAS software (version 8.2; SAS, Inc., Cary, NC).

Results
Antibiotic Production and Biocontrol are Associated with \( pqqA \) and \( pqqB \)
The \( pqqA \) and \( pqqB \) in-frame deletion mutants HX2ΔA and HX2ΔB lacked the ability to inhibit growth of \( A. \) \( \text{vitis} \) K308 on PDA (Table 2). Strains CHX2ΔA and CHX2ΔB, which were HX2ΔA and HX2ΔB strains complemented by plasmids containing the \( pqq \) gene cluster, displayed phenotypes of \( A. \) \( \text{vitis} \) K308 inhibition \( \text{in vitro} \) (Table 2). Strain HX2ΔA and HX2ΔB showed lesser biocontrol activity to crown gall disease on sunflower than wild-type strain HX2 and complement strain CHX2ΔA and CHX2ΔB (Table 2). Strain HX2ΔB showed higher biocontrol efficiency compared to strain HX2ΔA and EI of crown gall disease treated by HX2ΔB significantly increased by 41% compared to HX2ΔA treatment \( (P<0.05) \) (Table 2 and Fig. 1). The abilities to produce antibacterial substance, inhibit growth of \( A. \) \( \text{vitis} \) K308 \( \text{in vitro} \), and suppress gall development on sunflower were fully restored in the complemented strain CHX2ΔA and CHX2ΔB, respectively (Table 2 and Fig. 1).

Genes \( pqqA \) and \( pqqB \) are Related to Phosphate Solubilization and Plant Growth Promotion
Based on the growth conditions of the bacteria strains in NBRIP with agar after 7-day incubations, it was shown that there was a significant decrease \( (P<0.05) \) of phosphate-solubilizing halo diameters when either the \( pqqA \) or \( pqqB \) gene was disrupted (Table 3). When HX2ΔA or HX2ΔB was complemented with the \( pqq \) gene cluster (CHX2ΔA or CHX2ΔB), phosphate solubilization on NBRIP plates was restored. Wild-type HX2 excreted high levels of OA, especially GA and manifested much stronger MPS ability compared to strains HX2ΔB and HX2ΔA (Table 2). Strains CHX2ΔA and CHX2ΔB restored intrinsic MPS ability and secreted similar levels of GA compared to the wild-type strain HX2. The phosphate-solubilizing halo diameters of \( R. \) \( \text{aquatilis} \) HX2, CHX2ΔA, and CHX2ΔB colonies were about two-fold greater than that of HX2ΔA and HX2ΔB strains (Table 3). The phosphate-solubilizing halo of complemented strains
CHX2ΔA and CHX2ΔB were, in-fact, even greater than those of the wild type strain HX2 (significant at P<0.05) (Table 3).

To confirm that pqqA and pqqB were related to phosphate solubilization, additional tests were performed to quantify soluble P and lower culture pH in liquid media (Table 3 and S1 Figure). After 7-day incubations, the concentration of soluble P in culture solutions treated with CHX2ΔA, CHX2ΔB were 450.3 mg l\(^{-1}\) and 465.9 mg l\(^{-1}\), which were 3.7 and 3.5 times greater than mutant HX2ΔA and HX2ΔB (Table 3). There was significant decrease (P<0.05) in soluble P concentration between strains with a disrupted pqqA or pqqB gene compared to the wild type strain HX2. At the same time, the pH value of culture solutions increased from 3.45 to 4.68 and 4.53 when the pqqA or pqqB gene was disrupted (Table 3). Disruption of pqqA resulted in the lowest concentration of soluble P (99.7 mg l\(^{-1}\)) of the derivative strains tested and also had the highest culture pH (4.68). The largest quantity of soluble P (465.9 mg l\(^{-1}\)) was present in the

| Strains     | PQQ (ng/ml) | Inhibition zone diameter (mm) | EI (%) |
|-------------|-------------|-------------------------------|--------|
| HX2         | 8.21±0.02 a | 30.3±0.3 a                    | 89.4±2.9 a |
| HX2ΔA       | \           | \                             | 19.5±1.1 c |
| HX2ΔB       | \           | \                             | 27.4±1.0 b |
| CHX2ΔA      | 8.02±0.02 b | 27.3±0.2 a                    | 88.6±1.3 a |
| CHX2ΔB      | 7.93±0.03 b | 28.7±0.3 a                    | 88.9±1.8 a |

Mean ± standard error values followed by different letters indicate statistically significant differences (P<0.05). The effectiveness index (EI) was calculated using the following formula: EI (%) = [(C-T)/C]×100, where C is the mean fresh weight of the crown gall tumor of the positive control group and T is the mean fresh weight of the crown gall tumor in the treated group.

