Journal of Agricultural Science; Vol. 7, No. 8; 2015
ISSN 1916-9752 E-ISSN 1916-9760
Published by Canadian Center of Science and Education

# Cloning and Expression of Cellulosome-Integrating Protein from *Aspergillus niger* H1 Improves Phosphate Solubilization

Mingbo Gong[1] & Chaoxi Tang[1]

[1] Key Laboratory of Microbial Resources, Ministry of Agriculture/Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, Beijing, China

Correspondence: Mingbo Gong, Institute of Agricultural Resources and Regional Planning, Chinese Academy of Agricultural Sciences, No. 12, Zhongguancun South Street, Haidian District, Beijing, China. Tel: 86-136-9152-7883. E-mail: gongcaas@163.com

Received: May 11, 2015 Accepted: June 11, 2015 Online Published: July 15, 2015

doi:10.5539/jas.v7n8p156 URL: http://dx.doi.org/10.5539/jas.v7n8p156

**Abstract**

A primary cDNA library of *Aspergillus niger* H1 was constructed using SMART (switching mechanism at the 5′ end of RNA transcript) technique. A total of 169 clones had halos on the insoluble phosphate medium, and clone H-47 had clear halos. The full-length cDNA of clone H-47 was 625 bp, with a complete open reading frame (ORF) of 390 bp, encoding a protein of 129 amino acids. Multiple alignment revealed a high degree of homology between the ORF of the clone and other fungi cellulosome-integrating protein (CipC-like). The expression vector of ORF was constructed and transformed into *Escherichia coli* DH-5α. The transformant (ORF-1) with the CipC-like gene secreted more organic acid when grown in tricalcium phosphate (TCP) medium, with TCP as the sole source of phosphate. *E. coli* DH5α containing the *cipc-like* gene secreted methanoic acid, acetic acid, malic acid, and citric acid reached 81.2, 93.3, 50.6, and 147.7 μg $mL^{-1}$, respectively, within 28 h. These results showed that the expression of the *A. niger* H1 CipC-like gene in *E. coli* could enhance organic acid secretion and improve phosphate solubilizing ability.

**Keywords:** phosphate solubilizing, *Aspergillus niger*, SMART, organic acid

## 1. Introduction

Phosphorus is one of the major essential macronutrients for plants, and it is applied to soil in the form of phosphate (P). Most P are immobile and the available content of P is below the limit of plant growth in soils. Microorganisms play an important role in rendering insoluble P into soluble forms that are utilized by plants and prompt plant growth (Mehta et al., 2013; Xiao et al., 2009). Many studies have proven that acidification is the most process of P solubilization (Xiao et al., 2015; Sindhu et al., 2014; Rodríguez et al., 2006). Numerous studies have also focused on the organic acid secretion of microorganisms to solubilize P in soil. The main organic acids known to solubilize P in soil are gluconic, oxalic, citric, 2-ketoglutaric, lactic, acetic, formic, and succinic acids (Kumar et al., 2013; Bianco et al., 2010; Werra et al., 2009; Khan et al., 2007; Fomina et al., 2004; Altomare et al., 1999; Banik et al., 1982).

Highly efficient expression of exogenous P solubilization-related genes for organic acid production is an optimal strategy to improve P uptake. The critical step is obtaining P solubilization-related genes that can be heterologously expressed. *Enterobacter intermedium*, *Klebsiella pneumoniae*, *Rahnella aquatilis*, *Penicillium oxalicum*, and *Aspergillus niger* were used to clone genes that could solubilize P (Gong et al., 2014a; Lü et al., 2011; Kim et al., 1998; Meulenberg et al., 1992). In Gram-negative bacteria, many studies have focused on the *pqq* gene. Gluconic acid is produced by the direct oxidation of glucose mediated by a membrane-bound glucose dehydrogenase (GDH), which requires pyrroloquinoline quinone (PQQ) as a cofactor (Goldstein 1995). In fungi, many studies have focused on the gene that can be expressed in *E. coli* to improve organic acid secretion and P solubilization. Lü cloned mitochondrial malate dehydrogenase from *P. oxalicum* and expressed the gene in *E. coli* to improve P solubilization.

Although numerous studies have focused on the cloning and expression of genes from fungal species to solubilize P. Genes from fungi, especially *A. niger*, are rarely reported. In the present study, *A. niger* H1 was used for cloning and expression of genes. *A. niger* H1 secretes organic acids and has high P solubilization ability. We constructed a primary cDNA library of *A. niger* H1 and then screened clones with P solubilization ability in

TCP medium. We also demonstrated that overexpression of CipC-like in *E. coli* DH5α increased the secretion of organic acids and enhanced P solubilization ability.

