Canadian Journal of Microbiology
Revue canadienne de microbiologie

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Journal:	<i>Canadian Journal of Microbiology</i>
Manuscript ID	cjm-2015-0206.R1
Manuscript Type:	Article
Date Submitted by the Author:	29-Jul-2015
Complete List of Authors:	Giles, Courtney D.; The James Hutton Institute, Ecological Sciences Hsu, Pei-Chun Lisa; AgResearch Lincoln, Innovative Farm Systems Richardson, Alan E.; CSIRO, Agriculture Hurst, Mark R. H.; AgResearch Lincoln, Innovative Farm Systems Hill, Jane E.; Dartmouth College, Thayer School of Engineering
Keyword:	Pseudomonads, phytase, rhizosphere, phosphorus, gluconate

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Title

The Role of Gluconate Production by *Pseudomonas* spp. in the Mineralization and Bioavailability of Calcium-Phytate to *Nicotiana tabacum*

Author Names:

Courtney D. Giles ^a, Pei-Chun (Lisa) Hsu ^b, Alan E. Richardson ^c, Mark R. H. Hurst ^b, Jane E. Hill ^d

Author Affiliations:

- ^a The James Hutton Institute, Invergowrie, Dundee, Scotland, UK DD2 5DA
- ^b AgResearch Lincoln, Innovative Farm Systems, Christchurch, New Zealand 8140
- ^c CSIRO Agriculture, GPO Box 1600, Canberra ACT 2601
- ^d Dartmouth College, Thayer School of Engineering, Hanover, NH, USA 03755

Corresponding Author

Courtney D. Giles

Courtney.Giles@hutton.ac.uk

(p) +44 7933 546838

Abstract

Organic phosphorus (P) is abundant in most soils but is largely unavailable to plants. *Pseudomonas* spp. can improve the availability of P to plants through the production of phytases and organic anions. Gluconate is a major component of *Pseudomonas* organic anion production and may therefore play an important role in the mineralization of insoluble organic P forms such as calcium-phytate (CaIHP). Organic anion and phytase production was characterized in two *Pseudomonas* sp. soil isolates (CCAR59, Ha200) and an isogenic mutant of strain Ha200, which lacked a functional glucose dehydrogenase (Gcd) gene (strain Ha200 *gcd*::Tn5B8). Wild-type and mutant strains of *Pseudomonas* sp. were evaluated for their ability to solubilize and hydrolyze CaIHP, and to promote the growth and assimilation of P by tobacco. Gluconate, 2-keto-gluconate, pyruvate, ascorbate, acetate, and formate were detected in *Pseudomonas* sp. supernatants. Wild-type *Pseudomonads* containing a functional Gcd could produce gluconate and mineralize CaIHP, whereas the isogenic mutant could not. Plant inoculation with *Pseudomonas* improved the bioavailability of CaIHP to tobacco, but there was no difference in plant growth response due to Gcd function. Glucose dehydrogenase function is required for the mineralization of CaIHP *in vitro*, however further studies will be needed to quantify the relative contribution of specific organic anions such as gluconate to plant growth promotion by soil *Pseudomonads*.

Keywords: *Pseudomonads*, gluconate, phytase, rhizosphere, phosphorus

Introduction

A growing concern for the sustainable use of phosphorus (P) in agricultural systems has intensified interest in the identification of biogeochemical mechanisms that can be targeted to improve crop access to native or residual soil P (Sattari et al. 2012; Stutter et al. 2012). Increases in the efficient use of P by crop plants could lower external fertilization requirements (Arcand and Schneider 2006; Richardson et al. 2011) and the potential for P loss from agricultural soils (Kleinman et al. 2011).

Myo-inositol hexakisphosphate (IHP) and its metal ion precipitates (e.g., calcium or iron complexes of phytate) are among the most abundant forms of organic P identified in soil (Giles et al. 2011; Turner et al. 2002). To become bioavailable, IHP must be hydrolyzed by plant or microbial phytases to generate orthophosphate (Hill and Richardson 2007). The hydrolysis of IHP is often restricted by low substrate solubility and low phytase activity in the soil environment (George et al. 2007). Rhizosphere processes that enhance the solubility of phytate may therefore play a critical role in plant access to sparingly available phytate in soils.

Several studies have investigated the effect of plant and microbial phytases on the availability of phytate to plants (Azeem et al. 2014; Richardson et al. 2007; Richardson et al. 2009; Richardson et al. 2011). For example, the genetic modification of plants to exude exogenous phytase in excess of wild-type levels has proven useful for enhancing the bioavailability of phytate to *Arabidopsis* (Richardson et al. 2001b) and *Nicotiana tabacum* (George et al. 2005a; Giles et al. 2014; Giles et al. 2012). Phytase-producing wild-type soil bacteria (Hill and Richardson 2007) and genetically manipulated soil microbiota (e.g., *Pseudomonas* spp. [Richardson et al., 2001a], *Pantoea* sp. [Patel et al., 2010a], and *Citrobacter* sp. [Patel et al., 2010b]) have been shown to improve the mineralization and availability of

phytate-P to several grass and legume species. More recently, the inoculation of phytase-exuding transgenic plants with P-solubilizing and mineralizing bacteria improved the availability of sparingly soluble phytate to tobacco (Giles et al. 2014; Giles et al. 2012).

In plant inoculation studies, phytase-producing *Pseudomonas* spp. improved the assimilation of soil P by tobacco (Giles et al., 2012; Giles et al., 2014) and in pasture plants (Richardson et al. 2001a). In agar media, plant utilization of soluble phytate (NaIHP) was increased by up to 3.9-fold (Richardson et al., 2001a). In a similar study with mung beans, Patel et al. (2010b) demonstrated a 1.5-fold improvement in NaIHP utilization when plants were inoculated with a recombinant *Pseudomonad* over-expressing a *Citrobacter brakii appA* gene (histidine-acid-phosphatase; HAP-like phytase).

The efficacy of these strategies, however, has been limited in soils (George et al. 2008; George et al. 2005a), in part, due to a limited understanding of the specific biogeochemical interactions that control the solubilization and mineralization of phytate. For example, tobacco plants over-expressing the *Aspergillus niger* fungal phytase (*ex::phyA*; PHY-lines) did not display a growth benefit over wild-type plants, despite having significantly greater phytase activity in root exudates (George et al. 2005a). Similar results have been reported under controlled growth conditions, whereby the growth of PHY plants in a goethite-containing growth system was also minimal (Giles et al., 2012). The sorption and precipitation of enzyme and/or substrate with soil and mineral surfaces are considered to be the primary processes limiting the success of these strategies in soils (Celi et al. 2001; George et al. 2007).

