Cloning and Functional Analysis of *pqq* Genes Phosphorus Solubilizing From *Bacillus Mycoides* Gnyt1

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Abstract

[Background]

The *pqq* genes which encodes phosphorus solubilizing from *Bacillus mycoides* Gnyt1 has been cloned and characterized. The role of this action on the function of *pqq* genes were detected. The phosphorus soluble *pqq* genes can secrete some specific organic activity acids to work but don't destroy the living environment of plants such as soil. In contrast, this plants containing the *pqq* genes grew well, even better than other plants with lower phosphorus solubilization, in the phosphorus free medium even though its *pqq* genes activity and turned out phosphorus ring, while *pqq* dependent expression was induced.

[Results]

This reaserch that five *pqq* genes were cloned from *Bacillus mycoides* Gnyt1, phylogenetic analysis revealed that pBI-*pqqA*, pBI-*pqqB*, pBI-*pqqC* and pBI-*pqqE* similarly cluster with Phosphorus-soluble protein. Under the action of five promoters, each coding region of *pqqA*, *pqqB*, *pqqC* and *pqqE* from *Bacillus mycoides* Gnyt1 can directly participate in the phosphate solubilization of the strain, directly improving the phosphate solubilizing capacity of the plant, which have strong nitrogenase activity. Interestingly, changes in the phosphate solubilizing gene result in complete loss of the phosphate solubilization of the strain. However, the ability to dissolve the phosphorus ring and to dissolve the phosphorus was also exhibited in the medium having five phosphorus-dissolving genes.

[Conclusions]

The results indicated that the *pqq* genes were successfully cloned and a new specific phosphate-dissolving strain was formed. In this research, *pqq* genes were first cloned from *Bacillus mycoides* Gnyt1.

1. Background

Phosphorus soluble gene was discovered in 1979 as a new type of phosphate-soluble cofactor for several prokaryotic dehydrogenases^[1-3]. The phenomenon of phosphorus deficiency in soil is widespread and should be given great attention. *Pqq* genes have been founded and characterized from bactetia, cloned and sequenced of the *pqq* gene from *Acetobacter aceti* ^[4-5]. Several researches indicated that *pqq* is present in a variety of plants and animals. For example, Kumazawa T *et al.*^[6] and Mitchell et al. were found *pqq* in human and rat tissues. Kumazawa *et al.*^[7]reseach said that all kinds of food and vegetables contain *pqq*. The recent research showed that PQQ-lack of mouse liver has low mitochondrial content and elevated plasma glucose concentration^[8]. In addition, PQQ is used as a scavenger for reactive oxygen species (ROS)^[9-10]and prevents damage to cells of DNA fragments^[11].They are gram-negative of the bacteria famlily, which has a strong phosphorus dissolving ability^[12]. This enzyme can only the first

in our *Bacillus mycoides* Gnyt1, and they have been found in *Acinetobacter* sp. or *Pseudomonas putida*^[13-14].

Research progress showed the *pqq* gene most of the performance was *pqq-adhB*, which cloned *A. polyoxogenes*^[5]. Kosano *et al.*^[15] reported that PQQ inhibited α -MSH-stimulated melanin-producing B16 melanoma. This inhibition of melanin production is caused by PQQ, which inhibits the expression of tyrosinase mRNA. However, other roles of PQQ, such as the action of tyrosinase-related proteins and the action of Mitf transcription in the pigment system, are not well known^[16]. Whereas *K. pneumonia* is synthesized by gluconate dihydrogen dehydrogenase and coenzyme PQQ. Closely related intestinal bacteria and *Salmonella typhimurium* can only synthesize decoenzyme, while PQQ cannot^[17]. However, the addition of PQQ to *E. coli* and *S. typhimurium* cells produces active holoenzyme^[18]. Kumazawa *et al.*^[19] report said that all kinds of food and vegetables contained PQQ. Naito *et al.*^[20] reported that PQQ can stimulate DNA synthesis. PQQ is synthesized by a variety of microorganisms^[21] from the two amino acids glutamate and tyrosine encoded in the precursor peptide, and is shown as an effective growth factor for plants^[22], bacteria^[23] or higher organisms^[24]. Although PQQ is an effective growth factor in both in vitro and in vivo models, the detailed mechanism of action remains unclear.