## 2. Methods

### *2.1 Strains, Plasmids, and Media*

*A. niger* H1 was grown at 30 °C on PDA agar medium or in PDA broth with shaking. *E. coli* DH5α was grown at 37 °C on Luria–Bertani (LB) agar medium or in LB broth with shaking. Plasmid transformants of *E. coli* DH5α were grown on LB medium and TCP agar or broth medium containing 100 μg/mL ampicillin. TCP medium comprised the following components (in g/L of distilled water): glucose, 10 g; $MgSO_4 \cdot 7H_2O$, 0.3 g; NaCl, 0.3 g; KCl, 0.3 g; $(NH_4)_2SO_4$, 0.5 g; $FeSO_4 \cdot 7H_2O$, 0.03 g; $MnSO_4 \cdot 4H_2O$, 0.03 g; and $Ca_3(PO_4)_2$, 5 g (Nautiyal, 1999). The vector was pBluescript II SK(+) (TaKaRa).

### *2.2 RNA Isolation and Purification*

Total RNA was isolated from P-solubilizing fungus *A. niger* H1 after incubating on PDA medium using Trizol reagent (Invitrogen) according to the manufacturer's instruction. The extracted RNA was treated with DNase I to eliminate genomic DNA contamination. The mRNA was purified from total RNA using an Oligotex mRNA kit (Qiagen). The quality and concentration of mRNA samples were examined using EB-strained agarose gel electrophoresis and spectrophotometric analysis.

### *2.3 Construction of cDNA Library*

The cDNA library was constructed using a SMART cDNA Library Construction Kit (Clontech, USA). Total RNA concentration was determined using a spectrophotometer, and 1 μg of RNA was used with 1 μL of the CDS III/3′ PCR Primer and 1.0 μL of SMART IV Oligonucleotide for first-strand cDNA synthesis. Poly(A)$^+$ mRNA was incubated with PowerScript Reverse Transcriptase at 42 °C to synthesize the first chain of cDNA. Double-stranded cDNA was synthesized by LD PCR with CW III/3′ PCR Primer and 5′ PCR Primer with Advantage 2 Polymerase Mix. Double-stranded cDNA was digested with proteinase K and *Sfi* I enzyme. The size-fractionated cDNA was purified by a CHROMA SPIN-400 column. The cDNA was then ligated to the pBluescript II SK vector. Transformation of recombined plasmids was performed using DH-5α competent cells following the manufacturer's instructions.

### *2.4 Quality Assessment of the cDNA Library*

The colonies with 100-fold dilution were spread on LB medium plates containing IPTG (24 mg $L^{-1}$) and X-gal (4 mg $L^{-1}$). After 24 h of incubation at 37 °C, the number of clones on each plate was counted to calculate the library titer and clone capacity. The ratio of blue-to-white clones was calculated to determine the recombination efficiency. Fifty clones were randomly selected from the cDNA library and cultured in 1 mL of liquid LB medium at 37 °C overnight to determine the insert size of fragments. The target sequence was amplified using the T3 (5′-CCCAGTCACGACGTTGTAAAACG-3') and T7 (5′-AGCGGATAATTTCACACAGG-3') primers.

### *2.5 Screening of Phosphate-Solubilizing Clones*

Colonies with 100-fold dilution were spread on TCP agar medium containing 100 μg/mL ampicillin. After 3 d, clear halos appeared as a result of TCP being solubilized by the clones, and the clones were subcultured to confirm their stability. The cDNA of the clones with stable P-solubilizing function was sequenced, and the sequences were compared with the GenBank database using BLAST.

### *2.6 Expression Vector Construction*

Estimation of the ORF was based on cDNA sequence translation into protein by DNAMAN V6. The ORF sequence was amplified with the sense primer, (5′-TATTCGGAATTCATGGCCTGGGGCTGGGACC-3′) and antisense primer (5′-CAAGTACTCGAGTTACCAGCCACCACGGCGG-3′). The PCR reaction mix contained 1 μM of each primer, 10 μM deoxynucleotide, 10× buffer (NEB), 1 U of Taq Polymerase (NEB), and 1 μM primer 1 and primers. The following thermocycling conditions were used: initial denaturation at 94 °C for 5 min; 30 cycles of 94 °C for 30 s, 57 °C for 50 s, 72 °C for 50 s; and final elongation at 72 °C for 10 min (ABI 9700). The ORF sequence and pBluescript II SK vector were digested with the *EcoR* I and *Xho* I enzymes at 37 °C for 4 h. Digested products were linked to pBluescript II SK by T4 DNA ligase. Plasmids containing the ORF sequence were transformed into *E. coli* DH-5α. Transformants were selected on TCP plates with clear halos.