Table 2. Production of PQQ and inhibition effect of Rahnella aquatilis HX2 and its derivatives on the growth of Agrobacterium vitis strain K308 and tumor formation on sunflowers.

CHX2ΔA and CHX2ΔB were, in-fact, even greater than those of the wild type strain HX2 (significant at P<0.05) (Table 3).

![Biological control crown gall disease of sunflower](image)

Fig. 1. Biological control crown gall disease of sunflower with R. aquatilis HX2 and derivative strains. Crown gall on sunflower were caused by A. vitis K308 with wild-type HX2 and derivative strains including pqqA mutant strain HX2ΔA, pqqB mutant strain HX2ΔB, complemented mutants CHX2ΔA and CHX2ΔB whilst water as control.

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CHX2ΔB culture, corresponding to the lowest pH (3.38). There was a significant negative correlation ($R^2 = -0.97$, $P < 0.01$) between pH and soluble P.

The effects of strains HX2ΔA and HX2ΔB on plant growth promotion were monitored using pot experiments in the greenhouse. The details of the plant length, fresh and dry weight, total P and soluble P at 42 days post-inoculations are listed in Table 4. All plant growth parameters and conditions measured here were improved in the presence of HX2 as compared to the negative control. Specifically, maize plant length increased by 48%, fresh weight was 255% greater, dry weight increased by 168%, plant total P was 67% greater, and there was 182% more soluble P in the soil. The effects of HX2 and derivative strains on plant growth promotion are significant, as compared to a blank control. However, HX2, CHX2ΔA, and CHX2ΔB treatments were more effective in enhancing maize length, fresh weight, dry weight, total P and soluble P in soil than HX2ΔA or HX2ΔB mutant treatments. There was no significant difference in length or fresh weight among HX2ΔA and HX2ΔB mutant treatments. However, there was a significant difference between HX2ΔB treatment and HX2ΔA treatments in which HX2ΔB displayed 19% greater dry plant weight, 17% more total P in maize, and 18% more soluble P in soil than HX2ΔA. In contrast to the negative control, the effect of HX2ΔB also shows significant improvement with respect to plant dry weight and soil soluble P where as HX2ΔA treatment only resulted in significantly greater soil soluble P. There was a significant positive correlation ($R^2 = 0.87$, $P < 0.01$) between fresh weight and soil soluble P.

| Strains  | Phosphate solubilizing halo diameter (mm) | Soluble P (mg l⁻¹) | pH of medium | Gluconic acid (g l⁻¹) |
|----------|------------------------------------------|---------------------|--------------|-----------------------|
| HX2      | 22.7 ± 0.3 b                               | 438.7 ± 15.0 a       | 3.45 ± 0.01 c | 9.68 ± 0.34 ab        |
| HX2ΔA    | 12.7 ± 0.2 c                               | 99.7 ± 3.7 b         | 4.68 ± 0.03 a | 0.65 ± 0.01 d         |
| HX2ΔB    | 13.1 ± 0.1 c                               | 115.3 ± 6.3 b        | 4.53 ± 0.06 b | 2.01 ± 0.46 c         |
| CHX2ΔA   | 24.7 ± 0.3 a                               | 450.3 ± 7.0 a        | 3.45 ± 0.06 c | 8.75 ± 0.19 b         |
| CHX2ΔB   | 25.0 ± 0.6 a                               | 465.9 ± 21.8 a       | 3.38 ± 0.02 c | 10.84 ± 0.68 a        |

Mean ± standard error values followed by different letters indicate statistically significant differences ($P < 0.05$).

