

# The protons of gluconic acid are the major factor responsible for the dissolution of tricalcium phosphate by *Burkholderia cepacia* CC-A174

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## Abstract

*Burkholderia cepacia* CC-A174 with a high ability for solubilizing tricalcium phosphate (TCP) was used to study the P-solubilization mechanism. We collected filtrates able to solubilize TCP from the cultures of strain CC-A174 and demonstrated that the P-solubilization increased from  $0 \mu\text{g ml}^{-1}$  to  $200 \mu\text{g ml}^{-1}$  during exponential growth, when the pH decreased from 8 to 3. HPLC-analysis revealed that the solubilization of TCP was mainly caused by the release of 16.3 mM gluconic acid. At this concentration, gluconic acid was capable of solubilizing  $376 \mu\text{g ml}^{-1}$  of TCP whereas water at pH 3 only solubilized  $35 \mu\text{g ml}^{-1}$ . The difference is due to the final  $\text{H}^+$  concentrations which were 13.5 mM and 1 mM in 16.3 mM gluconic acid and deionized water, respectively at pH 3.

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**Keywords:** *Burkholderia cepacia*; Phosphate solubilizing; Bacteria; Solubilization mechanisms; Gluconic acid

## 1. Introduction

Phosphorus is an essential macronutrient for plants and is added to soil in the form of phosphatic fertilizers. It has been estimated that in some soil up to 75% of applied phosphate fertilizer may become unavailable to the plant because of mineral phase reprecipitation (Goldstein, 1986; Sundara et al., 2002). Phosphate-solubilizing bacteria (PSB) are able to convert insoluble phosphates into soluble forms (Illmer and Schinner, 1995; Hilda et al., 2000; Peix et al., 2002; Viverk and Singh, 2001; Sudhakara et al., 2002) and have therefore been used to enhance the solubilization of reprecipitated soil P for crop improvement. (Young et al., 1986; Young,

1990; Shekhar et al., 2000). Several studies have shown that the release of low molecular weight organic acids is responsible for solubilizing insoluble phosphate (Sperber, 1957; Goldstein, 1995; Kim et al., 1997; Hilda and Fraga, 1999). The most efficient mineral phosphate solubilization (MPS) phenotype in Gram-negative bacteria results from extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase to gluconic acid (Goldstein and Liu, 1987; Liu et al., 1992; Kpombekou-A and Tabatabai, 1994; Hilda and Fraga, 1999; Hilda et al., 2000). The resulting pH change and reduction potential are thought to be responsible for the dissolution of tricalcium phosphate in the culture medium.

In the current study, we collected filtrates from the PSB *Burkholderia cepacia* CC-A174 cultures grown under  $\text{Ca}_3(\text{PO}_4)_2$ -limited condition. Our objectives were to analyze the organic acid composition of the filtrates and to test if the organic acids present were capable of solubilizing calcium phosphate.

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## 2. Methods

### 2.1. Bacteria strain, media and growth conditions

The phosphate-solubilizing bacterium CC-A174 used in this experiment was isolated from soil in Pingdong, Taiwan and identified as *B. cepacia* based on its 16S ribosomal DNA (rDNA) sequence. Genomic DNA was extracted from strain CC-A174 by using the MO BIO UltraClean Microbial DNA Kit (MO BIO, USA) according to the protocol supplied by the supplier. The 16S rRNA genes were amplified by PCR with forward primer 1F (5'-GAG TTT GAT CAT GGC TCA G-3') and reverse primer 7R (5'-TGA CGG GCG GTG TGT ACA A-3') (Brosius et al., 1978; Edwards et al., 1989; Young et al., 2005). The PCR products were purified using the Montage DNA Gel Extraction Kit (Millipore, USA), and were used as template for cycle-sequencing. The sequencing reaction products were analyzed using an ABI PRISM 310 Genetic analyzer (Perkin-Elmer). The sequence was submitted to GenBank under accession number AY149233. Strain CC-A174 was deposited at the Bioresource Collection and Research Center (BCRC), Food Industry Research and Development Institute, Taiwan, ROC. The number of strain CC-A174 in BCRC is 14256.