### *2.7 P-Solubilizing Ability and Organic Acid Secretion*

The P-solubilizing ability of *E. coli* DH5α overexpressing the CipC-like gene of *A. niger* H1 was tested on TCP medium. *E. coli* DH5α containing pBluescript II SK and ORF sequence (ORF-1), *E. coli* DH5α containing

pBluescript II SK and cDNA sequence (cDNA-1), and *E. coli* DH5α containing pBluescript II SK(P) were grown in TCP medium. Changes in pH medium and phosphorus concentrations were measured at 0, 4, 8, 12, 24, and 28 h, with shaking at 200 rpm at 37 °C. About 30 mL of broth was dispensed into 150 mL bottles, followed by inoculation with 100 μL of each bacterial culture ($10^8$ cfu/mL) grown in broth. The pH medium was measured using a pH meter equipped with a glass electrode. Organic acids produced by the bacteria were determined by an ion chromatography system (ICS-3000, Dionex, USA). The soluble phosphorus concentrations of the culture filtrates were measured using a spectrophotometer at 660 nm.

## 3. Results

### *3.1 RNA Isolation and Purification*

Agarose gel (1%) electrophoresis of total RNA from the sample of *A. niger* H1 is shown in Figure 1. Total RNA extracted with the TRIzol reagent showed two bright and clear bands corresponding to ribosomal 28S and 18S RNA. The ratio of A260/A280 of total RNA was 1.991 upon checking RNA purity using spectrophotometric analysis. These data showed that high-quality total RNA was successfully isolated from *A. niger* H1.

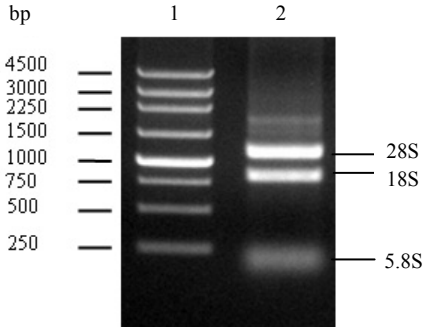


Figure 1. Gel analysis of total RNA recovered from *A.niger* H1. 2 μL of RNA on a 1% agarose gel. Lane 1, 250bp DNA Ladder marker (TaKaRa) , Lane 2, total RNA of *A.niger* H1

### *3.2 cDNA Library Construction*

The cDNA library of *A. niger* H1 was successfully constructed in pBluescript SK(+) starting with 2 μg of RNA by the SMART method. Gel analysis of the ds cDNA fragments showed that the cDNA was diffused, with a range of 250–4500 bp. After purification, cDNA fragments less than 500 bp were removed (Figure 2). Gel analysis of the ds cDNA indicated that the cDNA was intact and could ensure the integrity of the cDNA library.

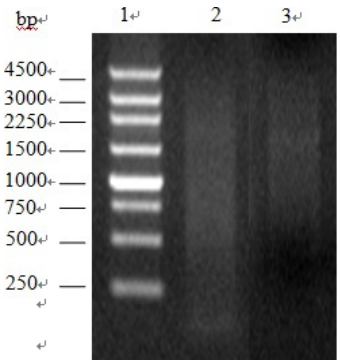


Figure 2. Unpurified and purified double-stranded cDNA. Lane 1, 250 bp Ladder DNA marker. Lane 2, unpurified double-stranded cDNA. Lane 3, purified double-stranded cDNA

### *3.3 Quality of the SMART cDNA Library*

The titer of the primary cDNA library was $1.95\times10^7$ cfu $mL^{-1}$, with a recombination rate of 99.15%. Fifty positive clones were randomly picked from the cDNA library, and the inserted cDNA fragments were confirmed

by PCR amplification, which revealed that most of the cDNA inserts ranged from 0.5 kb to 3 kb. Moreover, 90% clones were longer than 1000 bp (Figure 3). These results showed that a high-quality cDNA library of *A. niger* H1 was successfully constructed, and it was used for isolating and identifying full-length expressed genes.

