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Comparative proteomic studies of a *Scrippsiella acuminata* bloom with its laboratory-grown culture using a ^{15}N-metabolic labeling approach

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ABSTRACT

Comparative proteomic analysis was carried out using cells isolated from a natural bloom of *Scrippsiella acuminata* (formerly *Scrippsiella trochoidea*) in the early bloom (EB) and late bloom (LB) stages as well as with laboratory-grown cultures of cells isolated from the bloom in early growth (EG) and late growth (LG) stages. For quantitative proteomics, LG cells were grown for 20 generations in the presence of ^{15}N as a reference (i.e. common denominator) for all comparison. In comparisons with early growth laboratory grown cells (EG/LG), nearly 64% of proteins identified had similar abundance levels, with the remaining 36% mostly more abundant in EG cells. Calvin cycle, amino acid metabolism, chlorophyll biosynthesis and transcription/translation were among the up-regulated processes. Cells from the early bloom (EB/LG) had a greater abundance of transporters and enzymes related to light harvesting and oxidative phosphorylation, while the abundance of these proteins decreased in late bloom cells (LB/LG). All natural bloom samples showed either constant or lower abundance levels of enzymes involved in sugar synthesis and glycolytic pathways compared to laboratory grown cells. Our results represent the first examination of the proteomic changes in the development of a natural dinoflagellate bloom. Importantly, our results demonstrate that the proteome of cells grown in the laboratory is distinctively different from cells in a natural bloom.

1. Introduction

Harmful algal blooms (HAB) are natural phenomena commonly found in coastal waters. HABs occurring near highly populated coastal areas or aquaculture sites can cause serious economic losses (Habas and Gilbert, 1974; Jin et al., 2008; Lu and Hodgkiss, 2004; Yang and Hodgkiss, 2004). Because of this negative economic impact, many studies have been conducted to understand the mechanisms of HABs (Huisman et al., 1999; McGillicuddy et al., 2003; Sun et al., 2004), and proteomic technologies were used regularly in these studies (Chan et al., 2004, 2002; Wang et al., 2013, 2012). Proteins are the end-point of gene expression, and they directly interact with metabolites as enzymes and acceptors to carry out cellular events and to respond to the environment (Carretero-Paulet et al., 2010; Rubio et al., 2001). Thus, protein expression profiles will more accurately reflect the actual metabolic events happening in the cell than will RNA levels. However, as it is difficult to obtain field-collected bloom samples with sufficient purity and quantity for elaborate proteomic analysis, laboratory grown cultures were usually used as models. Approaches to investigating bloom-related mechanisms include the response to different environmental conditions, such as temperature and salinity (Kim et al., 2005; Lee et al., 2009; Shim et al., 2011), as well as to different nitrogen and phosphate levels (Lei and Lu, 2011; Sun et al., 2012; Zhang et al., 2015b).

It has been generally assumed that differentially expressed proteins found by comparing laboratory-grown cells in different growth phases or nutrient availabilities was a good strategy to identify initiators of the blooming processes (Chan et al., 2004, 2002; Lee et al., 2009; Lei and Lu, 2011; Sun et al., 2012; Wang et al., 2013, 2012; Zhang et al., 2015b). However, this approach has yet to reveal any confirmed bloom initiating factors and the mechanism underlying formation of the bloom is still unknown. Thus, we hypothesized that proteomic changes occurring during bloom progression in field-collected cells might be different from what is seen during growth of cultures in the laboratory. These types of studies are difficult to carry out as they require serial samples of a natural bloom. To date, only a single study has compared protein

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expressions in an algal bloom field sample (of the diatom *Skeletonema costatum*) with those of laboratory grown samples of the same strain using label-free shotgun proteomics (Zhang et al., 2015a), and no serial bloom samples were collected and analyzed. Zhang et al. reported a generally high abundance of proteins participating in light harvesting, photosynthetic pigment biosynthesis, photo-protection, cell division and redox homeostasis in both laboratory and field samples (Zhang et al., 2015a). They also found that levels of proteins involved in translation, amino acid and protein metabolic processes, as well as nitrogen and carbon assimilation differed between the field collected and laboratory grown diatom samples and concluded that diatoms had evolved adaptive mechanisms to acquire energy for survival in a changing environment (Zhang et al., 2015a).

In August 2012, in the coastal area around Tai Mei Tuk, Hong Kong, a *Scrippsiella acuminata* (formerly *Scrippsiella trochoidea*) (Kretschmann et al., 2015) bloom with ~95% purity occurred. Fresh field samples were collected daily from the area, and fortuitously, these include those from the 3rd of August, the first day of bloom, and from 6th August 2012 when cell populations were at their peak. The bloom disappeared totally on 8th August 2012. In this study, we report an analysis of serial samples of this dinoflagellate bloom. As *S. acuminata* was one of the HAB-causative species that bloom frequently in Hong Kong, we were particularly interested to find if the proteome of these cells changes in a similar manner during growth in the field and in the laboratory.

2. Materials and methods

Unless stated otherwise, all chemicals used were purchased from Sigma-Aldrich (USA) and were at least of analytical reagent (AR) grade. All solvents used were at least of HPLC grade. Except for the use of distilled water for culture medium preparation, water used was of ultra-pure grade and purified using a Milli-Q Type-1 system (Millipore, USA). Autoclaved or nuclease-free reagents and apparatus were used in RNA purification procedures. Safe-lock micro-centrifuge tubes (Eppendrof, USA) and Diamond pipette tips (Gilson, USA) were used in all steps of the proteomic manipulations.