For the assay of mineral phosphate solubilization (MPS), the strain was cultivated in modified liquid tricalcium phosphate (TCP) medium (Young, 1990) containing the following ingredients per liter: 10 g sucrose, 0.27 g  $\text{NH}_4\text{NO}_3$ , 0.2 g KCl, 0.001 g  $\text{MgSO}_4 \cdot 4.6\text{H}_2\text{O}$ , 0.001 g  $\text{Fe}_2(\text{SO}_4)_3 \cdot 7\text{H}_2\text{O}$ , 0.001 g  $\text{MnSO}_4 \cdot 4.6\text{H}_2\text{O}$  and 0.05 g  $\text{Ca}_3(\text{PO}_4)_2$ . The pH of the medium was adjusted to 6.8 with 0.1 M HCl before autoclaving. The concentration of  $\text{Ca}_3(\text{PO}_4)_2$  in liquid TCP medium was  $50 \text{ mg l}^{-1}$ , a limiting concentration that was assumed to allow detection of solubilizing exudates.

Strain CC-A174 was inoculated into 500 ml liquid medium in 1 L conical flasks (ca.  $10^3 \text{ cfu ml}^{-1}$ ) and incubated on a gyratory shaker (120 rpm) at 25 °C. Samples were taken at 0, 28, 52, 76, 100, 130, 151, 174 and 199 h. Autoclaved, uninoculated medium served as control. A 5-ml sample was removed from each flask and centrifuged at 10,000g for 5 min (HETTICH, MIKRO 22R, Germany). The supernatant was filtered through a 0.22  $\mu\text{m}$  Millipore filter and assessed for bacterial population, pH, organic acids, and P-solubilizing capability.

### 2.2. Effects of gluconic acid and 2-keto-gluconic acid on TCP solubilization

The pH of 16.3 mM gluconic acid and 3.8 mM 2-keto-gluconic acid was adjusted to pH 3 with HCl for the analysis of the P-solubilizing capability. Uninoculated medium and deionized water at pH 3 served as control and blank, respectively.

### 2.3. Analysis

Cell counts of strain CC-A174 were obtained by dilution-plating on solid TCP medium. The pH of culture filtrates was measured with a pH meter (SUNTEX, SP-2200, Taiwan). The determination of organic acids was carried out by HPLC (Hitachi L-5000, Japan; detector: Hitachi L-3000, Japan; column: Bio-Red aminex HPX-87H, US; solvent: 10.8% acetonitrile in 0.0035 M  $\text{H}_2\text{SO}_4$ ; flow:  $0.5 \text{ ml min}^{-1}$ ; temperature: 35 °C; UV: detector at 210 nm; during: 35 min; injection volume: 20  $\mu\text{l}$ ). The HPLC-system was capable of distinguishing 13 kinds of organic acids including citric, gluconic, 2-keto-gluconic, succinic, glycolic, lactic, fumaric, formic, acetic, butyric, isobutyric, valeric and isovaleric acid.

P-solubilization was tested by incubating 1 ml of filtrate at 25 °C with 10 mg TCP under shaking condition (120 rpm). After 1 h, the assay mixtures were centrifuged at 10,000g for 5 min. The supernatant was filtered through a 0.22  $\mu\text{m}$  Millipore filter and assessed for dissolved P. The P-solubilizing capability was calculated as the difference of dissolved P between added (10 mg TCP) and non-added sample.

Values are given as means  $\pm$  S.D. for triplicate samples. Data were analyzed using Microsoft Excel software or Duncan's multiple range test. Differences in the results were considered to be significant at the  $P < 0.05$  level.

## 3. Results and discussion

### 3.1. Properties of solubilizing TCP of *B. cepacia* CC-A174

The homology of 16S ribosomal sequence of strain CC-A174 to the reference isolate is 100%. It showed that strain CC-A174 was identified as *B. cepacia*.