Table 4. Green house pot experiment: effect of HX2 and derivative strains on maize plant height and weight and soil total P and soluble P.

| Strains | Length (cm) | Fresh weight (g plant⁻¹) | Dry weight (g plant⁻¹) | Total P (mg kg⁻¹) | Soluble P (mg kg⁻¹) |
|---------|-------------|---------------------------|------------------------|-------------------|---------------------|
| CK      | 54.2 ± 0.9 c | 8.12 ± 1.61 c             | 1.68 ± 0.07 e          | 1.28 ± 0.05 b     | 5.12 ± 0.30 e       |
| HX2     | 80.0 ± 1.9 a | 28.82 ± 2.57 a            | 4.51 ± 0.18 a          | 2.09 ± 0.01 a     | 14.46 ± 0.20 a      |
| HX2ΔA   | 70.0 ± 1.1 b | 16.24 ± 1.03 b            | 1.87 ± 0.08 e          | 1.00 ± 0.04 c     | 8.09 ± 0.06 d       |
| HX2ΔB   | 70.6 ± 0.6 b | 16.42 ± 0.99 b            | 2.22 ± 0.13d           | 1.17 ± 0.06 b     | 9.54 ± 0.06 c       |
| CHX2ΔA  | 78.0 ± 2.4 a | 25.73 ± 0.51 a            | 3.52 ± 0.20 c          | 2.09 ± 0.01 a     | 11.86 ± 0.10 b      |
| CHX2ΔB  | 76.2 ± 1.0 a | 23.94 ± 2.09 a            | 3.84 ± 0.16 b          | 2.08 ± 0.01 a     | 11.79 ± 0.12 b      |

Mean ± standard error values followed by different letters indicate statistically significant differences ($P < 0.05$).

5CK is negative control, LB medium only.

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soluble P treated by the strains was positively correlated as well ($R^2=0.72$, $P<0.01$).

Detection of PQQ
The level of PQQ production by HX2ΔA and HX2ΔB, determined with HPLC showed that \textit{pqq}A and \textit{pqq}B mutants lost the ability to biosynthesize PQQ (Table 2 and Fig. 2). Non-detectable levels of PQQ were shown for each \textit{pqq} mutant (HX2ΔA and HX2ΔB), but an average of 8.0 ng ml\(^{-1}\) and 7.9 ng ml\(^{-1}\) were detected in complemented strains CHX2ΔA and CHX2ΔB. However, PQQ produced by the complemented strains was significantly less than wild strain HX2 (Table 2).

OA Production
Gluconic acid was the main OA produced by \textit{R. aquatilis} HX2 which accounted for 94.3% of total OA production (Fig. 3). While HX2 produced GA and LA, HX2ΔA and HX2ΔB with their complemented strains only produced GA (Fig. 3). Strains HX2ΔA and HX2ΔB excreted low quantities of gluconic acid, as compared to all strains with intact \textit{pqq} gene clusters (Table 3). HX2ΔB produced significantly more GA than did HX2ΔA ($\text{P}<0.05$). Furthermore, CHX2ΔB GA production was significantly greater than that of CHX2ΔA ($\text{P}<0.05$). Production of GA shows a significant negative correlation ($r=−0.984$, $\text{P}<0.01$) to pH of culture solution and is positively correlated ($r=0.973$, $\text{P}<0.01$) to amount of soluble P in culture solutions.

Discussion
The most significant findings of this study are the different outcomes of \textit{pqq}A and \textit{pqq}B mutations on biocontrol capabilities, P solubilization, and plant-growth promotion by strain HX2. Although the \textit{pqq}A gene product is redundant for the synthesis of PQQ in some bacteria, its availability was essential for PQQ biosynthesis in HX2 (Table 2) [37]. Of the derivative strains, HX2ΔA exhibited the lowest amounts of soluble P and excretion of organic acid and cultures had the

Fig. 2. RP-HPLC detection of PQQ synthesized by \textit{R. aquatilis} HX2 and derivative strains. Arrows indicate 5-acetonyl-PQQ.