Bacillus mycoides Gnyt1 is a Gram-positive normal isolate from the rhizosphere of the alpine steppe plant of the alpine steppe, which is isolated and named in our laboratory, and has a high ability to dissolve phosphorus^[25]. At present, there is no known about the mechanism of phosphorus solubilization and the phosphorus-dissolving gene of this kind of bacteria. In this study, we aimed to clarify the functions of *Bacillus mycoides* Gnyt1 of *pqq* genes family, the four *pqq* genes were cloned and their activities and properties were characterized. We further validated the cloning of the phosphate-dissolving gene and its effect on the ability of the fraction to dissolve phosphorus.

2. Methods

2.1 Media and growth conditions

Bacillus mycoides Gnyt1 was cultured in medium, which contains: 5g yeast powder, 10g Peptone, 10g NaCl, PH 7.0. Optimal growth occurs at 28°C in aerobic conditions.

2.2 Cloning of *pqq* genes

The complete sequence of the *pqq* genes were selected from the baseline genome-wide database^[26] in our laboratory, all of which have accession numbers in NCBI. Primers are designed from conserved regions. All primers for this study were listed in Table 1. Primer pairs *pqqF* and *pqqR* were performed to obtain partially conserved regions. The amplified PCR product was purified and cloned into pMD-T19 vector (Takara, Japan). The expression vector pBI-121 was obtained from the wheat laboratory of Gansu Agricultural University, and after transformation in bacterial DH5 α competent cells, the plasmid was

isolated from the positive clone and inserted. DNA was obtained by PCR, and the remaining four clones were cloned by conventional PCR.

Table 1 List of primers used in the study

Primers	Oligonucleotide sequence (5'→3')	Purpose
<i>pqq1F</i>	AACAGGACGAGATTGGAGCA	<i>pqq1</i> amplification
<i>pqq1R</i>	TTGCGTGGATGCCCACAAAG	<i>pqq1</i> amplification
<i>pqq2F</i>	GTAGCGATGGTTGGTGCAGTAGG	<i>pqq2</i> amplification
<i>pqq2R</i>	TGCTGGAGCCGGATTCAACATAAC	<i>pqq2</i> amplification
<i>pqq3F</i>	AGGCGGAGCTGACTACATTC	<i>pqq3</i> amplification
<i>pqq3R</i>	TTCTGCCCCGCCAAATCCAG	<i>pqq3</i> amplification
<i>pqq4F</i>	AGCTCTTCGTGTCGCAGGAATTAC	<i>pqq4</i> amplification
<i>pqq4R</i>	ACTGCACCGCTCGTACCAATTG	<i>pqq4</i> amplification
<i>pqq5F</i>	TCATTGCGGGACAGTATGCTTCAG	<i>pqq5</i> amplification
<i>pqq5R</i>	CGGATCGGTTCTAGCTCTCCATTG	<i>pqq5</i> amplification

2.3 Sequence analysis and phylogeny prediction

ORF Finder found ORF (open reading frame)(<http://www.ncbi.nlm.nih.gov/g-orf/gorf.html>). The conserved domain of the *pqq* gene was detected by the Conserved Domain Database (CDD) search tool on the NCBI server (<http://www.ncbi.nlm.nih.gov/structure/cdd/wrpsb.cgi>). The physical and chemical parameters of the protein were determined using the ProtParam tool (<http://us.expasy.org/tools/protparam.html>).

2.4 Expression of *pqq* genes in *E. Coli*

The forward primer *pqq* with the built-in QuickCut™ SmaI cleavage site and the *pqq* clone ORF with the QuickCut™ XbaI site and five *pqq* genes (Table 2) were used. The PCR product was digested with SmaI and XbaI and ligated into the PBI-121 expression vector. Positive transformants were identified by PCR. The culture was cultured in Luria-Bertani (LB) medium with a positive concentration of 50 µg/mL. When the OD600 reached 0.5-0.7 at 37 ° C, the culture (5 mL) was transferred to an alternative 500 mL LB medium. In the 2000 mL flask, a 50 µg/mL pseudonym was added to the medium. When the OD600 reached about 0.6, the antibiotic thio-β-galactoside (IPTG) was added to the medium at a final concentration of 0.1 mmol / L. The bacteria were then centrifuged at 5,000 g for 30 minutes at 16 ° C, washed with PBS, and then cultured with binding buffer (20 mmol / L imidazole, 500 mmol / L NaCl, 20).

