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Cloning of Indigenous Bacillus licheniformis Pyrroloquinoline Quinone Gene and Its Role in Enhancement of Phosphate solubilization in Escherichia coli

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Abstract: Phosphorus fertilizer is precipitated in the soils where plants could not benefit from it. Many soil bacteria and fungi have the ability to solubilize phosphate (Pi) minerals and make it available to plants. Since phosphate-solubilizing microorganisms have an economically important role in agriculture, many research institutions have been investigating in this area to through lights on the solubilization mechanisms and to isolate efficient phosphate-solubilizng microorganisms. In this study attempts were done to isolate the *pqq* gene from an indigenous *Bacillus licheniformis* strain efficient in phosphate solubilization, and to study its effect on phosphate-solubilization. Polymerase Chain Reaction (PCR) technique was used to amplify the pyrroloquinoline quinone (*pqq*) gene using sequence available at in the EMBL Nucleotide Sequence Database. A method was described for *pqq* gene amplification and cloning. Results indicated the successful amplifying the *pqq* gene from the indigenous *Bacillus licheniformis* strain which indicates the similarity in *pqq* and its surrounding sequences between the indigenous strain and the sequenced *B. licheiniformis* strain (NCBI Reference Sequence: NC_006270.3). *Pqq* gene was expressed in *E. coli* JM107 after its transformation and three transformants were obtained. All transformants were able to drop the pH value of their cultures and were able to enhance phosphorus solubilizatiaon. The highest phosphate solubilization activity was found in PQQ-1 which was about 71% more than the original strain *E. coli* JM107 in the seventh day of incubation. This study confirmed the importance of pyrroloquinoline quinone in phosphate solubilization and its usage to improve available phosphorus to plants.
Keywords: Phosphate-solubilization, *Bacillus licheniformis*, Pyrroloquinoline Quinone, PCR gene cloning.

Introduction and Experimental

Phosphorus (P) is the second major nutrient after nitrogen that limits plant growth[1,2]. Chemical phosphorus fertilizer is the main source for plant, but almost 75 to 90% of added phosphorus is precipitated by iron, aluminum and calcium complexes present in the soils[1,3].

Many soil bacteria and fungi have the ability to solubilize phosphate (Pi) minerals and make it available to plants [4,5]. They have an important contribution to overall plant P nutrition and growth, and have increased yields of many crops[6-8].

Phosphate-solubilizing microorganisms produce organic acids to solubilize the bound phosphates [9,10]. The enzymes involved are glucose dehydrogenase, gluconic acid dehydrogenase and 2-ketogluconic acid dehydrogenase. Glucose dehydrogenase oxidizes glucose to gluconic acid, which is oxidized by gluconic acid dehydrogenase to 2-ketogluconic acid. The 2-ketogluconic acid dehydrogenase oxidizes 2-ketogluconic acid to 2, 5-diketogluconic acid[11-14].Glucose dehydrogenases (GDH) [15,16] require pyrroloquinoline quinone (PQQ) as a redox cofactor for activity.

Bacterial strains and culture conditions

E. coli was cultured in Luria-Bertani (LB) broth at 37°C[17] (Davis *et al*., 1980). An indigenous *Bacillus licheniformis* strain (Microbial Genetic Department, National Research Centre) was cultured in the same medium at 30°C. Ampicillin was used if required (AP) at 100 µg/ml.

Cloning of pyrroloquinoline quinone (*pqq*) gene

Bacillus licheniformis was cultivated on LB plates at 30°C for 19 hours. Two bacterial loops were added in 250 µl H_2O; heated at 95^0C for 20 minutes, incubated in ice for 10 minutes and centrifuged at 10000 rpm for 3 minutes. The supernatant was then used as crude DNA[18] (Ostuki *et al*., 1997). The crude lysate (fresh preparation) was subjected to PCR amplification using *pqq* forward and reverse primers.

The DNA sequence of the pyrroloquinoline quinone (*pqq*) gene was obtained from the available data in the EMBL Nucleotide Sequence Database of *Bacillus licheniformis* (NCBI Reference Sequence: NC_006270.3).

Primers derived from this sequence were obtained using Primer3 software. The forward primer was: 5'-TGCATTTCCGCTACGATACA-3' and the reverse primer was: 5'- ACCGTAATACAAGG AAG CTGAA- 3'.