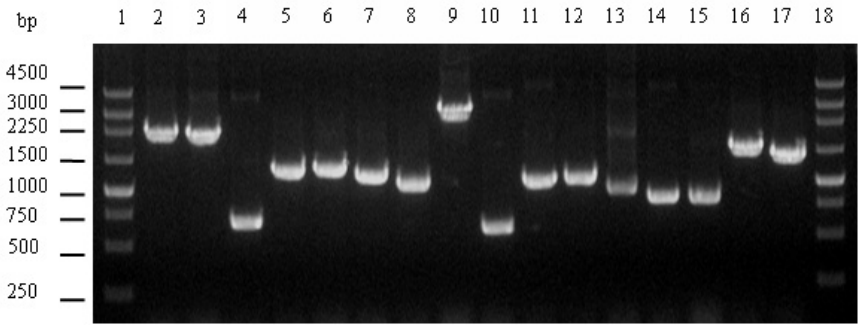


Figure 3. Analysis of PCR products from randomly picked clones. Lane1 and Lane 18: 250 bp Ladder DNA marker. Lane 2 to Lane 17, PCR products

*3.4 Screening Phosphate-Solubilizing Clones, Sequence Analysis, and Expression Vector Construction*

Clear halos appeared because of the solubilization of TCP by the clones after 3 d of incubation at 37 °C. A total of 162 positive clones were obtained, and the diameters of the clear halos ranged from 1.4 mm to 5.2 mm. H-47 was among the clones with clear halos. The diameter of the clear halo of H-47 was 2.34 mm. The full-length cDNA of clone H-47 was 625 bp-long containing an ORF of 390 bp, which encoded a polypeptide of 129 aa with a calculated molecular mass of 14.67 kDa. The nucleotide sequence had a 32 bp untranslated region at the 5′-region and another 203 bp untranslated region at the 3′-region. The sequence of H-47 cDNA has been deposited with GenBank under the accession number KR779001.

The cDNA sequence was analyzed with the GenBank database. H-47 showed 99% identity with *A. niger* CBS 513.88 partial mRNA and 98% identity with *A. niger* contig An07c0100 partial mRNA. The sequence from *A. niger* H1 also showed < 84% identity and 55% query cover with other fungi. The deduced amino acid sequence of *A. niger* H1 was analyzed using the Blast program (DNAMAN 6.0) with the GenBank database. Multiple alignment revealed a high degree of homology between the amino acid sequences of *A. niger* H1 and other CipC-like antibiotic response proteins (Figure 4). The sequence from *A. niger* H1 showed 100% identity with *A. niger* ATCC1015 (EHA23914.1) and 99% identity with both *A. niger* CBS 513.88 (XP_001391449.2) and *Aspergillus kawachii* IFO 4308 (GAA86201.1). The sequence from *A. niger* H1 also showed < 76% identity with other fungi (Figure 4).

The ORF of clone cDNA-1 was linked to pBluescript II SK, and the recombinant plasmid pBluescript II SK I-1 was transformed into *E. coli* DH-5α. Clear halos appeared as a result of TCP being solubilized by the transformed *E. coli* DH-5α in vitro, and a stable transformant I-1 was obtained (Figure 5).

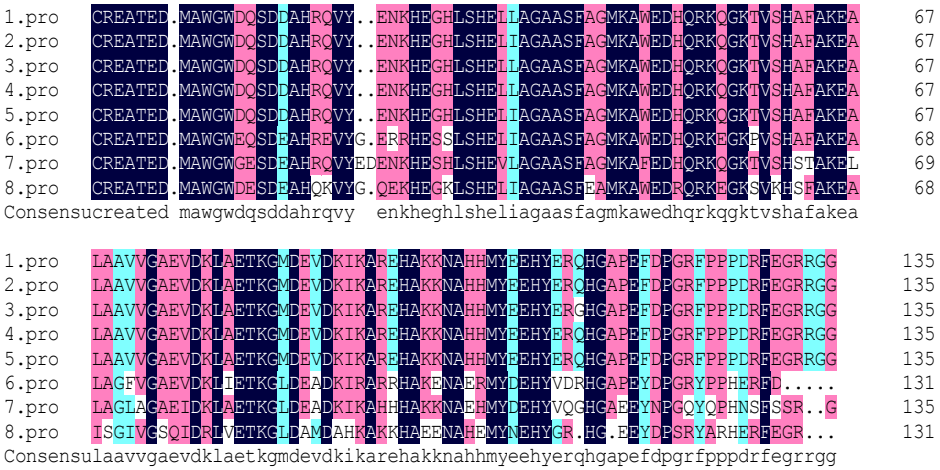


Figure 4. CLUSTALW alignment of the predicted amino acid sequence of the cloned *A. niger* H1 CipC-like gene, with other CipC-like and hypothetical protein sequences