Hours to induce expression of the DfSQS1 protein. Methylene / L Na₃PO₄, pH 7.4) resuspended. Then, a certain amount of lysozyme (0.1 mg / mL) and RDNase (20 µg / mL) were added to the cell suspension. The cell suspension was stirred and placed at 4 ° C for 30 minutes. The cell suspension was disrupted by sonication 20 and the supernatant was harvested by centrifugation at 4200 rpm for 20 minutes at 4 ° C and replaced by a 750 kDa hollow fiber column. After washing with binding buffer (20 mmol / L imidazole, 500 mmol / L NaCl, 20 mmol / L Na₃PO₄, pH 7.4), protein and exchange buffer (20 mmol / L Na₃PO₄, 250 mmol / L imidazole, 500 mmol)) combined / L NaCl, pH 7.4). All collected samples were analyzed by SDS-PAGE.

Table 2 List of enzyme cleavage site primers used in the study

Primers	Oligonucleotide sequence (5'→3')	Purpose
<i>pqq1F</i>	GCTCTAGAAACAGGACGAGATTGGAGCA	<i>pqq1</i> amplification
<i>pqq1R</i>	CGACCCGGGTTGCGTGGATGCCACAAAG	<i>pqq1</i> amplification
<i>pqq2F</i>	GCTCTAGAGTAGCGATGGTTGGTGCAGTAGG	<i>pqq2</i> amplification
<i>pqq2R</i>	CGACCCGGGTGCTGGAGCCGGATTCAACATAAC	<i>pqq2</i> amplification
<i>pqq3F</i>	GCTCTAGAAGGCGGAGCTGACTACATTC	<i>pqq3</i> amplification
<i>pqq3R</i>	CGACCCGGGTTCTGCCCGCCAAATCCAG	<i>pqq3</i> amplification
<i>pqq4F</i>	GCTCTAGAAGCTCTTCGTGTCGCAGGAATTAC	<i>pqq4</i> amplification
<i>pqq4R</i>	CGACCCGGGACTGCACCGCTCGTACCAATTG	<i>pqq4</i> amplification
<i>pqq5F</i>	GCTCTAGATCATTGCGGACAGTATGCTTCAG	<i>pqq5</i> amplification
<i>pqq5R</i>	CGACCCGGGCGGATCGGTTCTAGCTCTCCATTG	<i>pqq5</i> amplification

3. Results

3.1 Cloning of five *pqq* genes from *B. mycoides* Gnyt1

The five *pqq* genes clusters were isolated from *Bacillus mycoides* Gnyt1. Five phosphorus soluble genes were cloned by conventional PCR using individual two genes primers *pqqF* and *pqqR*, as the figure shows that the *pqq* genes related to phosphorus soluble gene after cloning and screening. Moreover, BLAST analysis and sequence comparison revealed that the size of *pqqE* is 1539bp, which is the longest gene fragment, *pqqA* is 924bp, *pqqB* is 897bp, and *pqqC* is 645bp, which is presumed to be the target fragment. The sequencing datas obtained in this study have been deposited in the GenBank database under the following accession numbers by CP020743. The total of five genes sequence involving the putative open reading frame(ORF) was obtained, the ORF was amplified by using primer pair.

3.2 Construction of cloned phospholysis gene and plasmid for expression vector

The plasmid for expression vector PBI-121 has been successfully constructed and stored in a laboratory cryogenic refrigerator, which measurement was far more than 15000bp (Fig.2.A). Four plasmids of four *pqq* genes were successfully extracted using a plasmid kit, and the sizes were all less than 2500 bp (Fig.2.B). As can be seen from the figure, the agarose gel electrophoresis showed that the band was clear and single, and it was proved that the plasmid of the gene was successfully extracted, and the next experiment can be carried out.

3.3 Double digestion of plasmid

The enzymatic digestion system was as follows: 10×QuickCut Buffer 3ul, plasmid DNA 6ul, QuickCut™ SmaI 1ul, QuickCut™ XbaI 1ul, five genes were successfully extracted, and the expression vector pBI121 plasmid DNA was added to the rapidly cleaved enzymes SmaI and XbaI for double digestion, ddH₂O is up to 30ul. The enzyme was digested at 37 ° C for 6 hours, electrophoresis was detected, and the target fragment was recovered. It can be cut from the figure and has successfully recovered 6 fragments of plasmid DNA (Fig.3).

