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Suppression subtraction hybridization analysis revealed regulation of some cell cycle and toxin genes in *Alexandrium catenella* by phosphate limitation

Chao Zhang [a,b], Senjie Lin [b,c,*], Liangmin Huang [a], Wang Lu [b], Meizhen Li [b], Sheng Liu [a]

[a] CAS Key Laboratory of Tropical Marine Bio-resources and Ecology, South China Sea Institute of Oceanology, Guangzhou, Guangdong, China
[b] Key State Laboratory of Marine Environmental Science and Marine Biodiversity and Global Change Research Center, Xiamen University, Xiamen, Fujian, China
[c] Department of Marine Sciences, University of Connecticut, Groton, CT, USA

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ABSTRACT

Molecular mechanisms regulating responses to phosphate (P) limitation in dinoflagellates are poorly understood. Here we investigated gene differential expression *Alexandrium catenella* strain ATHK using suppression subtractive hybridization (SSH) and reverse-transcription quantitative PCR (qRT-PCR) techniques. SSH revealed a score of genes responded to P-limitation, and comparison with EST sequencing results indicated superior efficiency of SSH in identifying differentially expressed genes. Among the P-limitation responsive genes were the up-regulated stress-related genes, the down-regulated phosphorus-transferring genes, and a number of other genes previously undocumented for dinoflagellates. Most notably, the previously undocumented cell cycle inhibitory regulator fizzy/cell division cycle 20-related protein gene was up-regulated while putatively cell cycle promoting genes (e.g. calcium-dependent protein kinase) down-regulated, consistent with the decrease in growth rate and cell cycle arrest in G1 phase under P-limitation observed microscopically and flow cytometrically respectively. Besides, both SSH and qRT-PCR showed that saxitoxin related genes, menaquinone biosynthesis methyltransferase, an unknown gene and some other genes were also significantly induced by P-limitation. Our results demonstrate the utility of SSH in gene regulation research and provide a set of promising genes for further studies to understand the regulation of cell division (and hence population growth) and toxin production by P-limitation in *A. catenella* and likely other dinoflagellates.

1. Introduction

Dinoflagellates are key contributors of harmful algal blooms (HABs) and biotoxins in the marine ecosystem. Among the many species of dinoflagellates, *Alexandrium catenella* is a producer of the potent neuromuscular toxin (saxitoxin) that causes paralytic shellfish poisoning (PSP) in humans and some marine animals consuming shellfish that accumulate the toxin. In recent years, *A. catenella* received increasing attention by researchers worldwide because of impact on human health and marine ecosystem (Bravo et al., 2008; Toulza et al., 2010). This is a species in the *Alexandrium tamarense* complex that belongs to Group IV based on 28S rDNA phylogeny or Clade IID based on 18S rDNA phylogeny, which was lately revalidated as *A. catenella* to be one of the five phylogenetic species in this complex (Wang et al., 2014).

It is widely believed that dinoflagellates are better able to survive in nutrient-limited environments, with low growth rates, than other phytoplankton, such as diatoms (Smayda, 1997). Among other mechanisms, dinoflagellates are able to compensate for nutrient deficiency, due to traits such as circadian nutrient-retrieval migrations, high prevalence of mixotrophy, production of allelochemicals and toxins to prevent interspecific competition and predation (Cembella, 2003). An increasing amount of work has been done in the areas of intrinsic regulation of growth, nutrient uptake and starvation responses of dinoflagellates (Bonnet et al., 2008; Thingstad et al., 2005; Van Mooy et al., 2006). In order to better understand the mechanisms that regulate bloom formation and toxin production, a combined investigation of physiological characteristics and differential gene expression under different nutrient conditions is really important. Phosphate (P) is an essential nutrient to support phytoplankton growth, global ocean

* Corresponding author at: Department of Marine Sciences, University of Connecticut, Groton, CT, USA. Tel.: +1 860 405 9168; fax: +1 860 405 9153.
E-mail addresses: senjie.lin@uconn.edu, senjie.lin@gmail.com (S. Lin).

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productivity, and N_2 fixation (Moutin et al., 2008). P is thus increasingly recognized as a growth-limiting factor for phytoplankton.

Physiological studies of dinoflagellates to P-limitation have revealed decreased cellular P content, reduced growth rate, enlarged cell size, and increased cellular content of toxin (for toxin-producing species), as well as frequent switch from photoautotrophic to mixotrophic mode (Burkholder et al., 2008; Legrand et al., 1998; Hardison et al., 2013). Nevertheless, the molecular mechanisms underlying these physiological responses of dinoflagellates to P-limitation are still poorly understood. Only few studies have specifically addressed molecular responses to P-limitation (Lei and Lu, 2011; Morey et al., 2011). Characterized in slightly greater details was alkaline phosphatase encoding gene (AP), which catalyzes the hydrolysis of phosphomonoester to release phosphate to supplement P nutrition under P-limitation condition (Lin et al., 2011, 2012a,b). Transcription of AP is up-regulated under P-limitation, making it one of the few genes documented as transcriptionally regulated in dinoflagellates (Lin et al., 2012b). However, a broader view of what other genes are involved, or how basic biochemical pathways respond to P-limitation in dinoflagellates, particularly harmful algal bloom species, is still obscure. A transcriptomic profiling would be a useful approach to address the inadequacy. In contrast to the majority of eukaryotic algae, whose genome sizes typically range from tens to hundreds of million bp (Mb), dinoflagellates have genomes of 3–245 gigabase (Gb) DNA contained in several to over 100 chromosomes. It was predicted that dinoflagellate genomes, equivalent to 1–80 fold of a human haploid genome, might contain 38,188–87,688 protein-coding (40,086–92,013 total, including rRNA and tRNA) genes. The extraordinarily high number of genes, along with the generally low level of transcriptional regulation in dinoflagellates (reviewed by Lin, 2011), makes it difficult to identify differentially expressed genes using transcriptome sequencing.

