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SHORT COMMUNICATION

Screening of genes of secreting acetic acid from *Aspergillus niger* H1 to improve phosphate solubilization

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Abstract

A primary cDNA library of *Aspergillus niger* H1 was constructed using the switching mechanism at the 5´ end of the RNA transcript (SMART) technique. A total of 169 clones exhibited halos when grown on tricalcium phosphate medium, and the H-46 clone displayed a clear halo. The full-length cDNA of the clone H-46 clone was 1 407 bp in length with a complete open reading frame (ORF) of 816 bp, and it encoded a protein that contained 272 amino acids. Multiple alignment analysis revealed a high degree of homology between the ORFs of the H-46 clone and the Bax inhibitor family (BI-1-like) proteins of other fungi. Acetic acid was secreted by *Escherichia coli* DH5α that express the *BI-1*-like gene. The level attained was 492.52 mg L^{-1}, which was associated with the release of 0.212 mg mL^{-1} of soluble phosphate at 28 h. These results showed that the heterologous expression of *BI-1*-like genes in *Eschericha coli* DH5α increased the secretion of acetic acid by altering the membrane permeability and enhancing the solubility of phosphate (P).

Keywords: phosphate solubilisation, *Aspergillus niger*, acetic acid

1. Introduction

Organic acids are important in terms of phosphate (P) solubilization in soil. P-solubilizing microbes (PSMs) secrete organic acids that increase P solubilization. Oxalic, lactic, acetic, propionic, malic, tartaric, citric, butyric, malonic, succinic, gluconic and fumaric acids are the main organic acids known to solubilize P (Banik *et al.* 1982; Altomare *et al.* 1999; Fomina *et al.* 2004; Khan *et al.* 2007; Werra *et al.* 2009; Bianco *et al.* 2010; Gulati *et al.* 2010).

Highly efficient expression of exogenous genes that are related to P solubilization, which increase organic acid production, is the optimal strategy to improve P uptake. It is critical to obtain and heterologously express genes that improve organic acid secretion. Many studies on genes that improve organic acid secretion have been conducted to determine a way to improve P solubilization (Misra *et al.* 2013). In Gram-negative bacteria, gluconic acid is produced by the direct oxidation of glucose. This process is mediated by the membrane-bound glucose dehydrogenase enzyme,

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which requires pyrroloquinoline quinone as a cofactor (Goldstein *et al.* 1999). However, few fungal genes have such functions. Gong *et al.* (2014a) cloned delta-1-pyrroline-5-carboxylate dehydrogenase gene from *Penicillium oxalicum* and expressed the gene in *Escherichia coli*, which enhanced the secretion of acetic acid and α-ketoglutarate.

The fungus, *Aspergillus niger* H1, secretes lactic acid and converts a wide range of insoluble forms of P into soluble forms (Gong *et al.* 2014b). In this study, we constructed a primary cDNA library of *A. niger* and screened for clones that could solubilize P when grown on tricalcium phosphate (TCP) medium. We obtained a gene, named as *bI-1-like* gene, which could be expressed in *E. coli* to enhance the secretion of organic acids. The heterologous expression of *bI-1-like* gene in *E. coli* DH5α increased the secretion of acetic acid by altering the membrane permeability and enhancing the solubility of P.

2. Materials and methods

2.1. Strains, plasmids and media

A. niger H1 (ACCC32579) was obtained from the Agricultural Culture Collection of China and cultured in potato dextrose agar medium at 30°C. Plasmid transformants of *E. coli* DH5α were grown in Luria-Bertani medium and in TCP containing 100 μg mL^{-1} of ampicillin at 37°C. The TCP medium contained 10 g of glucose, 0.3 g of $MgSO_4 \cdot 7H_2O$, 0.3 g of NaCl, 0.3 g of KCl, 0.5 g of $(NH_4)_2SO_4$, 0.03 g of $FeSO_4 \cdot 7H_2O$, 0.03 g of $MnSO_4 \cdot 4H_2O$, and 5 g of $Ca_3(PO_4)_2$ per liter of distilled water (Nautiyal 1999). The vector was pBluescript II SK (TaKaRa, Japan).

2.2. Construction of the cDNA library and screening for P-solubilizing clones

A cDNA library was constructed using the the switching mechanism at the 5′ end of the RNA transcript (SMART) method. Then, the cDNAs were ligated into the pBluescript II SK vector, and recombinant plasmids were transformed into competent *E. coli* DH5α cells. Cultures that were diluted 100-fold were spread onto the TCP medium containing 100 μg mL^{-1} of ampicillin. After 3 days, clear halos appeared. The clones were subcultured to confirm their stability. The cDNAs of the clones that had stable P-solubilizing properties were sequenced using T3 and T7 primers. These sequences were compared to those in the GenBank database.

2.3. Expression vector construction

Open reading frame (ORF) was identified based on cDNA sequences that were translated into proteins using DNAMAN ver. 6.0. The ORF sequence was amplified with a sense primer (5′-TATTCGGAATTCATGCAGCCCTCCTACAATGT-3′) and an antisense primer (5′-CAAGTACTCGAGTTACCTCCGGTTGTTGTTGCGCAT-3′). The ORF sequence and the pBluescript II SK vector were digested with *Eco*RI and *Xho*I at 37°C for 4 h. The digested products were linked to pBluescript II SK using T4 DNA ligase. Plasmids containing the ORF sequence were transformed into competent *E. coli* DH5α cells. Transformants that yielded clear halos were selected from the TCP plates.

2.4. P-solubilizing ability and organic acid secretion

The P-solubilizing ability of the *E. coli* DH5α heterologous expression of the *A. niger* H1 *BI-1*-like gene was explored *via* growth in the TCP medium. *E. coli* DH5α containing pBluescript II SK and the ORF sequence (ORF-1) or pBluescript II SK (pBlu) alone were grown in the TCP medium. Aliquots (up to 100 μL) of each bacterial culture (×10^8 cfu mL^{-1}) were grown in a 50-mL broth. The cultures were shaken at 120 r min^{-1} at 37°C. Changes in the medium pH and P concentrations were measured in culture filtrates obtained at 0, 4, 8, 12, 24 and 28 h. The pH was measured using a pH meter. Organic acid levels were measured using an Anion Chromatography System (ICS-3000; Dionex, USA). The concentrations of soluble P in the culture filtrates were measured at 660 nm using a spectrophotometer.

2.5. Assessment of the membrane permeability of *E. coli* DH5α

Changes in the membrane permeability of the *E. coli* DH5α heterologous expression of the *A. niger* H1 *BI-1*-like gene were explored after growth in the TCP medium. The procedure was the same as described above for assessing the organic acid secretion. Cells were collected at 28 h, and the membrane were observed using transmission electron microscopy (TEM). TEM was performed using an accelerating voltage of 800 kV (JEM-1230; JEOL, USA).

3. Results

3.1. Screening of P-solubilizing clones, sequence analysis, and expression vector construction

Clear halos appeared around the clones (because of TCP solubilization) after 3 days of incubation on the TCP medium at 37°C. A total of 169 clones were obtained, and the diameters of the clear halos ranged from 1.4 to 5.2 mm. H-46 was among the clones with the clear halos, and the diameter of its halo was 4.6 mm. The full-length cDNA of