3.4 Linked expression vector

The four *pqq* genes and the expression vector PBI121 were ligated, and the ligation system was: 10×T4 DNA Ligase Buffer 1ul, *pqqA* / *pqqB* / *pqqC* / *pqqE* 6ul, pBI121 DNA 1ul, T4 DNA Ligase 1ul, dd H₂O 10ul. Except T4 DNA Ligase, the reaction was incubated at 65 ° C for 3 minutes, then rapidly transferred to ice for a few seconds, then T4 DNA Ligase was ligated for 16 hours or more and stored at 4 degrees for conversion.

3.5 Phylogenetic analysis

The amino acid sequences corresponding to the four cloned gene products of the Gny1 variable are compared with similar sequences already in GenBank, and the software MEGA is used to generate the phylogenetic tree using the conversion method. The results show that the *pqq* genes family is derived from a single Gny1 *pqqA* phosphorus dissolving gene that is closer to bacterial phospholytic protein (WP 003194766.1) and clustered together with other distributions (Figure 5.5), and the phosphorus dissolving gene *pqqB* is closer to PqqB Thiazoline antibiotic biosynthetic protein (WP 018985147.1) (Figure 5.6), while *pqqC* is clustered closer to the *Bacillus pqqC* family protein group (Figure 5.7), and *pqqE* is closer to the *Bacillus* and *Bacillus cereus* dissolved phosphorus family protein cluster (Figure 5.8).

3.6 *E. coli* transformation

Using competent cells, each product was transformed into *E. coli*, and *E. coli* was used as a vector for propagation and growth, and the constructed PBI-*pqqA*, PBI-*pqqB*, PBI-*pqqC*, PBI-*pqqE* vectors were transformed into *Escherichia coli* DH5α in the large intestine. A large number of bacteria are amplified and positively transformed by bacterial liquid phase PCR.

White colonies cultured overnight were selected, and individual colonies were picked and cultured in LB liquid medium for about 13 hours. At the same time, blue spots were picked as a negative control (Fig.8), and bacterial cells were detected by PCR, and the target bands were amplified and correctly sequenced. It is used to detect the phosphate-dissolving activity of phosphate-dissolving bacteria.

3.7 Identification of phosphate-dissolving activity

Qualitative experiments have shown (Fig.9) that the strains of recombinant phosphorus-solubilizing genes can produce small phosphorus-solubilizing circles on the culture medium, and the phosphorus dissolving characteristics of PBI-*pqqA*, PBI-*pqqB*, PBI-*pqqC*, and PBI-*pqqE* were identified by liquid chromatography. Secreted organic acid content, standard solutions of different concentrations are purified and enriched by C18 column mobile phase, and analyzed under specific chromatographic conditions. The results showed that the four recombinant phosphorus-solubilizing gene strains can all secrete organic acids, and thus have a role in phosphorus dissolution (Fig. 10). The concentration peak of secreted organic acids can be clearly seen from the figure, successfully verifying that the four clones linked to the previous clones Phosphorus gene, indicating that the phosphorus dissolution gene dissolves phosphorus Among them, the peak of oxalic acid is the highest, and the peak of citric acid is the lowest. In addition (Table 3) , PBI-*pqqA* can secrete 6 kinds of organic acids, with the strongest phosphorus-dissolving effect, followed by PBI-*pqqB* and PBI-*pqqC*, and can secrete 4 kinds of organic acids, followed by phosphorus-dissolving effect. PBI-*pqqA* and PBI-*pqqC* secreted the highest lactate concentration, while PBI-*pqqB* and PBI-*pqqD* secreted the highest malate concentration.

