Organic acid production and phosphate solubilization by

*Enterobacter intermedium* 60-2G


**ABSTRACT**

A phosphate solubilizing bacterium, strain 60-2G, possessing a strong ability to solubilize insoluble phosphate was isolated from the rhizosphere of grass. On the basis of GC-FAME profile, carbon utilization pattern, and the DNA sequence of a conserved partial 16S rRNA gene, the 60-2G was identified as *Enterobacter intermedium*. The analysis by HPLC revealed that the strain 60-2G produced mainly gluconic and 2-ketogluconic acids with small amounts of lactic acid in broth culture medium containing hydroxyapatite. During the incubation period of the strain 60-2G in broth culture, pH of the medium decreased up to 3.8 while the soluble phosphate concentration increased. The reversed correlation between pH and soluble phosphate concentration indicated that the solubility of P was due to the produced organic acids. The sequence homology of the deduced amino acids suggested that *E. intermedium* 60-2G synthesized PQQ which is essential for the oxidation of glucose by glucose dehydrogenase.

**Key words**: Gluconic acid, Hydroxyapatite, 2-ketogluconic acid, Phosphorus, *Enterobacter intermedium*.

Phosphorus (P) is one of the major essential macronutrient for plant growth and development. However, the available P in natural soil is easily converted into insoluble complexes such as iron and aluminum hydrous oxides, crystalline and amorphous aluminum silicate, and calcium carbonate (Sample et al., 1980). Therefore, a large quantity of soluble forms of P fertilizers are applied.

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to achieve a maximum yield even though plants have evolved efficient mechanisms for absorbing P from soil. The applied soluble forms of P fertilizers are easily precipitated into insoluble forms, which lead to an excess application of P fertilizer to crop land. This unmanaged excess of P application is known to cause environmental and economic problems (Brady, 1990).

Soil and rhizosphere naturally harbor phosphate-solubilizing bacteria (PSB), which can solubilize poorly available inorganic P in crop land (Babenko et al., 1984, Illmer and Schinner, 1992). The solubilization mechanism by PSB was explained by the production of organic acids (Illmer et al., 1995, El-gibaly et al., 1977). Sperber (1957) has identified organic acids where lactic acid was predominant with smaller quantities of glycolic, fumaric, adipic and succinic acids in culture media containing hydroxyapatite. Leyval and Berthelin (1989) attributed the solubilization of inorganic phosphate to the production and release of organic acids, mainly malic and lactic acids. Liu et al. (1992) reported that phosphate solubilization by Erwinia herbicola was due to production of gluconic acid. Moghimi et al. (1978) and Moghimi and Tate (1978) found that 2-ketogluconic acid was a main organic acid responsible for phosphate solubilization.

The objectives of this study are to isolate and identify PSB having strong phosphate solubilization ability from rhizosphere soil, to identify the organic acids produced by PSB, to explain the relationships among soluble P concentration, pH and organic acids, and finally to identify pqq gene(s) that is essential for P solubilization.

Material and method

Isolation of PSB

A bacterium was isolated and identified from the rhizosphere soils in Chonnam Province, Republic of Korea. Rhizosphere soils were brought to the laboratory and standard serial dilutions were performed. An appropriate soil dilution was inoculated on a standard medium containing glucose, 15.00 g; yeast extract, 0.50 g; calcium chloride, 0.10 g; magnesium sulfate, 0.25 g; ammonium chloride 0.50 g; hydroxyapatite, 4.00 g; and agar, 15.00 g per liter of distilled water at pH 7.00. After 3-day incubation at 30°C, PSB developed clear zones (Kim et al., 1997). Among the isolate, a bacterium possessing a strong phosphate solubilization trait was selected and named as 60-2G. The bacterium was treated with 25% glycerol and kept at -70°C for further experiments.

Polymerase chain reaction (PCR) and sequence of 16S rRNA gene

Polymerase chain reaction was performed to amplify a part of the 16S rRNA gene of phosphate solubilizing bacterium, 60-2G. The forward primer Y1 (5'-TGG CTC AGA AGC AAC GCT GGC GGC-3') corresponded to the positions of 20 to 43 and the reverse primer Y2 (5'-CCC ACT GCT GCC TCC CGT AGG AGT-3') corresponded to the positions of 361-338 in the Escherichia coli 16S rRNA sequences. The sequences were highly conserved in a wide range of bacteria and chloroplast 16S rRNA sequences (Young et al., 1991). The PCR product was cloned using T4 cloning kit (Invitron). The nucleotide sequence of 16S rRNA gene of the 60-2G was determined by Biodye™ Terminator
cycle sequencing kit and compared with published 16S rRNA sequences using Blast search at NCBI.