Changes in the population, pH and P-solubilizing capability of the culture medium are represented in Fig. 1. The population reached a maximum cell density of about  $10^7 \text{ cfu ml}^{-1}$  after 52 h. The P-solubilizing capability increased from zero to  $200 \mu\text{g ml}^{-1}$  as the pH decreased from 8 to 3. Strain CC-A174 released two kinds of organic acids at 100 h and 130 h, namely 16.3 mM gluconic acid and 3.8 mM 2-keto-gluconic acid (Fig. 2). The gluconic acid and 2-keto-gluconic acid produced by strain CC-A174 potentially play a vital role in the P-solubilization, since the most efficient MPS phenotype in Gram-negative bacteria results from extracellular oxidation of glucose via the quinoprotein glucose dehydrogenase (Goldstein, 1995), resulting in acidification of the region adjacent to the cell (Goldstein and Liu, 1987; Liu et al., 1992; Hilda and Fraga, 1999; Hilda et al., 2000).

The concentration of gluconic acid and 2-keto-gluconic acid was very low until after 76 h whereas dissolved

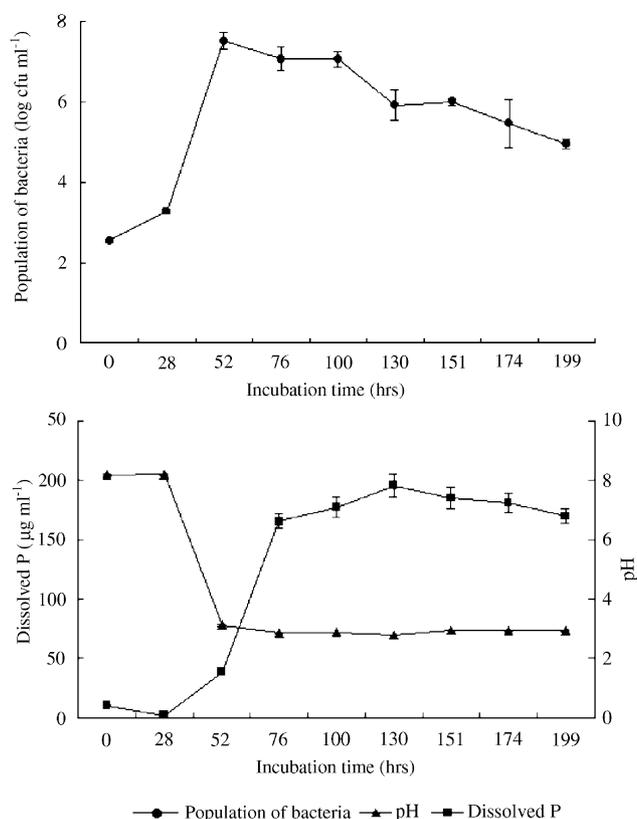


Fig. 1. Changes of the population of bacteria, the P-solubilizing capability and pH value in liquid tricalcium phosphate medium during incubation with *Burkholderia cepacia* CC-A174 for 199 h. Values are means of three replicates. Error bars ( $\pm$ S.D.) are shown when larger than the symbol.

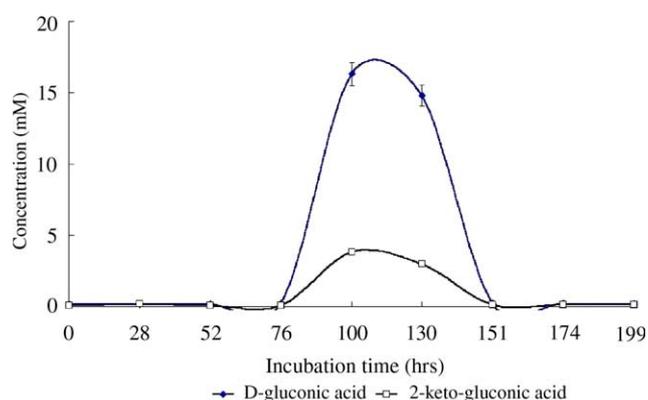


Fig. 2. Changes of the concentration of D-gluconic acid and 2-keto-gluconic acid in liquid tricalcium phosphate medium during incubation with *Burkholderia cepacia* CC-A174 for 199 h. Values are means of three replicates. Error bars ( $\pm$ S.D.) are shown when larger than the symbol.