Soil or media pH have a direct bearing on the solubility of mineral-bound or precipitated phytate (Celi and Barberis 2007). *In vitro* experiments have shown improved solubility and hydrolysis of precipitated forms of IHP at low pH, particularly in the presence of organic anions

97 (Tang et al. 2006). The influence of pH on the solubility of phytate was apparent in a study of
98 limed soils, in which the growth benefit of plants supplemented with soluble IHP was negated at
99 high pH due to likely precipitation of IHP with divalent cations (e.g., Ca^{2+} , Mg^{2+}) (George et al.
100 2005a). In plant-soil systems, changes in rhizosphere pH may occur due to the production and
101 exudation of low molecular weight organic anion acids by soil microorganisms or plant roots
102 (Hinsinger et al. 2003).

103 Previous studies have demonstrated that CaIHP hydrolysis by plant and microbial phytases is
104 improved in the presence of organic anions (e.g., gluconate, citrate, oxalate, acetate) (Dao 2007;
105 Patel et al. 2010a; Tang et al. 2006). Organic anions can mediate the availability of precipitated P
106 sources directly through the chelation of counter ions (e.g., Fe^{3+} , Al^{3+} , Ca^{2+}) or indirectly by acid
107 solubilization, which occurs when protons (H^+) are co-transported across the bacterial outer
108 membrane (Rodriguez and Fraga 1999). Gluconate production by *Pseudomonas* spp. is
109 associated with acidification, which has been shown to promote the solubilization of tricalcium
110 phosphates (Browne et al. 2009; Miller et al. 2010), but is less commonly studied with respect to
111 IHP solubilization and bioavailability (Patel et al. 2010a; Patel et al. 2010b; Richardson et al.
112 2011). Gluconate effectively binds Ca^{2+} and could therefore enhance the solubilization of
113 phosphate and IHP calcium salts (Miller et al. 2010; Patel et al. 2010a). *Pseudomonas* spp.
114 produce gluconate through the direct oxidation of glucose, a process which is mediated by the
115 periplasmic glucose dehydrogenase enzyme (*gcd*) (Buch et al. 2008).

116 Giles et al. (2014) reported improved utilization of CaIHP by tobacco plants that were
117 inoculated with an organic anion- and phytase-producing *Pseudomonas* sp. (strain CCAR59).
118 Gluconate and 2-ketogluconate were the primary organic anions produced by this strain, which
119 also produced a large zone of CaIHP solubilization and mineralization when grown on agar

media containing CaIHP as the sole source of P. We therefore hypothesized that gluconate production by *Pseudomonas* sp. could play a central role in the bioavailability of CaIHP to tobacco and that the ability of Pseudomonads to synthesize a functional glucose dehydrogenase will influence CaIHP solubilization, subsequent hydrolysis by bacterial or plant phytases, and ultimately the utilization of IHP by plants. To test this hypothesis, we (1) investigated the presence of functional glucose dehydrogenase genes in two phytate-mineralizing *Pseudomonas* spp., (2) made a *Pseudomonas* sp. mutated derivative unable to synthesize gluconate, and (3) determined the effect of this mutation on the ability of *Pseudomonas* sp. to produce organic anions, to solubilize and hydrolyze CaIHP and to subsequently affect the bioavailability of CaIHP to tobacco.

Materials and methods

Chemical sources

Myo-inositol hexaphosphate dodecasodium salt (Na_{12}IHP) and disodium phosphate (Na_2HPO_4) were purchased from Sigma (P-0810, Lot: 59H0559) and used for all experiments. When referring to the calcium salts of phosphate and IHP, we use the notation of CaPO_4 and CaIHP to represent all possible solid-state stoichiometries for the respective P forms. Organic anion calibration standards for High Performance Liquid Chromatography (HPLC) work were purchased from Supelco (oxalate, OxA; ascorbate, AsA; acetate, AA) and Sigma (gluconate, GA; pyruvate, PyrA; 2-ketogluconate, 2KGA).

Pseudomonas sp. strains and culture conditions

Wild-type soil isolates of *Pseudomonas* sp. CCAR59 (Richardson and Hadobas 1997) and *Pseudomonas* sp. Ha200 were obtained from CSIRO Plant Industry (Canberra, ACT) and the AgResearch Culture Collection (Lincoln, NZ), respectively. Bacterial strains and plasmids used

in this study are listed in Table 1. Bacteria were grown in Luria-Bertani (LB) broth or on LB agar, at 37°C for *Escherichia coli* and 28°C for *Pseudomonas* spp. Antibiotic concentrations ($\mu\text{g mL}^{-1}$) used for *Pseudomonas* sp. Ha200 and *E. coli* were ampicillin 100, gentamicin 20 and kanamycin 100, respectively. Cultures were incubated with shaking at 200 rpm in a Raytek orbital incubator (Raytek Instruments Ltd., Victoria, Australia). Calcium-IHP agar (20 g L⁻¹ BD Difco agar) for *Pseudomonas* sp. clearing-zone assays contained 20 mM MOPS (pH 7.2), D-glucose at 0, 1, 11 and 110 mM concentration, and 0.2 mM IHP with or without methyl red indicator (< 0.01% w/v) (Smith et al. 1994). Organic anion production was determined by HPLC analysis of *Pseudomonas* sp. cultures grown in minimal media (20 mM MES, pH 5.5, 0 to 110 mM glucose or carbon-equivalent as arabinose, 5 μM FeCl₃, Nitsch's trace elements) with 0.2 mM Na₂HPO₄ or without P provided (Richardson et al., 2011). The pH of each medium and culture supernatant was measured using a Fisher Accumet pH meter and a combination pH Ag/AgCl₂ electrode (Fisher Scientific, Waltham MA, USA).

Pseudomonas sp. Ha200 transposon mutagenesis

Standard techniques for manipulation of DNA were performed as described in Sambrook et al. (1989). Transposon mutagenesis was performed using the mini-Tn5 derivative Tn5 *KmI* as described by Delorenzo et al. (1990). To allow antibiotic selection of the recipient strain, the plasmid pBBR1MCS-5 (Table 1) was electroporated into *Pseudomonas* sp. strain Ha200 using a Biorad Gene Pulser (25 μF , 2.5 kV and 200 ohms; Dower et al., 1988). The resultant Tn5 *KmI* transposon mutants and the *Pseudomonas* sp. Ha200 WT (as the positive control) were patched onto CaIHP agar plates and left for seven days at 28°C (See '*Pseudomonas* clearing zone assays'). Transposon mutants with altered halo sizes were then patched to fresh CaIHP agar plates and the assay repeated.

The region including and flanking the transposon insertion was determined by touchdown PCR (Sarkar et al. 1993) using the Tn5 *KmI* specific primers Sp1, Sp2A and Sp2B, as the anchor primers (Table 1). For DNA template, genomic DNA was prepared using the MoBio Power Soil DNA Isolation kit in accordance with manufacturer's instructions (MoBio Laboratories, Inc. #12888-100, Carlsbad CA, USA). The amplicons were cloned into pGEM-T Easy vector (Promega #A1360, Madison WI, USA) followed by transforming into *E. coli* DH10B. The resultant clones were sequenced on both strands using M13F, M13R, or custom made primers on an Applied Biosystems 3730xl and 9 ABI 3700 sequencer, (<http://dna.macrogen.com/eng/>). Sequences were assembled using Geneious version 6.1 (<http://www.geneious.com>). Databases at the National Center for Biotechnology Information (NCBI) were searched using BlastX and BlastP (Altschul et al. 1997; Schaffer et al. 2001).