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highest pH. Notably, the HX2ΔB mutant displayed significantly greater GA production, soil soluble P and dry plant weight when compared to HX2ΔA (Table 3 and Table 4). This indicates a different role of pqqB in PQQ biosynthesis than that of pqqA. Furthermore, when HX2ΔA and HX2ΔB were complemented with the entire pqqABCDEF operon, the derivatives have additional pqq genes as compared to the wild-type strain. For example, CHX2ΔB now has one pqqB gene and duplicate pqqA genes. There are several differences between CHX2ΔA and CHX2ΔB. Specifically, CHX2ΔB produces significantly more GA than CHX2ΔA and the wild-type strain. However PQQ was not measured at higher levels for CHX2ΔB. All together, these findings indicate that pqqA is more important than pqqB in biocontrol capabilities, P solubilization, and plant-growth promotion by strain HX2. These results fall in line with those previously reported. Velterop et al. [22] found that the PqqA protein provided precursors for PQQ biosynthesis and that PqqB proteins facilitated the transport of PQQ across the cytoplasmic membrane into the periplasm in Klebsiella pneumonia. In this case PQQ biosynthesis was rate dependent on PqqA [22]. Hence, in HX2 the function of the pqqA g and pqqB genes may be similar to that in K. pneumonia.

Many researchers have shown that the abilities of gram-negative bacteria, such as R. aquatilis, Pseudomonas cepacia, and Enterobacter intermedium, to solubilize insoluble phosphates were dependent upon pqq genes [38, 39, 40, 41]. PQQ is
necessary for the assembly of the GDH holoenzyme, which acts in the oxidation of glucose to GA [42, 43]. The results described here indicate that the MPS ability of \textit{R. aquatilis} HX2 was mainly determined by GA and LA production, and GA was significantly more important (Table 3 and Fig. 2). The correlation between quantities of GA and soluble P concentration indicates that GA production by HX2 effectively reduces the medium pH to increase MPS ability (Table 3, Fig 2 and S1 Figure). The main mechanism of insoluble phosphate dissolution in strain HX2 relates to glucose metabolism, in which GA is produced under the action of GDH utilizing PQQ as cofactor. Also, GA production will be greater if PQQ biosynthesis is increased. In this strain, \textit{pqqA} and \textit{pqqB} are both required for PQQ biosynthesis and to have wild-type levels of GA production. Although, it was at a greatly reduced level, strain HX2ΔA still produced GA but PQQ production was non-detectable. This brings to question if there is a GDH-PQQ independent pathway for GA catabolism in HX2.

Plant growth promotion was directly related to strain P solubilization ability in soil and culture medium (Table 3 and Table 4). Maize growth promotion was greatest when treatments included HX2, CHX2ΔA, and CHX2ΔB, while HX2ΔA and HX2ΔB treatments demonstrated promotion to a significantly lesser extent. Likewise, strains HX2, CHX2ΔA and CHX2ΔB produced the highest amounts of GA, which released insoluble P into the soil solution (Table 4). This is consistent with previous findings where inoculation of phosphate solubilizing bacteria such as \textit{Serratia marcescens}, \textit{Pseudomonas fluorescens} and \textit{Bacillus} spp. improved the phosphorous uptake of shoots and grains in maize and peanut plants [44, 45, 46]. Even with the greatly reduced GA production levels, HX2ΔA and HX2ΔB had benefits with respect to soil P-solubilization as compared to negative controls. It has been reported that plant growth promotion can be achieved by direct and indirect interaction between beneficial microbes and their host plants [47]. Given that HX2ΔA and HX2ΔB promoted plant growth, it is likely that strain HX2 has other plant-growth promoting properties in addition to phosphate solubilization.

Derivative strain HX2ΔB showed more effective biocontrol than did HX2ΔA (Table 2). This is likely related to greater GA production by HX2ΔB. Gluconic acid can serve as an antifungal agent and has been associated with the regulation of other antimicrobial compounds, 2,4-diacetylphloroglucinol and pyoluteorin [11, 48]. Without PQQ production, HX2ΔA and HX2ΔB mutants had greatly reduced abilities to produce GA and disrupted the ability to produce antibacterial substance, but they were still able to suppress tumor formation on sunflowers significantly (Fig. 1). Hence, it is likely that production antibacterial substance and GA is not the sole mechanism involved in HX2 biocontrol of crown gall disease.

Further work should be done to identify the potential additional plant growth promoting factors and antibacterial substances produced by HX2. More information will provide insight to optimized growing conditions or modifications to the strain which can lend it improved benefits to plants.
Supporting Information

S1 Figure. Soluble P (a) and pH (b) in media of HX2 and derivative strain cultures.
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Author Contributions

Conceived and designed the experiments: YG WW. Performed the experiments: LL ZJ. Analyzed the data: YG LL ZJ LH. Contributed reagents/materials/analysis tools: LL ZJ LH WW YG. Wrote the paper: YG LH.

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