*Note*. 1: *A. niger* H1; 2: *Aspergillus niger* CBS 513.88 (XP_001391449.2); 3: *Aspergillus kawachii* IFO 4308 (GAA86201.1); 4: *Aspergillus niger* ATCC 1015 (hypothetical protein,EHA23914.1); 5: *Aspergillus niger* (unnamed protein. CAK48117.1); 6: *Aspergillus fumigatus* Af293 (XP_753706.1); 7: *Aspergillus ruber* CBS 135680 (EYE98460.1); 8: *Aspergillus oryzae* RIB40 (XP_001727642.1).

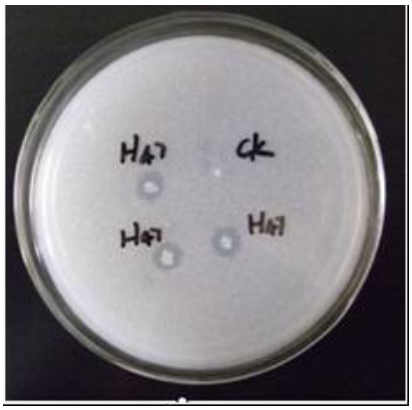


Figure 5. Phosphate-solubilizing activity of *E. coli* DH5α containing gene *psgT*. Appearance of clear zone around colonies on agar medium containing 0.3% TCP denotes solubilization of the insoluble P after three-day incubation. *E. coli* DH5α harboring the cloning vector without the inserts of *psgT* did not solubilize TCP

### *3.5 P-Solubilizing Ability and Organic Acid Secretion*

*E. coli* DH5α containing the CipC-like gene was grown in liquid medium to demonstrate the P solubilization ability of the recombinant. In the TCP liquid medium, the pH remarkably decreased by ORF-1 and cDNA-1 (Figure 6). Specifically, the pH decreased from 6.23 to 3.70 by ORF-1 and from 6.23 to 4.45 by cDNA-1 at 28 h. By contrast, the pH only decreased from 6.62 to 5.73 by pBlu at the same condition.

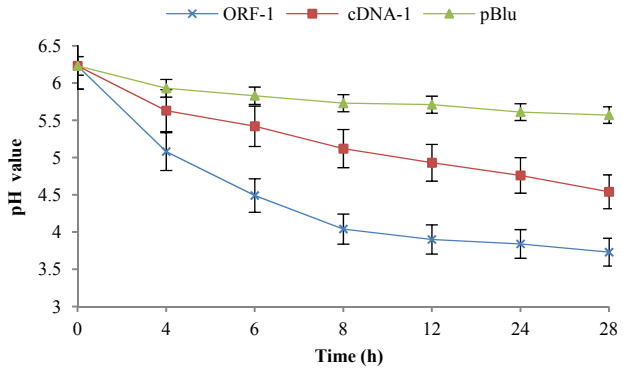


Figure 6. The pH change of culture filtrates after *E. coli* DH5α incubated in tricalcium phosphate liquid medium at 37 °C. *E. coli* DH5α containing ORF sequence(ORF-1), *E. coli* DH5α containing cDNA sequence(cDNA-1), and *E. coli* DH5α containing empty vector(pBlu)

Besides the drop in pH, a remarkable increase in the soluble P concentration was also observed in the culture medium in which ORF-1 and cDNA-1 were grown. The soluble P level in DH5α containing only the vector remained less than 18.8 μg/mL for up to 28 h. However, the soluble P concentration in *E. coli* DH5α (ORF-1) was 17.5 μg/mL after 4 h and then further increased to 117.7 μg/mL by 28 h. The soluble P concentration in *E. coli* DH5α (cDNA-1) was 14.8 μg/mL after 4 h and then further increased to 73.3 μg/mL by 28 h (Figure 7). *E. coli* DH5α (ORF-1) solubilized P more than *E. coli* DH5α (cDNA-1) by 37.7%.