Identification of a glucose dehydrogenase biosynthetic gene in *Pseudomonas* sp. CCAR59

To determine if *Pseudomonas* sp. CCAR59 encodes a *gcd* gene, degenerate primers (F1, R1; Table 1) were designed to a conserved region (~450 bp) of the *gcd* gene of disparate *Pseudomonad* orthologs, which were aligned using ClustalW2 in nucleotide mode. Primers were designed manually and checked for melting and self-complementarity using Primer3. The F1 and R1 primers were used to PCR-amplify *gcd* orthologues from the purified *Pseudomonas* sp. CCAR59 genomic DNA by temperature-gradient PCR (55.0 to 71.0 °C; 30 cycles, Invitrogen Taq Platinum Polymerase) using a thermal cycler (BioRad Corp., C1000TM 96-well block) with an optimal annealing temperature of 55°C determined. The predicted size fragment (~447 bp) was purified and sequenced as described above. The resultant sequence was trimmed to remove the degenerate primer sequence, leaving 406 bp.

188 *Pseudomonas* clearing zone assays

189 The solubilization and hydrolysis of CaIHP by *Pseudomonas* sp. CCAR59 and Ha200 WT
190 and mutant strains were assayed as previously described (Giles et al., 2014). Briefly,
191 *Pseudomonas* spp. were grown in LB media (16 h, 200 rpm, 28°C), cells pelleted (10000xg, 1
192 min) and washed (0.9% sterile saline) to remove residual phosphate. Ten µL of resuspended cells
193 (OD₆₀₀ ~ 0.5) were pipetted onto CaIHP agar with or without methyl red and grown for 72 h
194 (28°C) prior to photography. Red zones were observed around colonies that produced acid in
195 methyl red-containing media. The CaIHP plates were treated with cobalt chloride (CoCl₂) in
196 order to re-precipitate the IHP that had been solubilized due to bacterial acid production (Bae et
197 al. 1999). Zones that remained clear following CoCl₂-treatment were indicative of CaIHP
198 hydrolysis. Methyl red-free plates were photographed both before (No Treatment) and after
199 cobalt chloride treatment (2% w/v CoCl₂ flood, 1h) on the same day (Bae et al. 1999).

200 Analysis of organic anion production by *Pseudomonas* spp. using High-Performance Liquid 201 Chromatography

202 *Pseudomonas* cells were prepared as described for the clearing zone assays and inoculated
203 (1:100; OD₆₀₀ ~0.05) into minimal media. Cells were grown for 13 d (0 to 110 mM glucose, No
204 P; 28°C, 200 rpm) and supernatants harvested following centrifugation (10000xg, 1 min) and
205 filtration (0.2 µm Polyethersulfone, EMD Millipore, Darmstadt, Germany). Organic anions in
206 *Pseudomonas* spp. supernatants were measured as described previously (Agilent 1100 HPLC
207 system, Santa Clara CA, USA; Phenomenex Rezex-ROA Organic Acid column, Torrance CA,
208 USA) (Giles et al., 2014). Triplicate HPLC measurements were made per biological replicate and
209 a four-point calibration was prepared using a mixed organic anion standard in MES media.

Incubations of *Pseudomonas* spp. supernatants with insoluble calcium-phytate

Supernatants collected from cultures of *Pseudomonas* sp. CCAR59, Ha200 WT, and Ha200 *gcd::Tn5B8* were harvested after 13 d growth by centrifugation (5000xg, 10 min) and filtration (0.2 μ m). Biological supernatants and organic anion free media controls (pH 4.0 and 5.5; Ionic strength, $I=0.1$) were incubated with CaIHP (1 - 2 mM, 24 h) in 15 mL tubes placed at an angle of 45° to facilitate mixing (30°C, 100 rpm). Reaction supernatants were harvested by centrifugation and filtration (as above), and total P determined using inductively-coupled-plasma-optical-emission-spectroscopy (ICP-OES; JY-Horiba Ultima 2C, UVM Geology, Burlington, VT). Total P in reaction solutions was adjusted for the concentration of P in initial biological supernatants prior to conversion to IHP (1 mole IHP per 6 moles P).

Plant media

Plant nutrient solution (PNS) contained macronutrients (4 mM KNO₃, 4 mM Ca(NO₃)₂, 3 mM NH₄Cl, 1.5 mM MgSO₄, 0.1 mM Fe-Na-EDTA) and micronutrients (23 μ M H₃BO₃, 46 μ M MnCl₂, 15 μ M ZnSO₄·7H₂O, 1.6 μ M CuSO₄·5H₂O, 1.0 μ M (NH₄)₆Mo₇O₂₄·4H₂O, 1.0 μ M CoCl₂·6H₂O) at pH 5.5 (Richardson et al. 2001a). Ten g L⁻¹ BD Difco agar was included to prepare plant nutrient agar (PNA) for plant inoculation assays (see below). Five plant media conditions were achieved by adding PNS solution to molten agar (~42°C) either (i) without added P (No P) or P supplied as; (ii) Calcium phytate precipitate (CaIHP), (iii) Ca-phosphate precipitate (CaPO₄), (iv) soluble phytate as NaIHP, and (v) soluble phosphate as NaPO₄. Total P in each media was added as 0.8 mM phosphate or 0.13 mM IHP (i.e., equivalent to 0.8 mM phosphate). Calcium-precipitated P sources (CaPO₄, CaIHP) were prepared as described by Tang et al. (2006) using three quantitative washing steps. Total P in each wash solution was determined colorimetrically without or following autoclave-digestion for CaPO₄ and CaIHP

preparations, respectively (Hayes et al. 2000). The total amount of P precipitated with Ca^{2+} was calculated as the difference between the initial solution P added to the preparations and the cumulative P content of the wash solutions.

Plant exudate measurement and growth assays

Wild-type (WT) *Nicotiana tabacum* seeds (variety W38) were obtained from CSIRO Plant Industry (Canberra, ACT). Seeds were surface-sterilized for one hour in a sealed glass desiccator by placement above a reservoir containing 100 mL of 5% (v/v) concentrated hydrochloric acid and 4% (v/v) bleach solution. For exudate collection, 10-15 seeds were grown for 7-10 days in two mL of plant nutrient solution with constant swirling and light (mean photon flux $\sim 200 \mu\text{mol s}^{-1}$) at 24°C . The original growth solution was replaced with two mL of Fe- and P-free plant nutrient solution and exudates collected after 24 h (5 biological replicates per plant line). The concentration of organic anions ($50 \text{ nmol plant}^{-1} \text{ day}^{-1}$) and phytase activity ($0.2 \times 10^{-2} \text{ nkat plant}^{-1} \text{ day}^{-1}$) was previously determined in WT root exudates collected as described above (Giles et al. 2012). Exudate solutions were assayed for glucose enzymatically using a glucose oxidase-peroxide reaction, which yielded reaction products proportional to glucose concentration at 570 nm (Glucose Assay Kit; Eton Bioscience Inc.).