Mineral phosphate solubilization determination

The mineral phosphate was assayed according to[19] Koenig and Johnson (1942). Fifty ml of NBRIP medium[20] which consists of (g l^{-1}) glucose, 10.0; tricalcium phosphate (TCP), 10.0; $MgCl_2.6H_2O$, 5.0; $MgSO_4.7H_2O$, 0.25; KCl, 0.2; $(NH_4)_2SO_4$, 0.1, were inoculated with 5 ml of 24-hr-old culture of tested bacteria. Flasks were incubated at 37°C with shaking at 120 rpm. One milliliter samples were taken daily, centrifuged at 12000 rpm for 5 min. and the concentration of soluble phosphate of the supernatant was determined photometrically at 430 nm using SHIMADZU UV-1201 spectrophotometer[19]. The soluble phosphate concentrations were deduced from a standard curve of KH_2PO_4.

Results and Discussion

Up to 90% of phosphorus fertilizer is precipitated in the soils where plants could not benefit from it0[1,3]. Since phosphate-solubilizing microorganisms have an economically important role in agriculture, many research institutions have been investigating in this area to through lights on the solubilization mechanisms and to isolate efficient phosphate-solubilizng microorganisms. Organic acids production by these organisms has a very important role in solubilizing the bound phosphates[9,10]. Gluconic acid is produced by many of phosphate-solubilizing microorganisms. This organic acid is synthesized according to the action of glucose dehydrogenases (GDH)[15,16] which requires the pyrroloquinoline quinone (PQQ) as a redox cofactor for its activity.

In this study attempts were done to isolate the *pqq* gene from an indigenous *Bacillus licheniformis* strain efficient in phosphate solubilization, to clone it, to transform it to *E. coli* and to study its effect on phosphate-solubilization.

The DNA sequence of the pyrroloquinoline quinone (*pqq*) gene and 84 bp upstream and 83 bp downstream of the gene (1104 bp), was obtained from the available data in the EMBL Nucleotide Sequence Database of *Bacillus licheniformis*. This sequence was analyzed for its restriction endonuclease cutting using the

Webcutter 2.0 software (http://rna.lundberg.gu.se/cutter2/). Results indicated that among the endonucleases which do not cut this sequence, each of *Bam* HI, *Hind* III, *Sal* I and *Xho* I.

Amplification of pyrroloquinoline quinone (*pqq*) gene

The obtained sequence was analyzed with Primer3 software and the forward and reverse primers were selected to include the amplification of the whole *pqq* sequence. The expected PCR-product size is 1202 bp of the target *pqq* sequence.

In order to optimize the PCR product, three or six µl of cell lysate were amplified in a 50 µl reaction mixture by using DreamTaq Green PCR Master Mix (Fermentas) using 1.5 µl (15 pmoles) or 3 µl (30 pmoles) of each primer (Table 1).The PCR was performed by using DNA thermal cycler (NYXTECHNIK). The PCR program consisted of one cycle of DNA denaturation at 94°C (3 min.), 35 cycles of 94°C (1 min), 48°C (2 min) and 72°C (3 min), plus one additional cycle of a final chain elongation at 72°C (10 min).

Four mixtures of DNA/primers were used to identify the best one for *pqq* amplification. Samples of PCR product was analyzed using agarose gel electrophoresis and compared with 1Kb Ladder DNA marker (300 bp – 10000 bp) (AXYGEN), results are present in Figure 1. The best trial was that which contained 6 µl DNA (cell lysate) and 30 pmoles of each primer in a 50 µl reaction mixture.

Cloning of pqq gene

The PCR product was purified using Ron's PCR-Pure Kit (BIORON). The purified amplicon was cloned in pJET1.2 vector, which allowed positive selection after cloning (i.e, the vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site) Using Clone JET PCR Cloning Kit (Ferments).

The cloning mixture was used to transform *E. coli* JM 107; transformants were selected on LB plates containing ampicillin.

Different *E. coli* transformants were obtained. They were analyzed for the occurrence of *pqq* gene using PCR as mentioned above. Results (Fig. 2) confirmed the existence of *pqq* gene in the three tested *E. coli* transformants numbers PQQ-1, PQQ-7 and PQQ-20.