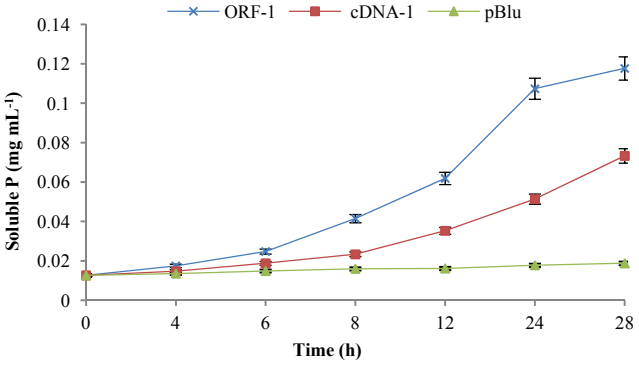


Figure 7. Changes in soluble phosphate concentration of culture filtrates after *E. coli* DH5α was incubated in tricalcium phosphate liquid medium at 37 °C

We tested whether the drop in pH observed with the *E. coli* is correlated with the secretion of organic acids. The result showed that *E. coli* DH5α (ORF-1, cDNA-1, and pBlu) could secrete organic acid. *E. coli* DH5α (pBlu) could secrete acetic acid only. *E. coli* DH5α (cDNA-1) could secrete methanoic acid and acetic acid. *E. coli* DH5α(ORF-1) could secrete methanoic acid, acetic acid, malic acid, and citric acid. The content of organic acids was significantly higher by *E. coli* DH5α (ORF-1) than that by *E. coli* DH5α (pBlu) and *E. coli* DH5α (cDNA-1). Acetic acid and methanoic acid were secreted in 4 h, and malic acid and citric acid were secreted in 24 h by E. *coli* DH5α (ORF-1). At 12 h, methanoic acid and acetic acid reached 81.2 and 93.3 μg $mL^{-1}$ respectively. At 28 h, malic acid and citric acid reached 50.6 and 147.7 μg $mL^{-1}$, respectively (Table 1).

Table 1. Organic acids production (μg $mL^{-1}$) by *E. coli* DH5α in tricalcium phosphate liquid medium at 37 °C

| | pBlu | ORF-1 | | | | cDNA-1 | |
|---|---|---|---|---|---|---|---|
| t/h | Acetic acid | Methanoic acid | Acetic acid | Malic acid | Citric acid | Methanoic acid | Acetic acid |
| 4 | 9.3 | 53.2 | 66.4 | - | - | 11.3 | 23.4 |
| 8 | 16.4 | 69.4 | 84.1 | - | - | 45.2 | 66.4 |
| 12 | 21.6 | 81.2 | 93.3 | - | - | 56.3 | 68.6 |
| 24 | 26.9 | 95.9 | 99.8 | 45.3 | 125.9 | 67.2 | 70.5 |
| 28 | 30.5 | 85.4 | 101.6 | 50.6 | 147.7 | 79.6 | 88.5 |

*Note*. pBlu: *E. coli* DH5α containing pBluescript; ORF-1: *E. coli* DH5α containing pBluescript II SK and open reading frame ORF sequence; cDNA-1:pBluescript II SK and cDNA sequence.

## 4. Discussion

Experiments proved that *A. niger* H1 exerted good effects on P solubilization and inhibited the transformation of soluble P to insoluble P. In this study, lactic acid was excreted and reached 377.9 μg $mL^{-1}$ at 30 h in culture by *A. niger* H1 (Gong et al., 2014b). The expression of certain genes could secrete organic acid in the *A. niger* H1 genome. The most important process of P solubilization is acidification (Sindhu et al., 2014; Rodríguez et al., 2006). The pBluescript II SK(+) is a cloning vectors designed to simplify commonly used cloning and sequencing procedures. The sequences include a multiple cloning site sequence, the multiple cloning site sequence is located within a LacZ controlled gene designed to provide a blue coloration when expressed in bacteria. Flanking the polylinker are T7 and T3 RNA polymerase promoters that can be used to synthesize RNA *in vitro* (Morris et al.,1986), The pBluescript II SK(+) can be used a plasmid to overexpress in *E. coli* (Gahlot et al., 2015). So it was a good way to screen gene that clear halos appeared because of the solubilization of TCP by the clones (Gong et al., 2014a). A cDNA library with SMART technique was constructed to screen cDNA sequences that could express in *E. coli* DH5α to solubilize P. A total of 169 positive clones showed clear halos in TCP by this. In this study, *E. coli* DH5α containing the *cipc-like* gene secreted methanoic acid, acetic acid, malic acid, and citric acid at levels of 81.2, 93.3, 50.6, and 147.7 μg $mL^{-1}$, respectively, within 28 h. Lü (2011) cloned a full-length gene encoding mitochondrial malate dehydrogenase from *P. oxalicum*, which was expressed in *E. coli* to secrete malate, lactate, acetic, and citrate acids, and the amount of organic acids was < 60 mg $L^{-1}$. Gong (2014) cloned the delta-1-pyrroline-5-carboxylate dehydrogenase gene that secreted acetic acid and α-ketoglutarate, and the acetic acid concentration was 389.81 μg $mL^{-1}$.