Agar slants (10° from vertical, 25 mL PNA) for plant inoculation assays with *Pseudomonas* spp. were prepared as described previously (Giles et al. 2012). Briefly, *Pseudomonas* sp., strains CCAR59, Ha200 WT, and Ha200 *gcd::Tn5B8* were prepared as described for CaIHP clearing-zone assays and added to molten agar (40°C) at $\sim 10^7 \text{ CFU mL}^{-1}$. Seed placement, growth (32 d, $24^\circ\text{C}/16 \text{ h light}/8 \text{ h dark}$; mean photon flux $\sim 400 \mu\text{mol m}^{-2} \text{ sec}^{-1}$), harvesting, and shoot P determinations for plants grown in sterile and inoculated agar media were carried out as described previously (Giles et al. 2014; Giles et al. 2012). A minimum of five agar

slants were prepared for each media and inoculation condition, and triplicate analyses of shoot P were conducted for each harvested plant.

Statistical analyses

Data are presented as the mean of between three and fifteen replicates with associated standard errors provided. Analysis of variance was used to establish significant differences (* $p < 0.05$; ** $p \leq 0.01$) between treatments. Student's t-test was used to calculate significance in pair-wise comparisons between inoculum conditions within a single media condition. Linear regression was used to generate pair-wise correlations between bacterial organic anion production, media pH, and CaIHP solubilization measurements.

Results

Identification and mutation of the glucose dehydrogenase gene in *Pseudomonas*

In total, 2496 putative mutants were screened for alterations in halo size after seven days growth on CaIHP agar. One mutant, designated *Pseudomonas* sp. Ha200 *gcd*::Tn5B8, was unable to form a zone of clearing on CaIHP agar (Fig. 1). BlastX analysis of the DNA sequence derived from primer-walking of the mini-Tn5 *Km1* B8 insertion point revealed the transposon had inserted into the glucose dehydrogenase (*gcd*) gene. Through a combination of genome-walking and DNA sequencing, the complete *gcd* gene and associated regions were sequenced. In total, 3475 bp were sequenced and deposited in GenBank under the accession number JX282600.

Annotation of the completed DNA sequence revealed that the Tn5 *Km1* had inserted at nucleotide 1818 of the predicted 2409 bp *gcd* gene. The translated product of the *Pseudomonas* sp. Ha200 *gcd* gene showed 96% similarity to the Gcd protein of *P. fluorescens* and contained a membrane-bound PQQ-dependent glucose dehydrogenase domain (cd10280). Proteins with this

domain convert D-glucose to D-glucono-1,5-lactone involved in the synthesis of gluconic acid (Table S1)..

The presence of a *gcd* orthologue in *Pseudomonas* sp. CCAR59 was assessed to determine if gluconate biosynthesis could occur by a pathway similar to that identified for *Pseudomonas* sp. Ha200. The degenerate *gcd* primers F1 and R2 (Table 1) were used to amplify a 437 bp amplicon from *Pseudomonas* sp. CCAR59 genomic DNA and the PCR product sequenced. The resultant DNA sequence (406 bp) was deposited in GenBank under accession number KJ733922. BlastN analysis of this sequence revealed 88% DNA identity to the *P. fluorescens* A506 *gcd* gene. BlastX revealed that the translated product of this DNA sequence shared 97% amino acid similarity to a PQQ-dependent Gcd protein from *Pseudomonas* sp. R81 and contained a PQQ-dependent Gcd domain. Based on this information it is likely that the *Pseudomonas* sp. CCAR59 also produces a PQQ-dependent Gcd.

Solubilization and hydrolysis of CaIHP by *Pseudomonas* spp. grown on agar

Calcium-IHP clearing and organic anion-production by *Pseudomonas* spp. strains CCAR59, Ha200 WT, and *gcd::Tn5B8* were assessed at multiple glucose concentrations (0, 1, 11, 110 mM). Glucose concentrations were selected to encompass the total daily exudation of glucose from roots as measured in 24 h tobacco exudate solutions ($0.2 \mu\text{mol glucose mg}^{-1}$ root dry wt.; $0.45 \pm 0.1 \mu\text{mol glucose mL}^{-1}$). In the absence of glucose, there were no observable zones of clearing associated with any of the strains tested on CaIHP agar plates (Fig. 1). Wild-type *Pseudomonas* sp. Ha200 and CCAR59 soil isolates produced acidic zones of clearing on methyl red plates containing 11 and 110 mM glucose, whereas the transposon insertion mutant (Ha200 *gcd::Tn5B8*) did not. Only the wild-type strains produced zones of hydrolysis on CoCl_2 -treated plates, with the largest zones of hydrolysis observed for Ha200 WT colonies grown with 11 and

110 mM glucose (Fig. 1). The Ha200 *gcd*::Tn5B8 strain had similar phytase activity to the wild-type Ha200 strain (1.9×10^{-2} μ kat mL⁻¹), despite its inability to hydrolyze CaIHP in agar.

Organic anion production and media acidification by *Pseudomonas* spp.

Pseudomonas sp. strains CCAR59, Ha200 WT, and Ha200 *gcd*::Tn5B8 were grown in liquid culture for 13 d under four glucose conditions (0, 1, 11, 110 mM). Strains reached stationary phase after 2-4 d and remained viable after 13 d of growth in the phosphate-limited minimal media (Fig. 2). The growth (OD₆₀₀) of all strains was dependent on glucose concentration with the highest glucose concentration (110 mM) leading to OD₆₀₀ of ~0.40 and 0.16 for CCAR59 and Ha200 strains, respectively (Fig. 2). Growth by these strains over 13 days at 11 and 110 mM glucose was accompanied with a significant decrease in pH of the media, whereas no change in media pH was observed for the Ha200 *gcd*::Tn5B8 mutant despite growth (OD₆₀₀) that was comparable to the wild-type strain (Table 2; Fig 2).

Both *Pseudomonas* sp. CCAR59 and Ha200 predominantly produced gluconate and 2-ketogluconate, with lesser amounts of acetate, ascorbate, pyruvate and formate being detected after 13 days growth depending on strain and growth conditions (Table 2). Only the wild-type strains produced significant concentrations of gluconate and 2-keto-gluconate, which was associated with acidification of the media (Fig. 2; Table 2). The production of these organic anions (i.e., gluconate, 2-keto-gluconate) occurs as a direct result of the oxidation of glucose, and thus was only evident when the cultures were provided with glucose (Fig. 2, Table 2). This is consistent with CaIHP plate assays in which only CCAR59 and Ha200 WT supplied with glucose formed zones of clearing, whereas strains grown in the absence of glucose did not (Fig. 1).

