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Short communication

Identification of negative regulator for phosphate-sensing system in *Anabaena* sp. PCC 7120: A target gene for developing phosphorus removal

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ABSTRACT

A negative regulator for phosphate-sensing system in *Anabaena* sp. PCC 7120 is encoded by *all4501*. The phosphorus availability and total cellular phosphorus content were enhanced in the Δall4501 strain lacking this negative regulator, while, growth curve was similar to the wild type. The initial rate of phosphate uptake and cellular phosphorus content of the Δall4501 strain were 8-times and 2-times higher than the wild type strain. Increasing of cellular phosphorus content was clearly shown that phosphorus was stored as polyphosphate granules. Phosphorus removal from recirculating aquaculture system using the *Anabaena* sp. PCC 7120 strain Δall4501 was performed in a 5 L photobioreactor. Separation of treated water and cyanobacterial cells could be achieved spontaneously via simple settle down method. With single starter cell inoculation, the photobioreactor could be repeatedly used for phosphorus removal up to 10 cycles with the average phosphorus removal efficiency of 57.4% and 96.9% for wild type and Δall4501 strains, respectively.

1. Introduction

Recirculating aquaculture systems (RASs) are environmental friendly with high production yield and less water demand. Without water exchange, RAS technology requires the appropriate technology to minimize solids and nutrients accumulation in the water [1–3]. The removals of either solids or nitrogenous compounds in the RASs have been achieved via several treatment technologies [1–4]. In contrast, removal of phosphorus from the RAS is more complex, the practical phosphorus removal technology is not yet available [2]. Phosphorus is the major nutrient causing eutrophication [5]. Discharge of phosphorus into the natural water resources must be well regulated. Previous study on phosphorus accumulation in the RAS shown that over 94% of phosphorus was in form of inorganic phosphates [6]. Traditional chemical phosphorus removal process is not suitable with the RAS due to high toxicity of heavy metal for chemical precipitation processes that may harm to fish. Biological phosphorus removal using heterotrophic polyphosphate accumulating organisms (PAOs) have been extensively studied [7,8]. They require a complicated reactor for anaerobic process and well regulated organic carbon addition. Although, the removal efficiency is not stable as the microorganisms responsible for PAOs are uncultured and there are many bacteria completing the mechanisms of PAOs [9,10].

Alternatively, phototrophic organisms such as algae and cyanobacteria are more attractive for phosphorus removal [6,11–15]. The photosynthetic phosphate removal process requires neither addition of carbon source, nor enclosed system for anaerobic condition. They take up phosphate directly and massively store in the cells as polyphosphate granules. The cyanobacterial phosphate specific transport system (Pst system) was previously studied and clearly shown that this process was energy-dependent requiring only light energy [16]. In addition, the affinity for phosphate of Pst system was very high with the transport constant (K_s) of 4 μg P/L [16]. In prokaryotic organisms, two-component phosphate sensing system is used for monitoring the extracellular phosphate concentration [17]. Inactivation of a negative regulator for phosphate sensing system (PhoU or SphU) resulted in constitutive expression of genes involving in phosphorus metabolism or Pho regulon genes [17,18]. Alkaline phosphatase and phosphate

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Table 1
Oligonucleotide sequences for PCR amplification.

name	sequence	Product size (kb)
f Upstream	TCAAAATCTGTCTCTCTCCT	0.4
r Upstream	CTTGTAGTCTCAAACGTGAA	
f Downstream	GAGTATTTTTAAGCTCATCCCA	0.5
r Downstream	GGTAGCTATTTTCAAACATGAG	
f Check	AGCTCACGGAGGTTCCATC	0.8 (WT)/1.4 (MT)
r Check	GGGAGGGATGGGATGAGCTT	

Table 2
Plasmids constructed in this study.

Name	Description
pUall4501	pGEM-T easy vector containing upstream region of *all4501*
pDall4501	pGEM-T easy vector containing downstream region of *all4501*
pUall4501: Nm^R	pUall4501 inserted with neomycin-resistance cassette at *Spe* I site
pΔall4501: Nm^R	pDall4501 inserted with upstream region of *all4501* and neomycin-resistance cassette at *Sph* I site
pRLΔall4501: Nm^R	pRL271 inserted with upstream-neomycin-resistance cassette-downstream region of *all4501* fragment at *Pst* I site

specific transport system genes are member of the Pho regulon in which alkaline phosphatase is commonly used as an indicator of the Pho regulon expression. A previous study has shown that the cyanobacterium *Synechocystis* sp. PCC 6803 strain ΔSphU efficiently removed phosphate in the RAS [6]. However, the separation of the treated water and cyanobacterial cells with less than 2 μm in size was complicated as it cannot be separated by regular filtration device. In addition, using filamentous cyanobacterium *Anabaena* sp. PCC 7120 could overcome the cell separation problem. With the auto-flocculation property of the *Anabaena* sp. PCC 7120, cell separation was able to simply perform via settle down method [19]. As mentioned above, inactivation of negative regulator *sphU* in *Synechocystis* enhanced phosphate removal efficiency [6]. The negative regulator of *Anabaena* sp. PCC 7120, however, has not been identified. Homologous study of the *Anabaena* sp. PCC 7120 genome indicates high sequence similarities of the genes involving in phosphate sensing system [20]. An open reading frame (ORF) of *all4501* shares strong similarity with the *slr0741* (*sphU*) of *Synechocystis* sp. PCC 6803 to 77% in deduced amino acid sequences (64% identity) [21].