Alternatively, mRNA differential display reverse transcription PCR, representation difference analysis (RDA), and suppression subtractive hybridization (SSH) (Zhang et al., 2012) can be used to identify differentially expressed genes. These approaches screen out constitutively expressed genes and identify differentially expressed genes for sequencing, enhancing chance of obtaining transcriptionally regulated genes while minimizing sequencing cost. Of these methods, SSH has superior sensitivity to recognize the differentially expressed genes in low transcript abundance (Diatchenko et al., 1999). In this study, SSH libraries were constructed for *Alexandrium catenella* cultures grown under P-replete and P-depleted conditions to reveal genes that are differentially expressed under the contrasting P conditions. The cDNA libraries constructed for the two P-conditions were sequenced at similar scales for comparison to the SSH method. In addition, reverse northern blot hybridization and quantitative reverse transcription PCR (qRT-PCR) were employed to verify the expression pattern of a selection of genes based on their differential expression pattern observed from the SSH library and their putative functions predicted by the blastx results.

2. Materials and methods

2.1. Algal cultures

Alexandrium catenella strain "ATHK" was obtained from the Collection Center of Marine Bacteria and Algae, in Xiamen University, China. The *A. catenella* cells were grown in K culture medium at 20 °C under a 14:10 h light:dark photoperiod at a photon flux density of 100 ± 10 μE m^{-2} S^{-1} provided by fluorescent lamps. Cell concentration was counted daily using Sedgewick-Rafter counting chamber (Phycotech, St. Joesph, MI, USA) (Lin et al., 2011).

2.2. P-limitation treatment and physiological measurements

The P-limited batch cultures were grown under the same conditions as P-replete cultures (modified K medium, with beta-glycerophosphate replaced with 22 μM $Na_2HPO_4{\cdot}12H_2O$), except that the dissolved inorganic phosphate (DIP), $Na_2HPO_4{\cdot}12H_2O$, was supplied at 2 μM. All treatments were set up in triplicates. Samples were collected for measurements of cell and alkaline phosphatase (AP) activity, 3 h after the light turned on every day. The bulk AP activity of the cultures was measured as described previously (Lin et al., 2011).

2.3. Culture synchronization and flow cytometric cell cycle analysis

Cultures of *Alexandrium catenella* were synchronized using dark induction. Cultures maintained in the exponential growth phases were kept in continuous dark for 48 h, and then transferred into the original L/D cycle with an initial cell density about 3000 cells/ml. Both the P-replete group and P-depleted group were grown in the same modified K medium as described above, except that the initial concentration of phosphate was supplied at 22 μM and 2 μM respectively. The samples for cell count, flow cytometric analysis, and DIP measurement were collected every 2 h.

For flow cytometric analysis, cells were pelleted by centrifugation at 2000 × *g* at 4 °C for 10 min and fixed in 1 ml of 70% ethanol at −20 °C. Cells were then pelleted again and washed in phosphate buffer saline (PBS). Next, cells were resuspended in 2 ml DAPI (Partec OmbH, Germany) to incubate for more than 10 min. The stained cells were then subjected to flow cytometric analysis on CyFlow Space (Partec OmbH, Germany). The data were analyzed using Modfit to resolve cell cycle composition.

Cell size was measured for samples fixed in Lugol's solution from both the DIP-replete and depleted groups. The measurement was carried out microscopically (calibrated with a stage micrometer) for the samples collected at hour 24, as the cell width along the cigulum. About 50 cells were analyzed in each sample.

2.4. SSH library construction and reverse northern blot hybridization

Cells were harvested from each culture by centrifugation at 4000 × *g*, 20 °C for 15 min. Cell pellets were ground on liquid nitrogen using a micro pestle (Handy Pestle, Code No: HMX-301; TOYOBO Life Science, Shanghai, China) and resuspended in 1 ml Trizol and stored at −80 °C until RNA extraction. The supernatant (culture medium) recovered was used to measure DIP concentration with the molybdenum blue method (Parsons et al., 1984).

Total RNA was extracted and treated as previously reported (Zhang et al., 2007). A SMARTer PCR cDNA Synthesis Kit (Clontech, Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) was used to reverse transcribe mRNA to cDNA from 1.0 μg total RNAs from P-replete and P-depleted cultures respectively, according to the manufacturer's protocol. Forward (P-depleted RNA as tester, P-replete RNA as driver) and reverse libraries (P-replete RNA as tester, P-depleted RNA as driver) were constructed by SSH using a PCR-Select cDNA subtraction kit (Clontech) following the manufacturer's instructions. Briefly, the tester and driver cDNA were digested with the restriction enzyme Rsal and then ligated to different adaptors, followed by two rounds of hybridization and PCR-select. The amplified product of the secondary PCR was cloned into the vector PMD18-T (Takara Biotechnology (Dalian) Co., Ltd., Dalian, China) transformed into *Escherichia coli* JM109 cell line. Finally, clones were randomly selected for PCR-based screening for