Supernatants from strain CCAR59 contained larger amounts of gluconate and were more acidic than those collected from the Ha200 cultures. For example, when 11 and 110 mM glucose was provided, supernatants from strain CCAR59 contained 8.5 and 25.1 mM gluconate, whereas Ha200 cultures contained 3.7 and 11.8 mM gluconate (Table 2). On average, the final pH values of the CCAR59 cultures were 0.5 units lower than in the Ha200 cultures (Table 2). Acetate (0.5 to 2.0 mM) was present in all cultures for all glucose concentrations (Table 2). Other organic anions detected in *Pseudomonas* sp. cultures included formate (0.4 to 0.5 mM), which was only detected in Ha200 *gcd::Tn5B8* cultures grown on 11 and 110 mM glucose (Table 2). Pyruvate was detected in CCAR59 (1.0 to 1.1 mM), Ha200 (0.1 to 0.2 mM), and the mutant (0.1 mM) cultures, whereas ascorbate was only detected in CCAR59 cultures (0.7 to 2.5 mM; Table 2).

The solubilization of CaIHP in *Pseudomonas* sp. supernatants

Supernatants obtained from the *Pseudomonas* sp. cultures were incubated with insoluble CaIHP (1 mM, 24 h, 30°C) in order to demonstrate the influence of gluconate production and media acidification on the solubilization of CaIHP (Table 2). Inclusive of all supernatant sources, CaIHP solubilization, measured as soluble IHP (mM) in reaction supernatants, increased proportionally to pH change ($R^2=0.91$) as well as log-transformed gluconate concentrations ($R^2=0.82$). In the 110 mM glucose condition, CCAR59 supernatants solubilized significantly more CaIHP (0.79 mM) than Ha200 WT (0.58 mM IHP; $p < 0.05$; Table 2). There was no significant difference in CaIHP solubilization between WT strains for the remaining glucose conditions (0, 1, 11 mM; $p > 0.05$).

The solubilization of CaIHP by supernatants from *Pseudomonas* sp. Ha200 *gcd::Tn5B8* (0.13-0.15 mM IHP at pH 5.5) was not different than that observed for organic anion free blank media (0.12 mM IHP at pH 5.5) or supernatants from WT strains grown in the absence of

glucose (0.16 mM IHP at pH 5.5; $p > 0.05$; Table 2). When incubated with CaIHP, blank media at pH 4.0 yielded four times more soluble IHP (0.46 mM) than pH 5.5 solutions (0.12 mM; Table 2). The pH-dependent solubilization trends were consistent with visual plate assays in which media acidification and CaIHP hydrolysis were only possible for WT strains with the ability to produce gluconate (Fig. 1). Supernatants collected from the CCAR59 strain grown on 110 mM glucose solubilized more CaIHP (0.79 mM) than organic anion-free blank media at the same pH (0.46 mM at pH 4; Table 2). Supernatants from the strain Ha200 WT yielded soluble IHP concentrations, which were not significantly different from the ones of the pH 4.0 blank media (0.58 mM versus 0.46 mM, $p > 0.05$; Table 2), although in absolute terms the supernatants from Ha200 WT reached pH 4.5. Gluconate concentrations were significantly lower in supernatants from Ha200 WT (11.8 mM) compared to CCAR59 (25.1 mM), despite similarities in supernatant pH. This suggests that the large concentration of gluconate in CCAR59 supernatants may have increased the solubilization of CaIHP beyond the effects of pH alone.

Root inoculation of tobacco with organic anion-producing *Pseudomonas* spp.

Although CaIHP is generally of limited availability to tobacco plants, inoculation with *Pseudomonas* sp. strains HA200 and HA200 *gcd::Tn5B8* resulted in significantly greater shoot dry weight and increased assimilation of phytate-P into shoots (Fig. 3). Sterile, phosphate-replete media (i.e., CaPO_4 , NaPO_4) and soluble IHP (NaIHP) provided sufficient P for shoot growth (Fig. 3a) and P uptake (Fig. 3b) in plants (0.33% to 0.75% P content; shoot DW basis), while CaIHP represented a limited source of P (0.19% P; Table S1; Reuter and Robinson, 1997). Wild-type tobacco plants inoculated with the WT and mutant Ha200 strains showed ~ 4 times greater biomass and incorporated approximately six-fold more shoot P (~20 μg) from CaIHP than uninoculated plants (3 μg ; Fig. 3b). However, there was no significant difference in shoot P

accumulation by plants grown on CaIHP and inoculated with the Ha200 WT (0.18% P; 19 ± 1 μ g P) and *gcd::Tn5B8* (0.17% P; 24 ± 4 μ g P) strains ($p < 0.05$; Fig. 3b, Table S2). Therefore, although the presence of the *Pseudomonas* sp. inocula improved plant assimilation of P from CaIHP, we could not conclude that it was gluconate production, specifically, that afforded this growth benefit.

Pseudomonas cells in the plant culture were checked for viability after plants had been harvested for shoot materials and streaked on CaIHP agar media to confirm that the CaIHP solubilization patterns of the inocula (Fig. 1) had been retained during the 30 d growth period. Cells remained viable in the plant growth media of all treatments and replicates and retained the expected CaIHP-solubilization characteristics (Fig. S1). All strains grew on CaIHP agar with and without methyl red added (Fig. S1). Consistent with our preliminary assessment of CaIHP solubilization phenotype, wild-type CCAR59 and Ha200 strains formed acidic zones of clearing on CaIHP agar, whereas the *gcd::Tn5B8* strain did not. Therefore, the different CaIHP solubilization phenotypes in the wild-type and *Gcd* mutant strains were retained through the plant growth period and a change in the expression of this trait can be ruled out as contributing to the lack of differentiation between plants inoculated with these strains.

Discussion

Pseudomonas gluconate production and solubilization of CaIHP

This study demonstrates that the production of gluconate and media acidification by *Pseudomonas* spp. contributes to CaIHP solubilization and hydrolysis *in vitro*. Culture supernatant from the *Pseudomonas* strain (CCAR59) with the highest gluconate concentration and lowest pH was the most effective in solubilizing CaIHP. This appears to be primarily controlled by acid-solubilization and the association of gluconate with Ca^{2+} , which

synergistically leads to higher concentrations of soluble IHP in the reaction supernatants. Two gluconate anions are required to bind one Ca^{2+} , making this association weak relative to other polycarboxylate anions, which chelate at 1:1 or higher cation to anion ratios (e.g., EDTA, citrate). Previous abiotic assays confirm that CaIHP solubilization increases with decreasing pH, and that holding pH constant, occurs proportionally to gluconate concentration and therefore increasing gluconate to Ca^{2+} ratios (Giles et al., 2014). Similar effects have been reported for tricalcium phosphate solubilization by *Pseudomonas* spp., where bacterial gluconate and 2-ketogluconate production occurred in concert with acid production and phosphate solubilization (Browne et al. 2009; Buch et al. 2008; Goldstein 1995; Miller et al. 2010). We have demonstrated that *Pseudomonas* sp. CCAR59 contains a *gcd* that is similar to the PQQ-dependent *gcd* in *Pseudomonas* sp. Ha200. It is therefore likely that the solubilization and hydrolysis of CaIHP by both WT *Pseudomonas* is associated with the ability of these strains to produce gluconate via the Gcd biosynthetic pathway.