Figure 1. PCR product of amplified *pqq* sequence of *Bacillus licheniformis*. Lane 1: 100 bp Ladder DNA marker (AXYGEN) (100 bp- 3000 bp), lane 2: Amplified *pqq* gene of *Bacillus licheniformis*

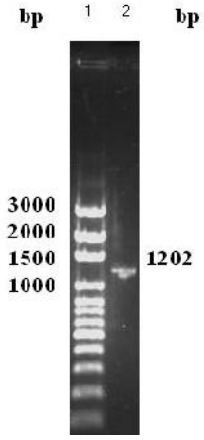
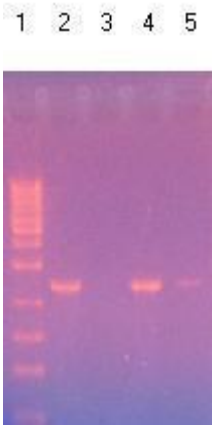


Table 1. Trials for PCR optimization of Pyrroloquinoline Quinone (*pqq*) gene amplification

Ingredients	Trial 1	Trial 2	Trail 3	Trail 4
Master Mix	25 µl	25 µl	25 µl	25 µl
DNA (cell lysate)	3 µl	6 µl	3 µl	6 µl
Primer R	1.5µl	1.5 µl	3 µl	3 µl
Primer L	1.5 µl	1.5 µl	3µl	3 µl
H_2O	19 µl	16 µl	16 µl	13 µl
Total volume	50 µl	50 µl	50 µl	50 µl

Figure 2. PCR product of *pqq* among *E. coli* JM107 transformants.Lane1, 1Kb Ladder DNA marker AXYGEN (300 bp – 10000bp); lane 2, *E. coli* PQQ-20; lane 3, *E. coli* JM107 (control); lane 4, *E. coli* PQQ-7; lane 5, *E. coli* PQQ-1



Pyrroloquinoline quinine expression

Changes in culture pH

E. coli JM107 and its three PQQ transformants were cultured overnight in LB. 1.5 ml of each culture were inoculated into 20 ml NBRIP medium, every 24 h samples were withdraw from each culture, centrifuged at 12000 rpm for 5 min and pH values were determined. Results are present in Table 2 and Fig. 3.

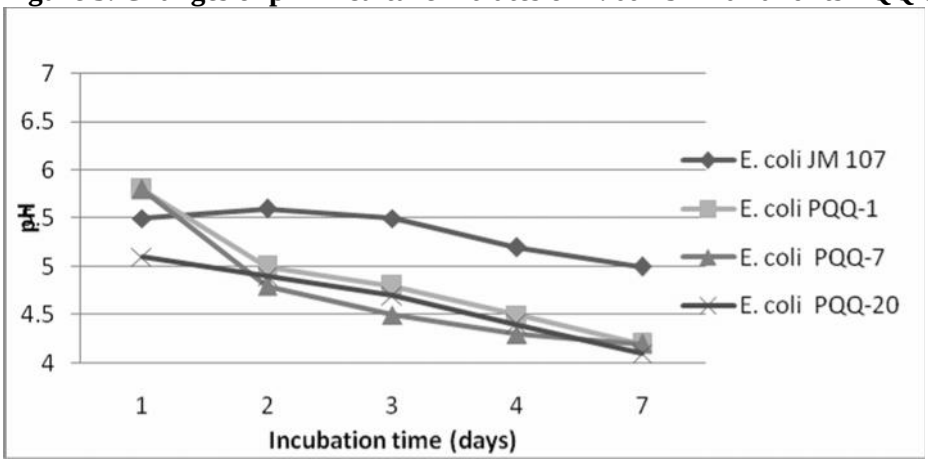
The initial pH value of NBRIP medium was 7.0[20]. All tested bacteria gradually lowered the pH values during incubation time. *E. coli* JM107 reduced the pH to 5.0, while the three transformants showed higher ability to reduce the pH values up to pH 4.1.

The higher reduction of pH values of the transformants is more probably due to synthesis and secretion of gluconic acid due to expression of *pqq* gene.