2.1. Cell strains, culturing and harvesting

On 3rd August 2012, a *Scrippsiella acuminata* (dinoflagellate) bloom of at least 95% purity as judged by cell counting occurred at the pontoon of Tai Mei Tuk Water Sport Centre (N22.467224, E114.232713), Hong Kong. This species was formerly called by its heterotypic synonym *Scrippsiella trochoidea*. In the midst of the bloom area, daily seawater samples from around 5 cm underneath the water surface were collected at midday from 3rd to 8th of August 2012. Cells were counted as described previously (Lee et al., 2009). Further, two liters of concentrated algal biomass were collected on 3rd of August (early bloom stage, denoted as EB) and 6th of August (late bloom stage, denoted as LB). The samples were centrifuged at 2,700 × *g* for five minutes and the cell pellets were frozen immediately after harvesting. Total phosphate, ammonia and nitrate in the seawaters on 3rd and 6th of August 2012 were measured with the PhosVer 3 Phosphate Reagent Kit, the Ammonia Salicylate Reagent Kit and the NitraVer 5 Nitrate Reagent Kit (all from HACH, USA), respectively, using the instructions provided.

The dominant dinoflagellates in the biomass were identified as *Scrippsiella acuminata* by a PCR-based ITS sequencing technique as described previously (Lee et al., 2008). Briefly, the genomic DNA were extracted using the High Pure PCR Temperate Preparation Kit (Roche, USA). The ITS region of the genomic DNA sample was amplified using PCR with two sets of primers. The primer set called ITS1/4 consisted of a forward primer 5′-TCCGTAGGTGAACCTGCGG-3′ and a reverse primer 5′-TCCTCCGCTTATTGATATGC-3′. Another set of primers, ITSF/R, consisted of a forward primer 5′-TGAACCTTAYCACTTAGAGGAAGGA-3′ and a reverse primer 5′-GCTRAGCWDHTCCYTSTTCATTC-3′. The amplified PCR products were then cloned into pGEM-T vectors (Promega, USA) and were sequenced by BGI (Shenzen, China). The identity of the ITS region was determined by searches against GenBank (Benson et al., 2013).

To generate a laboratory culture, a hundred single cells were hand-picked using an auto-pipette under a phase contrast microscope at 40× magnification (Olympus, Japan). These single cells were cultivated as single clones in 100 μl of Sterile L1-Si medium (Lee et al., 2009) in a 96-well microplate (Nunc, USA) placed in a plant growth chamber (Sanyo, Japan) at 24 °C, with 12 h of 4000 K fluorescent light and 12 h dark for seven days. Clones that survived were then transferred into 50 ml conical flasks containing 10 ml of L1-Si medium. The clones were sub-cultivated for at least 10 generations (14 days per generation) before constituting a stable laboratory culture. The laboratory grown strains were also confirmed as *S. acuminata* using PCR-based ITS sequencing. One of the laboratory cultures was then grown for ~20 generations in modified L1-Si medium, in which the normal ^{14}N-$NaNO_3$ was replaced by 98% ^{15}N-$NaNO_3$ at the same concentration. The growth of the laboratory strain was monitored by cell number counting with biological triplicates. An unlabeled cell culture at Day 4 of growth (in early growth phase, EG) and an ^{15}N-labeled culture at Day 14 (in late growth phase, LG) were collected in the middle of the light period by centrifugation at 2,700 × *g* for five minutes. Successful ^{15}N-replacement of N atoms of proteins in the laboratory culture was confirmed by mass spectrometry (Supplementary Fig. 1).

2.2. RNA purification for transcript library construction

The dinoflagellates pellets were disrupted by 10 rounds of quick freeze-thawing in 1 ml of Trizol reagent (BioRad, USA) under alternating treatments with liquid nitrogen and water at room temperature. Trizol was used for RNA extraction as described (Simms et al., 1993). RNA pellets in 80% ethanol/diethylpyrocarbonate (DEPC)-water (Qiagen, USA) were sent to BGI (Shenzen, China) on dry ice for construction of a transcriptome library using their standard methods (Xie et al., 2012). Messenger-RNA in the samples were prepared from the total RNA by passage through oligo (dT) beads before synthesis of the EST library. Sequencing was performed using an Illumina HiSeq 2000 platform. The data acquired by the HiSeq 2000 were assembled by Groken Bioscience (Hong Kong). Adaptors were removed and reads with more than 5% unknown nucleotides were discarded. The cleaned reads were assembled using the Trinity software (Grabherr et al., 2011). Only bases with at least 50-fold coverage were used during sequence assembly. The final library contained 174,903 unigene sequences (Supplementary Table 1A), in which 89,881 of them were annotated to commercially available databases (Supplementary Table 1B) using Blast v.2.2.26+ (Camacho et al., 2009). A histogram showing the length distribution of the unigenes in the FASTA file was shown in Supplementary Fig. 2. The FASTA file was then converted into a nucleotide database in Mascot Server 2.5 (Matrix Science, USA) for proteomic identifications.

2.3. Protein purification and digestion

Algal cell pellets were homogenized in 6 M urea, 50 mM dithiothreitol and 10 mM Tris. Debris were removed by centrifugation at 10,000 × *g* for 4 min. Proteins in the supernatants were precipitated using TCA-acetone (Isaacson et al., 2006). Protein concentrations were measured using Bradford's protein assay (BioRad, USA). Subsequently, 50 μg of proteins extracted from the