The solubilization of mineral phosphate due to organic anion production by *Pseudomonas* spp. is well-characterized, with several studies linking the function of a periplasmic glucose dehydrogenase to gluconate production and plant growth promotion (Buch et al. 2008; de Werra et al. 2009; Miller et al. 2010; Patel et al. 2011). In the current study, we have demonstrated that the hydrolysis of CaIHP is also dependent on the function of Gcd and the ability of *Pseudomonas* sp. Ha200 to produce gluconate (e.g., Fig. 3). Comparison of *Pseudomonas* sp. Ha200 and its *gcd* mutated derivative *gcd::Tn5B8* showed similar levels of cell-associated phytase activity, however enzymatic clearing was only observed for the WT colonies. This suggests that the Gcd-dependent production of gluconate is necessary for CaIHP solubilization and the subsequent enzymatic hydrolysis of IHP by phytases.

Pseudomonas glucose dehydrogenase function and plant utilization of CaIHP

The inoculation of tobacco plants with the Ha200 WT and *gcd::Tn5B8* strains led to an increase in CaIHP utilization, which was comparable to that measured in previous growth assays with the CCAR59 inoculum (Giles et al., 2014). Contrary to our hypothesis, the ability of the WT *Pseudomonas* strain Ha200 to produce gluconate did not appear to influence the availability of CaIHP to tobacco plants. This lack of response in the plant system may be due to constraints of the assay and potentially limited effects associated with the modification of a single trait.

Methodological constraints of the plant inoculation assay

The conditions necessary to elicit the expected plant-microbe interaction and resulting plant growth response may not have been fully achieved, including (1) insufficient or minor levels of glucose production by plants or (2) low bacterial cell densities in the plant growth media. As demonstrated in CaIHP clearing assays with *Pseudomonas* spp., gluconate production was dependent on the concentration of glucose provided (Fig. 1). Wild-type tobacco grown for 10 days in plant nutrient solution produced $0.45 \pm 0.1 \mu\text{mol glucose mL}^{-1} \text{d}^{-1}$, which was expected to be sufficient to support gluconate production by strain Ha200 WT (Lugtenberg et al. 1999). Gluconate was not detected in culture supernatants from strains grown on 1 mM glucose and these cultures only solubilized 30% of the total CaIHP provided in abiotic incubations (0.3 mM; Table 2). This amount of soluble IHP would exceed that provided as NaIHP in plant experiments (0.13 mM); by contrast, the lower threshold of CaIHP solubilization for cultures grown on less than 1 mM glucose was not determined. In tomato, glucose exudation varied with seed development stage and was found to be a minor component of exudate sugars (Lugtenberg et al. 1999). Whilst we did not measure the production of sugars other than glucose in the tobacco root exudates, it is possible that alternative carbon sources (e.g., fructose, maltose) could have

contributed to the production of organic anions by *Pseudomonas* sp., which were not directly under the control of glucose oxidation by the pentose phosphate pathway (Chavarria et al. 2012). As suggested by Adhikary et al. (2014), gluconic acid production and the subsequent solubilization of mineral phosphate by *Pseudomonas* spp. may rely heavily on the amount of aldose sugars present in the root exudates. Alternatively, seed colonization could have varied between wild-type *Pseudomonas* sp. and strains that were inhibited in the ability to grow on simple sugars (e.g., *zfw*, glucose-6-phosphate dehydrogenase) (Lugtenberg et al. 1999), leading to differences in the extent of plant and bacterial interaction between treatments.

Insufficient bacterial cell densities in the plant growth system may have caused limitations to the production of gluconate and root zone acidification by *Pseudomonas*. In liquid culture, the production of gluconate by Ha200 WT was dependent on cell density and growth phase, with production beginning in mid-exponential (10^8 CFU mL⁻¹; OD₆₀₀ ~0.1) and continuing into stationary growth phase (10^{10} CFU mL⁻¹; Fig. 3). In this study, the inoculum concentration (10^7 CFU mL⁻¹) in tobacco growth tubes was selected to minimize bacterial over-growth and nutrient competition during seed germination. Populations of *Pseudomonas* spp. in plant culture that did not undergo exponential phase growth (despite remaining viable for the 30 d duration of the experiment; Fig. S1) may have had limited ability to produce gluconate.

Other factors effecting CaIHP availability to tobacco

The fundamental complexity of the rhizosphere may limit the efficacy of single trait manipulations, such as gluconate production, to improving plant P availability. Plants exude multiple sugars, organic anions, and enzymes, which are expected to directly (e.g., through solubilization and mineralization of P) and indirectly (e.g., through bacterial conversion of root carbon to organic anions) influence the fate of P in the rhizosphere. In the current study, it is

possible that bacterial gluconate production did not play a major role in the response of plants to beneficial bacteria when compared to other plant growth promoting factors (e.g., plant hormones indole-3-acetic acid (IAA), 2,4-diacetylphloroglucinol, pyoluteorin, de Werra *et al.*, 2009) or the exudation of organic anions by roots. Similar levels of IAA have been detected in WT and mutant cultures of *Pseudomonas* sp. Ha200 (P.-C. L. Hsu, unpublished data) and could have contributed to the similar growth response observed in plants inoculated with these strains in the current study. In addition, acetate was detected in Ha200 WT and *gcd::Tn5B8* cultures, representing an additional organic anion source which was produced across all glucose concentrations and at the largest concentration by *gcd::Tn5B8* (Table 2). The increased level of acetate production by the mutant strain could indicate differences in glucose utilization by these strains and the partitioning of carbon to alternative metabolic pathways. Patel *et al.* (2010a) reported four-times greater CaIHP hydrolysis by phytases in incubated mixtures containing acetate compared to gluconate (50 mM, unbuffered pH 4.5), presumably due to intermediate solubilization by this organic anion. In abiotic incubations, the addition of acetate to CaIHP led to soluble IHP concentrations similar to, or greater than, those measured for gluconate ($p < 0.05$), though this phenomenon was only observed at pH 4.0 (Giles *et al.*, 2014).

Root exudation of organic anions could have exceeded or masked the effects of bacterial organic anion production if glucose were limiting gluconate production. Giles *et al.* (2012) reported concentrations of oxalate ($27 \text{ nmol plant}^{-1} \text{ d}^{-1}$), acetate ($12 \text{ nmol plant}^{-1} \text{ d}^{-1}$), citrate ($5 \text{ nmol plant}^{-1} \text{ d}^{-1}$), and pyruvate ($5 \text{ nmol plant}^{-1} \text{ d}^{-1}$) in root exudates from 10 d old wild-type tobacco. In tobacco grown for 30 d, similar rates of succinate and malate exudation have been measured (Wu *et al.* 2015). At these levels, root exudation of organic anions was an order of magnitude less than that of glucose, although it should be noted that certain organic anions such

as citrate and oxalate bind calcium more strongly than gluconate and are therefore expected to solubilize CaIHP more effectively (Giles et al. 2012, 2014). Therefore, we could not determine whether the solubilization of CaIHP was due to root exudation of organic anions or bacterial production of other organic anions such as acetate.