In this study, the *all4501* of the *Anabaena* sp. PCC 7120 was target gene to identify and characterize its role as the negative regulator for phosphate sensing system. Deletion of the whole *all4501* ORF was constructed. The application of the deletion strain for phosphorus removal in the RAS was also studied.

2. Materials and methods

2.1. Culture conditions and strain construction

Anabaena sp. PCC 7120 was grown in 250 mL flask containing 100 mL BG-11 medium under continuous shaking at 120 rpm and white light illumination at 6000 lx under room temperature 30 ± 3 °C. Cell density was measured at an optical density of 730 nm (OD_{730}), and total chlorophyll *a* concentration was determined by spectrophotometer (Hitachi U-1800, Japan) at 663 nm after extracted with 90% methanol [22]. The target gene for deletion mutagenesis was *all4501* encoding a putative negative regulator for phosphate sensing system. The 1.2 kb of neomycin-resistance cassette was replaced between 1 bp upstream of the GTG start codon and 9 bp downstream of the TAA stop codon of the 0.67 kb of *all4501* gene. Primers and plasmids used in this study were shown in Tables 1 and 2 respectively. Both upstream and downstream fragments of all4501 were amplified by PCR (Px2 Thermal cycler, Thermo Electron Corporation, USA) and ligated into pGEM-T easy vector (Promega, USA), yielding pUall4501 and pDall4501, respectively. The neomycin-resistance cassette was inserted into at *Spe I* site, yielding pUall4501:Nm^R. The DNA fragment containing upstream region and neomycin-resistance from pUall4501:Nm^R was cut with *Eco RI* and then inserted at the *Sph I* site of pDall4501, creating pΔall4501:Nm^R. The Δall4501 fragment was then cut with *Pvu II* and inserted to pRL271 at the *Pst I* site, yielding pRLΔall4501. The pRLΔall4501 was then transformed into *Anabaena* sp. PCC 7120 via triparental conjugation [23]. Briefly, the *Anabaena* cells were mixed with the *Escherichia coli* HB 101 cargo strain containing pRLΔall4501 and the *E. coli* helper strain and incubated at 30 °C under light illumination for 1 h. The transformants were screened on BG-11 plate containing 20 μg/mL neomycin. The complete segregation of the *Δall4501* gene in genomic DNA was confirmed by colony PCR with specific primers shown in Table 1. For phosphate-limiting BG-11 medium, K_2HPO_4 was replaced by KCl at the same concentration [18,24].

2.2. Analytical methods

Phosphate concentration was measured spectrophotometrically via ascorbic acid method [25]. Total phosphorus was digested to phosphate by persulfate autoclave digestion method and analyzed as phosphate [26]. Intracellular polyphosphate granules were detected under fluorescence microscopy (Olympus BX50, Japan), staining with 4′6-diamidino-2-phenylindole (DAPI) (Sigma, Israel) [6,27]. Alkaline phosphatase activity was determined by the hydrolysis of *p*-nitrophenyl phosphate (PNPP) (Sigma, USA) [18,24]. The intact cells were collected and resuspended in assay solution (0.2 M Tris/HCl pH 8.5/2 mM $MgCl_2$/120 mM PNPP). The assay mixture incubated at 37 °C for 20 min. The assay reaction was stopped by addition of NaOH solution and the clear supernatant was measured spectrophotometrically at 400 nm. Phosphate uptake study was done according to Burut-Archanai et al. by determination of the residual phosphate in the medium [16].

2.3. Phosphate removal in a photobioreactor

A photobioreactor in this study was a clear acrylic cylinder with working volume of 5 L (12 cm diameter and 60 cm height) operated under 6000 lx continuous illumination at 30 ± 2 °C. Starter culture of *Anabaena* in BG-11 medium was inoculated to the initial concentration of 1.8 mg Chl *a*/L. The photobioreactor was operated manually as a bubble column mode with 23 h of continuous aeration. Once a day, the aeration was paused for 1 h allowing 45 min of cell settling and 15 min of water draining and refilling with the water from RAS. The RAS in this study was a 4000 L indoor tilapia tank operated with bioflocs procedure [28]. To evaluate phosphate removal efficiency, 7 L of the raw water containing high phosphate concentration from the RAS was withdraw to 10 L settling tank and left for 1 h settlement. Water from upper layer was then transferred into the photobioreactor. The cells density and phosphate concentration in the photobioreactor was measured using water sampling from the center of the reactor. The residual phosphate in the water was measured after the sample filtration through 0.45 μm membrane filter.