Table 3 Content of organic acids in recombinant strains

Stains	Organic acid content [mg/L]					
	oxalic acid	tartaric acid	Malic acid	Citric acid	Lactic acid	Acetic acid
<i>pqqA</i>	14.93±0.41	14.10±0.90	14.81±0.69	14.39±0.46	18.23±0.94	18.18±0.05
<i>pqqB</i>	3.21±0.15	5.12±0.64	106.82±2.75	13.94±0.76	—	—
<i>pqqC</i>	24.24±0.34	—	—	20.17±0.97	0.48±0.01	0.29±0.01
<i>pqqD</i>	32.09±0.77	—	119.58±7.30	—	0.63±0.14	—

4. Discussion

In the present study, we found that the same pathway, *Bacillus* was used as beneficial bacteria in our research because it has high phosphorus dissolving ability^[27-28]. PQQ genes stably catalyzes sustained redox cycling as a cofactor and does so more efficiently than most other redox active cofactors^[29]. However, like all redox active compounds, PQQ can still promote oxidation under certain conditions and induce oxidative protein modification, including oxidation of cysteamine thiol^[30-32]. It has been demonstrated that terpenoids including PQQ undergo an electron redox cycle in the presence of ascorbate, NAD(P)H and thiol compounds (such as glutathione), thereby forming corresponding

semiquinone free radicals^[33-36]. In addition, PQQ oxidizes the redox regulatory site of the N-methyl-D-aspartate (NMDA) receptor, thereby protecting NMDA or glutamate-mediated neuronal cell damage^[37]. Therefore, the permanent phosphorus solubilization gene may have a certain basic significance for supplementing the ability of plants to dissolve phosphorus.

In this report, we successfully identified the four *pqq* genes in *Bacillus* for use as a phosphorus solubilization. We obtained conventional PCR clone of *pqqA*, *pqqB*, *pqqC* and *pqqE*. Compared with other known *pqq* genes, three key genes were found in our strain^[38-39]. The phosphorus-dissolving family is an important gene for bacterial phosphorus solubilization. We identified the *pqq* genes of *Bacillus mysoides* Gnyt1. This is the first clone and complete sequence description of the phosphate solubilizing gene in *Bacillus mysoides* Gnyt1. The plasmids of the four genes extracted were all within 15000 bp, and were well aligned with the partial sequence of *Bacillus* (partial sequence 94% identical). A high level of protection indicates that the Gnyt1 strain has a phosphate soluble gene similar to the reported corresponding phosphate-dissolving family gene^[40-41].

The cloning of the *pqq* genes of *Bacillus* provides interesting and important suggestions for the regulation of phosphorus and phosphorus-soluble organic acid metabolites. *Bacillus mysoides* Gnyt1 has the largest total number of acidic and basic residues for each protein. Explaining the mechanism of phosphorus solubilization is of great significance for the biotechnological application of such proteins. In this regard, the *Bacillus* Gnyt1 *pqq* genes may be a good model for research. Studies have shown that micro-spatial analysis of gene expression shows that changes in different PQQ genes can significantly affect metabolites, cell signaling and immune function-related genes^[42]. Strict control of the activity and expression of the phosphate-dissolving gene, transcription or post-translational regulation depends on the specific environment of the host organism. *B. Gnyt1* is at ambient temperature under aerobic and anaerobic conditions and uses hydrogen hydride as the electron donor. The amino-acid sequences of *pqqA* and *pqqB* of *B. Gnyt1* are more similar to the corresponding proteins in bacterial phospholytic protein and PqqB quinolinone biosynthetic protein (WP 003194766.1, WP 018985147.1).

Although the research was carried out in antibiotics, the results showed important information. The expression of four phosphorus-dissolving genes increased the expression rates of PBI-*pqq*, PBI-*pqq*, PBI-*pqq*, and PBI-*pqq* by increasing the temperature to 37 °C. Finally, we will obtain a single, expressible phosphate soluble gene that is recombined. In one study, the phosphate solubilizing gene was also present in other plants or phosphate solubilizing bacteria. However, other studies have shown that the phosphorus-dissolving family includes many genes, and different ones contain different phosphorus-dissolving genes. Among them, *pqqA*, *pqqC* and other reports have the same length^[43-45], and *pqqE* are rarely reported in other studies and can be used as a resource for further research.

There are few reports on phosphorus solubilization in *Bacillus mysoides*, our research will enrich the knowledge of phosphorus solubilization in these bacteria.

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phosphorus-dissolving family includes many genes, and different ones contain different phosphorus-dissolving genes. Among them, *pqqA*, *pqqC* and other reports have the same length^[43-45], and *pqqE* are rarely reported in other studies and can be used as a resource for further research.