Bacterial phytase activity was similar between the WT and mutant *Pseudomonas* sp. cultures in this study (0.2×10^{-2} nkat plant⁻¹ day⁻¹). Furthermore, plant growth promotion by *Pseudomonas* sp. was greatest when plants were provided with CaIHP in comparison to soluble IHP and inorganic phosphate as the sole P source (Fig. 3). Whilst the mechanism of CaIHP solubilization in our study system is unclear, our results are consistent with those of previous studies in synthetic soils amended with soluble phytate (Richardson and Hadobas 1997) and in agar media with insoluble phytate (Giles et al. 2014), which show that bacterial phytase activity improves the availability of organic P to plants, particularly under conditions of limited P solubility.

Understanding the effect of single organic anions on organic phosphorus availability to plants.

Additional *in situ* analysis of bacterial interactions in CaIHP-containing plant growth systems could be used to clarify the specific conditions under which *Pseudomonas* gluconate production will enhance plant growth. Current molecular techniques such as fluorescence *in situ* hybridization have been used to visualize viable bacteria and/or gene expression in the rhizosphere (Sorensen et al. 2009). Anion exchange membranes may also be employed for *in situ* collection of root exudates and subsequent analysis of organic anions via HPLC (Shi et al. 2011a; Shi et al. 2011b). Additional extractions of plant growth media (e.g., agar, soil) for the determination of microbial P could elucidate whether root-associated microbes are accumulating P from insoluble organic P, but not necessarily ‘giving’ it to the plant, and the perturbations that

may be necessary to promote the release of microbial P to plants (e.g., freeze-thaw or drying-rewetting cycles, (Blackwell et al. 2010); nematode grazing and soil trophic interactions, (Becquer et al. 2014). The application of these methods to visualize viable cell density, *gcd* expression, and composite plant and bacterial metabolite production would complement plant growth assays, the combination of which would help to demonstrate the influence of specific organic anions on CaIHP solubilization, hydrolysis, and bioavailability.

Conclusions

The combination of organic anion and phytase production by plants and microorganisms in the rhizosphere is one possible approach to improving the bioavailability of insoluble phytate in soils. In this study, we examined the role of bacterial gluconate production in the solubilization and mineralization of CaIHP and subsequent uptake of released P by plants. We showed that the ability of *Pseudomonas* sp. to produce gluconate, acidify the growth media, and hydrolyze CaIHP was dependent on the presence of a functional glucose dehydrogenase gene. However, the presence of a functional *Gcd* did not have a significant effect on the ability of tobacco plants to assimilate P from CaIHP when grown under controlled conditions in an agar culture system. The lack of P assimilation could be caused by the experimental plant growth conditions, specifically, if the appropriate levels of glucose exudation by tobacco or *Pseudomonas* sp. cell density were not achieved. It is also possible that the production of other plant growth promoting factors (e.g., hormones, other organic anions, sugar source) or microbial sequestration of P may ultimately have a larger effect on organic P availability and outweigh any growth benefits gained from *Pseudomonas* gluconate production alone. Further work is needed to understand the interaction of microbial and plant-derived exudates in the rhizosphere and the relative contribution of these processes to the mobilization of P in more complex soil systems.

Acknowledgements

We thank Matthew Wargo (UVM Microbiology and Molecular Genetics), David C. Lewis and Tina Rathjen (CSIRO Plant Industry), Milko Jorquera (Universidad de La Frontera, Chile), Johanna Mayerhofer (2009-2010, UVM Barrett Scholar), Brook Clinton (UVM School of Engineering), and the James M. Jeffords Center (2010-2011, UVM Graduate Research Fellowship) for their technical expertise and support. We would also like to acknowledge MBIE Grant C10X0904 lead by Dr. Carolyn Mander, enabling the isolation and manipulation of *Pseudomonas* sp. Ha200, as well as support provided by Vermont EPSCoR with funds from the National Science Foundation Grant (EPS-1101317) and a BBSRC responsive mode grant (BBK0170471).

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774 **Table 1** Description of plasmids, strains and primers used in this study.

Bacterial strains & plasmids	Description	Reference
pUTKm1	Kn ^R ; mini-Tn5Km1 transposon delivery vector	DeLorenzo et al. 1990
pBBR1MCS-5	GmR; broad host range vector	Kovach et al. 1995
<i>Escherichia coli</i> DH10B	F- <i>mcrA</i> Δ <i>mrr-hsdRMS-mcrBC</i> Δ 80d <i>lacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>endA</i> 1 <i>recA</i> 1 <i>deoR</i> Δ <i>ara</i> , <i>leu</i> 7697 <i>araD</i> 139 <i>galU</i> <i>galK</i> <i>nupG</i> <i>rpsL</i> Δ -.	Lorow and Jessee 1990
<i>Pseudomonas</i> sp. CCAR59	Wild-type soil isolate (Australia)	Richardson and Hadobas 1997
<i>Pseudomonas</i> sp. Ha200 WT	Wild-type soil isolate (Haast, Native forest, New Zealand, AgResearch culture collection)	This Study
<i>Pseudomonas</i> sp. Ha200 <i>gcd</i> ::Tn5B8	Gm ^R Kn ^R ; <i>gcd</i> ::Tn5Km1 insertion at 1818 bp 3' of the initiation codon of <i>gcd</i> , derivative of <i>Pseudomonas</i> sp. Ha200	This Study
Primer name	Oligonucleotides (5'→3') *	Reference
Uni1	AATACGACTCACTATAGN10GATC	Sarkar et al. 1993
Uni2	AATACGACTCACTATAGN10GAATTC	Sarkar et al. 1993
Uni4	AATACGACTCACTATAGN10GCGC	Sarkar et al. 1993
Uni5	GTAATACGACTCACTATAGGGCN10GCAGC	Sarkar et al. 1993
T7	TAATACGACTCACTATAGGG	Sarkar et al. 1993
Sp1	CAGGCTGACCCTGCGCGCTGCGCA	This study
Sp2A	GGATCCTCTAGAGTCGACCTGCAG	This study
Sp2B	CCCCGGGTACCGAGCTCGAATTCGG	This study
B8B11-Gcd-RF	ATCTATCCGGGTAACGTCGG	This study
B8B11-Gcd-R1	TABTGCTTDCRTCCTTGC	This study
B8B11-Gcd-LR	ATGTTCCGGCGAGTTGCGG	This study
B8B11-Gcd-L2	ATNNTGCTNCTGCTRATGGGC	This study
F1	GARGTRGGCCTGGACTGGTG	This study
R2	GTARAGCATGCCGTTGRCCT	This study

775 * Underscore denotes specific oligonucleotide from the Restriction Site Oligomers corresponding to the internal T7 sequence.