Cloning and homology of pqq gene

The primers for PCR amplification and sequencing were chosen carefully. The forward primer X1 (5'-GGC TGC TGG CCG AAC TGA CTT-3') and the reverse primer X2 (5'-GTC CGC AAG AAG CAT TAT TAG -3') were exactly matched the sequences (ID AF007584) found in ORF2 of Rahnella aquatilis pqq genes (Kim et al., 1998). The total DNA of E. intermedium 60-2G and primers (X1 and X2) were mixed, and amplified by PCR. The PCR product was cloned using T-easy vector (Promega). The nucleotide sequences of the pqq gene of E. intermedium 60-2G were determined by Biodye™ Terminator cycle sequencing kit. The deduced amino acid sequences were obtained from the nucleotide sequences, and then compared with known pqq amino acid sequences using Blast search at NCBI.

Cellular fatty acid analysis and carbon nutrition

A PSB, 60-2G, was grown in Tryptic Soy Broth agar (TSBA). About 100 mg of biomass of 60-2G was taken from TSBA plate and the fatty acid was extracted. The GC-FAME analysis revealed fatty acid profile and compared it with known fatty acid profile of the various bacteria which have been deposited in GC-FAME database. Biolog™ was used for microbial identification and characterization by carbon utilization patterns.

Bacterial growth, medium pH, and P solubilization.

Bacterial growth, pH changes of the medium, and P concentrations were measured in the standard broth culture medium at 0, 12, 24, 48, 72, 120, and 240 hours with shaking at 200 rpm at 30°C. One hundred ml of standard medium was dispensed into each of 250-ml bottles followed by inoculation with 10 μl of E. intermedium 60-2G culture (10⁶/ml) grown in LB broth at 30°C for one day. Medium pH was measured using a pH meter equipped with a glass electrode. The P concentration solubilized by the bacterium was measured using a spectrophotometer at 660 nm (Olsen et al., 1982).

Determination of organic acids

For the organic acid determination, the culture solution was filtered through a 0.2 μm Whatman membrane filter into a 1.5 ml Eppendorf tube. The organic acids in the purified solution were determined using a Shimazu SPD-10A HPLC with a UV-Vis detector at 210 nm and a RS pak KC-811 (7.8 mm ID x 300 mm L) column made by Shodex. The operating conditions consisted of 0.1% H₃PO₄ as the mobile phase, a constant (isocratic) flow rate of 0.5 ml min⁻¹, column temperature of 25°C, and a sample injection of 20 μl. The organic acids were identified by comparing the retention times and peak areas of chromatograms with those of the standards.

Results and discussion

Identification of PSB, strain 60-2G

The isolate, strain 60-2G, has strong phosphate solubilization ability in standard agar medium containing 0.4% hydroxyapatite as visualized by clear zones around colonies developed after 3-day incubation at 30°C (Fig. 1). Biolog™ analysis results revealed that this bacterium utilized N-acetyl-D-
glucosamine, L-arabinose, D-arabitol, D-galactose, D-manno, D-raffino and D-trehalose. This bacterium was identified as *Enterobacter intermedium*, which is a gram negative, rod-shaped, and has a motility characteristic. The bacterium was confirmed as *E. intermedium* using GC-FAME analysis with 80.7% possibility and named as *E. intermedium* 60-2G. Part of the 16S rRNA gene of *E. intermedium* 60-2G amplified by PCR was compared to the corresponding sequences of other bacterial 16S rRNA gene using Blast search at NCBI. Within 327 bp that were compared, there was only one base difference between *E. intermedium* 60-2G (ID AF297470) and *E. intermedium* 16S rRNA sequences (ID AB004747).

**Fig. 1.** Clear zones of phosphate solubilization around colonies of *Enterobacter intermedium* 60-2G on agar medium containing 0.4% hydroxyapatite at 30°C for 3-day incubation.

*E. intermedium* 60-2G released three kinds of organic acids, among which gluconic and 2-ketogluconic acids were predominant with a small amount of lactic acid. The concentration of gluconic acid remarkably increased to 5,900 mg/l at 24 h, but was followed by a marked decrease to 2,526 mg/l at 48 h (Fig. 3). After 48 h, the amount of gluconic acid slowly increased. The concentration of 2-ketogluconic acid was only 370 mg/l at 12 h, but suddenly increased to 2,300 and 6,370 mg/l at 24 h and 48 h, respectively, with a maximum concentration of 8,465 mg/l at 240 h. Similar results were observed by Svitel and Sturdik (1995) pointing that the concentration of gluconic acid produced by *Acetobacter pasteurianus* markedly increased in a culture medium containing glucose the first day of incubation and then decreased with the function of time. Meanwhile the concentration of 2-ketogluconic acid continuously increased in the culture medium. These authors reported that the 2-

![Absorbance Time Graph](image)

**Fig. 2.** Identification of organic acids produced by *E. intermedium* 60-2G in culture medium containing 0.4% hydroxyapatite at 30°C during 1 day incubation.

a: 2-ketogluconic acid, b: gluconic acid, c: lactic acid
Fig. 3. Concentration of organic acids produced by *Enterobacter intermedium* 60-2G in culture medium containing 0.4% hydroxyapatite at 30°C during 10 day incubation.
- ●: gluconic acid, ■: 2-ketogluconic acid, ▲: lactic acid

Ketogluconic acid was produced from glucose, via glucose dehydrogenase, to gluconic acid, which was subsequently dehydrogenated to 2-ketogluconic acid, via gluconate dehydrogenase. As shown in Fig. 3, the rapid increase of the gluconic acid concentration from 12 h to 24 h, indicated that *E. intermedium* 60-2G produced glucose dehydrogenase, which converted the glucose to gluconic acid. When gluconic acid was present in the culture medium, *E. intermedium* 60-2G released gluconate dehydrogenase, which oxidized gluconic acid to 2-ketogluconic acid. These may be the reason why 2-ketogluconic acid continuously increased, while gluconic acid decreased after the first day (Fig. 3).