Table 2. pH values during the incubation of *E. coli* JM107 and its PQQ transformants

Bacterial strains	Incubation time (days)				
	1	**2**	**3**	**4**	**7**
***E. coli* JM 107**	5.5	5.6	5.5	5.2	5.0
***E. coli* PQQ-1**	5.8	5.0	4.8	4.5	4.2
***E. coli* PQQ-7**	5.8	4.8	4.5	4.3	4.2
***E. coli* PQQ-20**	5.1	4.9	4.7	4.4	4.1

Figure 3. Changes of pH in culture filtrates of *E. coli* JM107 and its PQQ transformants



Solubilization of phosphate by *E. coli* transformants

Escherichia coli is capable of synthesizing the apo-glucose dehydrogenase enzyme (GDH) but not the cofactor pyrroloquinoline quinone (PQQ), which is essential for formation of the holoenzyme, and hence does not produce or secrete gluconic acid[15,16]. The obtained *E. coli* PQQ transformants were tested for their efficiency in phosphate solubilization. Table 3 and Fig. 4 show the amount of soluble phosphate released from tricalcium phosphate medium by two PQQ transformants.

Table 3 showed the release of high phosphate concentrations in the second day of incubation. It also presented less phosphate concentration during the 3rd and 4th day of incubation. The latter finding may reflect more phosphate utilization by these bacteria during this growth period. The highest phosphate solubilization activity was found in PQQ-1 which was about 71% more than the original strain *E. coli* JM107 in the seventh day of incubation.

The present results are in agreement with[21-23] they find a significant decline in the pH of medium during the solubilizatiaon of phosphate, which suggested secretion of organic acids by the bacterial isolates.

Table 3. Soluble phosphate concentration released by *E. coli* JM107 and its PQQ-transformants*

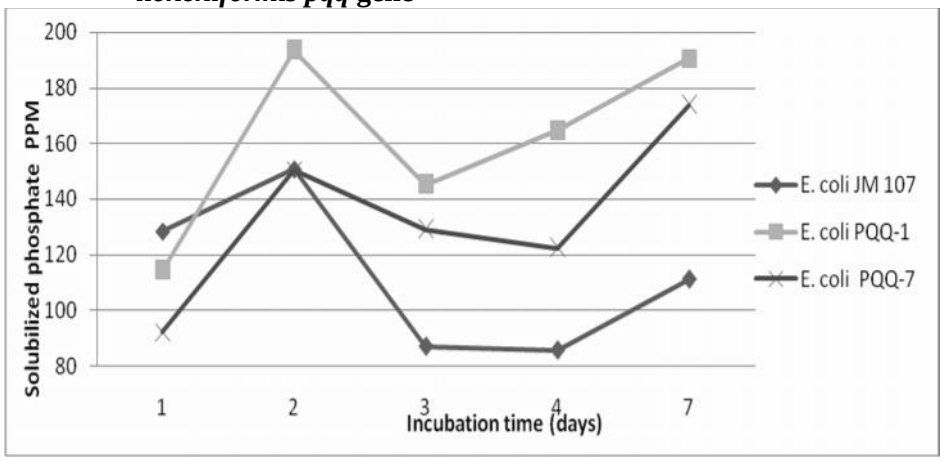
Bacterial strains	Incubation time (days)				
	1	**2**	**3**	**4**	**7**
***E. coli* JM 107**	128.50	150.75	87.08	85.55	111.07
***E. coli* PQQ-1**	114.35	193.48	145.14	164.57	190.56
***E. coli* PQQ-7**	92.11	150.40	129.1	122.315	173.93

- PPM

Conclusions

In this study a method was described for *pqq* gene cloning from an indigenous *Bacillus licheniformis* strain using primers deduced from the published data. This indicates the similarity in *pqq* and its surrounding sequences between the indigenous strain and the sequenced *B. licheiniformis* strain (NCBI Reference Sequence: NC_006270.3). This gene was expressed in *E. coli* and endows it the ability to solubilize phosphate through acidification of the medium by gluconic acid production, which is made possible by the complementation of endogenous glucose dehydrogenase by PQQ. This complementation of function in *E. coli* substantiates the key role for PQQ in solubilization of phosphate.

Figure 4. Phosphate-solubilizing activity of *E. coli* JM107 and its transformants containing *Bacillus licheniformis pqq* gene



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