CipC-like is a member of the DUF3759 superfamily. This family of proteins is found in eukaryotes, but their functions remain unknown. Proteins in this family are typically between 107 and 132 aa in length. A single completely conserved residue H may be functionally important (Marchler-Bauer et al., 2015, 2011, 2009, 2004). In this study, we obtained the 625 bp-long full-length cDNA containing an ORF of 390 bp, which encoded a polypeptide of 129 aa. The cDNA sequence shared 99% identity with *A. niger* CBS 513.88 partial mRNA and 98% identity with *A. niger* contig An07c0100 partial mRNA, and below 84% with other fungi CipC-like mRNA. However, the deduced amino acid sequence identities were high at 99% with *A. niger* CBS 513.88 (XP_001391449.2) and *A. kawachii* IFO 4308 (GAA86201.1) amino acid sequences of CipC-like genes. Although many studies about the characteristics of CipC-like genes have been conducted, the overexpression of CipC-like in *E. coli* DH5α to secrete organic acid and improve P solubilization has not been reported. CipC-like could influence the organic acid metabolic pathways of *E. coli* DH5α. Consequently, if the host possesses both cycles, introduction of the CipC-like gene into the host can enhance organic acid secretion and P-solubilizing ability.

**Acknowledgements**

Project 41440008 supported by National Natural Science Foundation of China.

**References**

Altomare, C., Norvell, W. A., Bjorkman, T., & Harman, G. E. (1999). Solubilization of phosphates and micronutrients by the plant growth promoting and biocontrol fungus *Trichoderma harzianum* Fifai 1295-22. *Appl Environ Microbiol, 65*, 2926-2933.

Banik, S., & Dey, B. K. (1982). Available phosphate content of an alluvial soil as influenced by inoculation of

some isolated phosphate-solubilizing micro-organisms. *Plant Soil, 69*, 353-364. http://dx.doi.org/10.1007/BF02372456

Bianco, C., & Defez, R. (2010). Improvement of phosphate solubilization and medicago plant yield by an indole-3-acetic acid-overproducing strain of *Sinorhizobium meliloti*. *Appl. Environ. Microbial, 76*, 4626-4632. http://dx.doi.org/10.1128/AEM.02756-09

Fomina, M., Hillier, S., Charnock, J. M., Melville, K., Alexander, I. J., & Gadd, G. M. (2004). Role of oxalic acid overexcretion in transformations of toxic metal minerals by *Beauveria caledonica*. *Appl. Environ. Microbial, 71*, 371-381. http://dx.doi.org/10.1128/AEM.71.1.371-381.2005

Gahlot, S., Joshi, A., Singh, P., Tuteja, R., Dua, J. M. A., Kumar, M., ... Tuteja, N. (2015). Isolation of genes conferring salt tolerance from *Piriformospora indica* by random overexpression in *Escherichia coli*. *World J Microbiol Biotechnol*. http://dx.doi.org/10.1007/s11274-015-1867-5

Goldstein, A. H. (1995). Recent progress in understanding the molecular genetics and biochemistry of calcium phosphate solubilization by Gram-negative bacteria. *Biol Agric Hort, 12*, 185-193. http://dx.doi.org/10.1186/1471-2164-10-40

Gong, M. B., Du, P., Liu, X., & Zhu, C. X. (2014b). An Effective Method for Screening and Testing the True Phosphate-Solubilizing Fungus That Enhances Corn Growth. *J. Agri. Sci, 9*, 60-70. http://dx.doi.org/10.5539/jas.v6n9p60

Gong, M. B., Tang, C. X., & Zhu, C. X. (2014a). Cloning and Expression of Delta-1-pyrroline-5-carboxylate Dehydrogenase in *Escherichia coli*DH5α Improves Phosphate Solubilization. *Can. J. Microbiol, 60*, 1-5. http://dx.doi.org/10.1139/cjm-2014-0412

Khan, M. S., Zaidi, A., & Wani, P. A. (2007). Role of phosphate-solubilizing microorganisms in sustainable agriculture - A review. *Agron. Sustain. Dev, 27*, 29-43. http://dx.doi.org/10.1051/agro:2006011

Kim, K. Y., Jordan, D., & Krishnan, H. B. (1998). Expression of genes from *Rahmella aquatilis* that are necessary for mineral phosphate solubilization in *Escherichia coli*. *FEMS Microbiol Lett, 159*, 121-127. http://dx.doi.org/10.1007/s00284-003-4068-7