The organic acids produced by *E. intermedium* 60-2G lowered the pH of the broth culture medium to 3.8, which resulted in a marked increase in soluble P concentration (Fig. 4). During the growth period of *E. intermedium* 60-2G, the soluble P concentration was inversely correlated with pH in culture medium, which was concomitant with increased organic acid concentrations (Fig. 3 and 4). Our previous results (Kim et al., 1997, 1998) revealed that phosphate solubilization was due to production of gluconic acid in broth culture medium containing hydroxyapatite. Moghimi et al. (1978) and Moghimi and Tate (1978) studied the release of P from calcium phosphate by rhizosphere products where P solubilization was due to the large amounts of 2-ketogluconic acid. Bar-Yoself et al. (1999) found that both of gluconic acid and 2-ketogluconic acid produced by *Pseudomonas cepacia* were responsible for the solubilization of insoluble phosphate in kaolinite and montmorillonite suspension.

**Fig. 4. Changes in pH and phosphorus concentration mediated by *Enterobacter intermedium* 60-2G in culture medium containing 0.4% hydroxyapatite at 30°C during 10 day incubation.**
- ●: P concentration, ◆: pH

**Homology of *pqg* gene**
In most phosphate solubilizing bacterial species,
Gluconic acid is produced by a direct extracellular oxidation of glucose by glucose dehydrogenase. To become an activated form, the enzyme requires glucose dehydrogenase needs a cofactor such as pyroloquinoline quinone (PQQ). However, some soil bacteria such as *Rhizobium leguminosarum* and *R. meliloti* have the capability to synthesize apo-glucose dehydrogenase, but not the cofactor, PQQ, which gives very little phosphate solubilization trait (Van Schie et al., 1987, Boiard et al., 1996). Therefore, PQQ is very essential for the soil bacteria to actively convert insoluble P into soluble form in soil environment. Our previous data (Kim et al., 1997, 1998) showed that *Rahnella aquatilis* exhibited a strong ability to solubilize hydroxyapatite and dicalcium phosphate in the external culture medium. From the results of HPLC analysis, we have identified gluconic acid as the main organic acid released by *R. aquatilis* which synthesized PQQ as a cofactor of glucose dehydrogenase. To ensure that *E. intermedium* 60-2G has the ability to synthesize PQQ, homology study was performed. A partial *pqq* gene of *E. intermedium* 60-2G was sequenced and then deduced amino acid sequences were obtained. As

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</tr>
<tr>
<td>E.herbicola</td>
<td>14 AA...S..................................................</td>
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</tr>
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<td>E.herbicola</td>
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<td>83 V.....G.....E.....I.....E...I.....Q...............</td>
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<tr>
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<td>R.aquatilis</td>
<td>133 E.....NA.....A.....M..............................</td>
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<tr>
<th>60-2G</th>
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<tr>
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<td>R.aquatilis</td>
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K.pneumoniae : Identities = 202/223 (90%)
E.herbicola : Identities = 195/223 (87%)
R.aquatilis : Identities = 191/223 (85%)

**Fig. 5. Comparison of the deduced amino acid sequences.**
shown in Fig. 5, the deduced amino acid sequences of *E. intermedium* 60-2G had a strong homology to the *pqq* genes of *R. aquatilis* (85%), *K. pneumoniae* (90%) and *E. herbicola* (87%) and confirming that *PQQ* is certainly involved in gluconic acid production by *E. intermedium* 60-2G (Kim et al., 1998, Meulenberg et al., 1992, Goldstein et al., 1987).

*Enterobacter* sp. is commonly found in rhizosphere of crops. *E. intermedium* 60-2G which was isolated from the rhizosphere of grass can be used as a strong phosphate solubilizer in agricultural environments. Moreover, 2-ketogluconic acid (pKa =2.66) increasing with time has more phosphate solubilization ability than gluconic acid (pKa = 3.41). The organic acids produced by *E. intermedium* 60-2G may replace the inorganic acids currently used in super-phosphate production. Currently we are investigating solubilization of rock phosphate using this bacterium and trying to transfer the *pqq* gene(s) to *Rhizobium* spp.

References


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*Enterobacter intermedium* 60-2G


Enterobacter intermedium 60-2G의 유기산 생성과 불용성인의 가용화

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**Key words** : gluconic acid, hydroxyapatite, 2-ketogluconic acid, phosphorus, *Enterobacter intermedium*.