Table 2 Organic anion concentrations and pH of supernatants collected from *Pseudomonas* spp. (strains CCAR59, Ha200 and Ha200 *gcd*::Tn5B8) and IHP released from calcium-phytate (CaIHP) following 24 h incubation with bacterial supernatants.

Organic Anions *	Glucose (mM)			
	0	1	11	110
<i>Pseudomonas</i> sp. CCAR59 WT				
Ascorbate	nd	nd	0.7 (0.1)	2.5 (0.1)
2-keto-gluconate	nd	nd	2.7 (0.2)	7.1 (0.3)
Pyruvate	nd	nd	1.0 (0.1)	1.1 (0.2)
Gluconate	nd	nd	8.5 (0.1) ^A	25.1 (1.9) ^B
Formate	nd	nd	nd	nd
Acetate	0.5	1.1 (0.1)	1.2 (0.1)	1.2 (0.2)
pH [†]	5.5	5.5	4	4
IHP release [‡]	0.25(0.04) ^{A,e}	0.32(0.06) ^{A,e}	0.63(0.05) ^{B,e}	0.79(0.03) ^{C,e}
<i>Pseudomonas</i> sp. Ha200 WT				
Ascorbate	nd	nd	nd	nd
2-keto-gluconate	nd	nd	1.2 (0.2)	3.6 (0.5)
Pyruvate	nd	nd	0.1 (0)	0.2 (0)
Gluconate	nd	nd	3.7 (0.1) ^A	11.8 (0.3) ^B
Formate	nd	nd	nd	nd
Acetate	0.6 (0.1)	0.9 (0.1)	0.8 (0.1)	1.5 (0)
pH	5.5	5.5	4.5	4.5
IHP release	0.16(0.03) ^{A,f}	0.20(0.04) ^{A,f}	0.57(0.04) ^{B,e}	0.58(0.03) ^{B,f}
<i>Pseudomonas</i> sp. Ha200 <i>gcd</i>::Tn5B8				
Ascorbate	nd	nd	nd	nd
2-keto-gluconate	nd	nd	nd	nd
Pyruvate	nd	nd	0.1 (0)	nd
Gluconate	nd	nd	nd	nd
Formate	nd	nd	0.5 (0)	0.4 (0)
Acetate	0.9 (0.1)	0.8 (0.2)	1.8 (0)	2.0 (0)
pH	5.5	5.5	5.5	5.5
IHP release	0.13(0.00) ^{A,f}	0.13(0.01) ^{A,g}	0.14(0.01) ^{AB,f}	0.15(0.01) ^{B,g}

*Values represent the average organic anion concentration with 1 standard deviation shown in parenthesis; nd = none detected. [†]pH measured in bacterial culture supernatants following 13 d growth in 15mM MES media (initial pH 5.5 with 0 to 110 mM glucose). [‡] Final IHP concentration (mM) in 24 h reaction supernatants of CaIHP incubated with *Pseudomonas* sp. culture supernatants. Statistically difference means (p<0.05) between columns are indicated by capital letters, or between rows by lower-case letters.

Figure Captions

Figure 1 Zones of clearing formed by *Pseudomonas* spp. soil isolate strain CCAR59 (A), strain Ha200 WT (B) and an isogenic mutant in the glucose dehydrogenase gene (C; Ha200 *gcd::Tn5B8*). Growth on calcium-phytate screening media with glucose added at 0, 11, or 110 mM. No treatment (NT), methyl red (MR, 0.01% w/v), and cobalt chloride (CoCl₂, 2% w/v) treatments are shown.

Figure 2 *Pseudomonas* sp. CCAR59 wild-type (A-D), Ha200 WT (E-H) and transposon insertion mutant in the glucose dehydrogenase (*gcd::Tn5B8*) grown in 15 mM MES-buffered minimal media (pH 5.5) with 0, 1, 11, or 110 mM glucose supplied. Growth (OD₆₀₀, dashed grey line), culture pH (solid grey line), concentration (mM) of gluconate (GA, solid black line), and acetate (AA, dashed black line) over time are shown. Organic anion concentrations were determined by HPLC. *Note scale change for panel D. Where shown, error bars indicate 1x SE.

Figure 3 Shoot dry weight (A) and total shoot P content (B) of *Nicotiana tabacum* plants after 32 days growth in sterile media (None) or inoculated with *Pseudomonas* sp. strain Ha200; either wild-type (WT) or a isogenic mutant in the glucose dehydrogenase gene (*gcd::Tn5B8*). Media P was supplied as insoluble (CaIHP, CaPO₄), soluble (NaIHP, NaPO₄), or No P. For each P source, significant differences between inocula are shown as ** $p < 0.01$ (n=5).

Figure S1 *Pseudomonas* sp. cell viability tests on CaIHP agar (110 mM glucose, 48 h, 30°) following 32 d plant growth inoculation study. Stab cultures from (1) uninoculated plant growth media or plant growth media inoculated with (2) *Pseudomonas* sp. Ha200 wild-type or (3) Ha200 *gcd::Tn5B8* are shown. Letters indicate phosphorus source: A, CaIHP; B, CaPO₄; C, NaIHP; D, NaPO₄; E, No P. CaIHP agar plates with (+) or without (-) methyl red are shown to indicate media acidification and the ability of strains to solubilize CaIHP.

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Figure 1. Zones of clearing formed by *Pseudomonas* spp. soil isolate strain CCAR59 (A), strain Ha200 wild-type (B) and an isogenic mutant in the glucose dehydrogenase gene (C; Ha200 *gcd::Tn5B8*). Growth on calcium-phytate screening media with glucose added at 0, 11, or 110 mM. No treatment (NT), methyl red (MR, 0.01% w/v), and cobalt chloride (CoCl₂, 2% w/v) treatments are shown.
196x76mm (150 x 150 DPI)

Figure 2. *Pseudomonas* sp. CCAR59 wild-type (A-D), Ha200 wild-type (E-H) and transposon insertion mutant in the glucose dehydrogenase (*gcd::Tn5B8*) grown in 15 mM MES-buffered minimal media (pH 5.5) with 0, 1, 11, or 110 mM glucose supplied. Growth (OD600, dashed grey line), culture pH (solid grey line), concentration (mM) of gluconate (GA, solid black line), and acetate (AA, dashed black line) over time are shown. Organic anion concentrations were determined by HPLC. *Note scale change for panel D. Where shown, error bars indicate 1x SE.

241x149mm (150 x 150 DPI)

Figure 3. Shoot dry weight (A) and total shoot P content (B) of *Nicotiana tabacum* plants after 32 days growth in sterile media (None) or inoculated with *Pseudomonas* sp. strain Ha200; either wild-type (WT) or a isogenic mutant in the glucose dehydrogenase gene (*gcd::Tn5B8*). Media P was supplied as insoluble (CaIHP, CaPO₄), soluble (NaIHP, NaPO₄), or No P. For each P source, significant differences between inocula are shown as ** $p < 0.01$ (n=5).

168x124mm (150 x 150 DPI)