Kumar, C., Yadav, K., Archana, G., & Kumar, G. N. (2013). 2-Ketogluconic Acid Secretion by Incorporation of *Pseudomonas putida* KT 2440 Gluconate Dehydrogenase (gad) Operon in *Enterobacter asburiae* PSI3 Improves Mineral Phosphate Solubilization. *Curr Microbiol, 67*, 388-394. http://dx.doi.org/10.1007/s00284-013-0372-z

Lü, J., Gao, X. R., Dong, Z. M., & An, L. J. (2012). Expression of mitochondrial malate dehydrogenase in *Escherichia coli* improves phosphate solubilization. *Ann. Microbiol,* 1-8. http://dx.doi.org/10.1007/s13213-011-0297-3

Marchler-Bauer, A., & Bryant, S. H. (2004). CD-Search: Protein domain annotations on the fly. *Nucleic Acids Res, 32*(W), 327-331. http://dx.doi.org/10.1093/nar/gkh454

Marchler-Bauer, A., et al. (2009). CDD: Specific functional annotation with the conserved domain database. *Nucleic Acids Res, 37*(D), 205-10. http://dx.doi.org/10.1093/nar/gkn845

Marchler-Bauer, A., et al. (2011). CDD: A Conserved Domain Database for the functional annotation of proteins. *Nucleic Acids Res, 39*(D), 225-9. http://dx.doi.org/10.1093/nar/gkq1189

Marchler-Bauer, A., et al. (2015). CDD: NCBI's conserved domain database. *Nucleic Acids Res, 43*(D), 222-6. http://dx.doi.org/10.1093/nar/gku1221

Mehta, P., Walia, A., Chauhan, A., & Shirkot, C. K. (2013). Plant growth promoting traits of P-solubilizing rhizobacteria isolated from apple trees in trans Himalayan region of Himachal Pradesh. *Arch Microbiol, 195*, 357-369. http://dx.doi.org/10.1007/200203-013-0881-y

Meulenberg, J. J. M., Sellink, E., Riegman, N. H., & Postma, P. W. (1992). Nucleotide sequence and structure of the *Klebsiella pneumonia pqq* operon. *Mol Gen Genet, 232*, 284-294. http://dx.doi.org/10.1007/BF00280008

Morris, C. E., Klement, J. F., & McAllister, W. T. (1986). Cloning and expression of the bacteriophage T3 RNA polymerase gene. *Gene, 41*, 193-200. http://dx.doi.org/10.1016/0378-1119(86)90098-3

Nautiyal, C. S. (1999). An efficient microbiological growth medium for screening phosphate solubilizing microorganisms. *FEMS Microbiol. Lett, 170*, 265-270.

http://dx.doi.org/10.1111/j.1574-6968.1999.tb13383.x

Rodríguez, H., Fraga, R., Gonzalez, T., & Bashan, Y. (2006). Genetics of phosphate solubilization and its potential applications for improving plant growth-promoting bacteria. *Plant Soil, 287*, 15-21. http://dx.doi.org/10.1007/s11104-006-9056-9

Sindhu, S. S., Phour, M., Choudhary, S. R., & Chaudhary, D. (2014). Phosphorus Cycling: prospects of using rhizosphere microorganisms for improving phosphorus nutrition of plants. *Geomicrobiol .Biogeochem, 39*, 199-237. http://dx.doi.org/10.1007/978-3-642-41837-2_11

Werra, P., Tarr, M. P., Keel, C., & Maurhofer, M. (2009). Role of gluconic acid production in the regulation of biocontrol traits of pseudomonas fluorescens CHA0. *Appl. Environ. Microbial, 75*, 4162-4174. http://dx.doi.org/10.1128/AEM.00295-09

Xiao, C. Q., Chi, R., He, H., Qiu, G. Z., Wang, D. Z., & Zhang, W. X. (2009). Isolation of Phosphate-Solubilizing Fungi from Phosphate Mines and Their Effect on Wheat Seedling Growth. *Appl. Biochem. Biotech, 15*, 330-342. http://dx.doi.org/10.1007/s12010-009-8590-3

Xiao, C. Q., Fang, Y., & Chi, R. (2015). Phosphate solubilization *in vitro* by isolated *Aspergillus niger* and *Aspergillus carbonarius*. *Res. Chem. Intermed, 41*, 2867-2878. http://dx.doi.org/10.1007/s11164-013-1395-6