P increased from zero to  $160 \mu\text{g ml}^{-1}$  as the pH decreased from 8 to 3. The process of acidification and chelation by gluconic acid and 2-keto-gluconic acid dissolved TCP in cultural medium. The chelation property of gluconic acid enables it to form insoluble complex

with  $\text{Ca}^{2+}$  liberating phosphates (Kpombrekou-A and Tabatabai, 1994; Reyes et al., 1999; Shekhar et al., 2000).

### 3.2. Effects of gluconic acid and 2-keto-gluconic acid on TCP solubilization

The P-solubilizing capability of bacterial filtrate reached a plateau at pH 3 (Fig. 1), when 16.3 mM gluconic acid and 3.8 mM 2-keto-gluconic acids were detected in the filtrates of strain CC-A174 (Fig. 2). Therefore we further explored the P-solubilizing capability of 16.3 mM gluconic acid and 3.8 mM 2-keto-gluconic acid at pH 3. The P-solubilizing capability of 16.3 mM gluconic acid and 3.8 mM 2-keto-gluconic acid were  $376 \mu\text{g ml}^{-1}$  and  $67 \mu\text{g ml}^{-1}$ , respectively at pH 3 (Table 1). The final pH of gluconic acid and 2-keto-gluconic acid after equilibration were 4.7 and 5.7, respectively. The P-solubilizing capability of gluconic acid was much higher compared to 2-keto-gluconic acid in the filtrate from strain CC-A174 culture.

The P-solubilizing capability in culture solution was  $200 \mu\text{g ml}^{-1}$ , and that of 16.3 mM gluconic acid in deionized water was  $376 \mu\text{g ml}^{-1}$  at pH 3 (Table 1). Therefore, the filtrates of strain CC-A174 were capable of solubilizing only 53% of the total P released by gluconic acid in deionized water. Presumably the metals present in liquid TCP medium such as Fe, Ca, Mn, Mg, K reacted with dissolved P resulting in the conversion of soluble phosphate in the filtrates of CC-A174 into insoluble forms (Corbridge, 1980; Snoeyink and Jenkins, 1982; Stumm and Morgan, 1996). Similarly, the P-solubilizing capability in deionized water was greater than that of uninoculated medium (Table 1), which can also be attributed to the reaction of metals in medium with soluble phosphate.

In addition, the P-solubilizing capabilities of 16.3 mM gluconic acid and deionized water were  $376 \mu\text{g ml}^{-1}$  and  $35 \mu\text{g ml}^{-1}$  at pH 3 (Table 1). When the pH of 16.3 mM gluconic acid and deionized water were modified to 3, the theoretical concentrations of protons were 13.5 mM and 1 mM, respectively, showing clearly that the

Table 1  
P-solubilizing by *B. cepacia* CC-A174 culture filtrate, 16.3 mM D-gluconic acid, 3.8 mM 2-keto-gluconic acid, uninoculated medium and deionized water at pH 3

Treatment	Dissolved P ( $\mu\text{g ml}^{-1}$ )
Culture filtrate	$200 \pm 4.8\text{b}^{\text{a}}$
16.3 mM D-gluconic acid	$376 \pm 4.9\text{a}$
3.8 mM 2-keto-gluconic acid	$67 \pm 3.3\text{c}$
Uninoculated medium (CK)	$9 \pm 0.5\text{e}$
Deionized water (BK)	$35 \pm 1.8\text{d}$

<sup>a</sup> Values (means  $\pm$  S.D.,  $n = 3$ ) within a row followed by different letters are significantly different ( $P < 0.05$ ) by Duncan's multiple range test.

concentration of protons in the solution is an important factor for P-solubilization.

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