There are few reports on phosphorus solubilization in *Bacillus mysoides*, our research will enrich the knowledge of phosphorus solubilization in these bacteria.

5. Conclusion

PqqA, *pqqB*, *pqqC* and *pqqE* genes were the first clone analysis and *E. coli* transformation. In this case, four genes cloned from B. Gnyt1 were full-length DNA encoding *pqqA*, *pqqB*, *pqqC* and *pqqE*, and were named pBI-*pqqA*, pBI-*pqqB*, pBI-*pqqC* and pBI-*pqqE*. Bioinformatics analysis of pBI-*pqqA*, pBI-*pqqB*, pBI-*pqqC* and pBI-*pqqE* have been completed. Phylogenetic analysis trees reflect the accumulation of the *pqq* genes in higher clade, slightly closer to Phosphorus-soluble protein. Four *pqq* genes were expressed in *E. coli*, and four recombinant phospholytic genes were obtained, which proved that PBI-*pqqA*, PBI-*pqqB*, PBI-*pqqC* and PBI-*pqqE* have phosphorus-dissolving properties. This is the first report on the cloning and identification of the phosphate solubilizing gene from *Bacillus mycooides* Gnyt1. Finally, the B. Gnyt1 phosphate-dissolving gene a promising model for studying the mechanism of phosphorus solubilization by specific phosphorus-dissolving bacteria, a feature that makes it an attractive candidate for biotechnology applications.

Abbreviations

B. Gnyt1—*Bacillus mycooides*Gnyt1

Declarations

Declarations

Not applicable

Ethics approval and consent to participate

Approve and agree to participate

Consent for Publication

All authors agree to publish content

Availability of data and material

Not applicable

Competing interests

The authors declare that there are no competing interests.

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Authors' contributions

Xiaomei Yang cloned the gene and drafted the manuscript. Tuo Yao designed the research and project outline and finalized the manual. Xiaomei Yang was genetically analyzed. All authors have read and approved the final manuscript.

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Figures

Figure 1

pqqA, pqqB, pqqC and pqqE of clone B. mycoidesGnyt1 strain. M: Marker 2000, 1: pqqA924bp, 2: pqqB897bp, 3: pqqC645bp, 4: pqqE1539bp

Figure 2

PCR detection of PBI-121, pqqA, pqqB, pqqC and pqqE plasmids. A: PCR detection of PBI-121 plasmid. M: marker 15000, 1: PBI-121 plasmid B: PCR detection of pqqA, pqqB, pqqC and pqqD plasmids. M: marker 15000, 1: pqqA plasmid, 2: pqqB plasmid, 3: pqqC plasmid, 4: pqqEplasmid

Figure 3

Double digestion of PBI-121, pqqA, pqqB, pqqC and pqqE plasmids. A: Double-digested plasmid recovery of plasmid PBI-121. M: Marker 15000, 1: PBI-121 Plasmid B: PCR detection of pqqA, pqqB, pqqC and pqqE double digested plasmids. M: marker 15000, 1: double digestion of pqqA plasmid, 2: double digestion of pqqB plasmid, 3: double digestion of pqqC plasmid, 4: double digestion of pqqE plasmid

Figure 4

PBI-121, pqqA, pqqB, pqqC and pqqE plasmid ligation

Figure 5

B. mycoides Gnyt1-pqqA of the phylogenetic tree

Figure 6

B. mycoides Gnyt1-pqqB of the phylogenetic tree

Figure 7

B. mycoides Gnyt1-pqqC of the phylogenetic tree

Figure 8

B.mycoidesGnyt1-pqqEof the phylogenetic tree

Figure 9

Blue and white spots of recombinant genes transformed into E. coli. A: PBI-pqqA blue and white spots. B: Blue and white speckle diagram of PBI-pqqB. C: Blue and white speckle diagram of PBI-pqqC. D: Blue and white speckle diagram of PBI-pqqE.

Figure 10

Qualitative determination of phosphorus-solubilizing gene strains

Figure 11

Determination of the content of organic acids produced by the phosphorus-dissolving gene strains. A: Determination of oxalic acid production of phosphorus-solubility gene strains. B: Determination of six organic acids produced by the phosphate-dissolving gene strain's dissolution characteristics.