Functional Differences in the Blooming Phytoplankton *Heterosigma akashiwo* and *Prorocentrum donghaiense* Revealed by Comparative Metaproteomics

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ABSTRACT Phytoplankton blooms are natural phenomena in the ocean, which are the results of rapid cell growth of some phytoplankton species in a unique environment. However, little is known about the molecular events occurring during the bloom. Here, we compared metaproteomes of two phytoplankton *Heterosigma akashiwo* and *Prorocentrum donghaiense* in the coastal East China Sea. *H. akashiwo* and *P. donghaiense* accounted for 7.82% and 4.74% of the phytoplankton community protein abundances in the nonbloom sample, whereas they contributed to 60.13% and 78.09%, respectively, in their individual blooming samples. Compared with *P. donghaiense*, *H. akashiwo* possessed a significantly higher abundance of light-harvesting complex proteins, carbonic anhydrase and RuBisCO. The blooming *H. akashiwo* cells expressed more proteins related to external nutrient acquisition, such as bicarbonate transporter SLC4, ammonium transporter, nitrite transporter, and alkaline phosphatase, while the blooming *P. donghaiense* cells highly expressed proteins related to extra- and intracellular organic nutrient utilization, such as amino acid transporter, 5'-nucleotidase, acid phosphatase, and tripeptidyl-peptidase. The strong capabilities of light harvesting, as well as acquisition and assimilation of inorganic carbon, nitrogen, and phosphorus, facilitated the formation of the *H. akashiwo* bloom under the high turbidity and inorganic nutrient-sufficient condition, whereas the competitive advantages in organic nutrient acquisition and reallocation guaranteed the occurrence of the *P. donghaiense* bloom under the inorganic nutrient-insufficient condition. This study highlights the power of metaproteomics for revealing the underlying molecular behaviors of different coexisting phytoplankton species and advances our knowledge on the formation of phytoplankton blooms.

IMPORTANCE A deep understanding of the mechanisms driving bloom formation is a prerequisite for effective bloom management. Metaproteomics was applied in this study to reveal the adaptive and responsive strategies of two coexisting phytoplankton species, *H. akashiwo* and *P. donghaiense*, during their bloom periods. Metabolic features and niche divergence in light harvesting, as well as carbon, nitrogen, and phosphorus acquisition and assimilation likely promoted the bloom occurrence under different environments. The molecular behaviors of coexisting bloom-causing species will give clues for bloom monitoring and management in the oceans.

KEYWORDS formation mechanism, metaproteomics, nutritional niche, phytoplankton bloom

Phytoplankton blooms are seasonal natural phenomena in the ocean and are generally associated with physical, chemical, and biological factors (1–3). Of particular importance are harmful algal blooms (HABs), which involve toxic or otherwise

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harmful phytoplankton species and have become a global concern due to their negative impacts on the ecosystems, economics, and public health (4–6). In the past few decades, the frequency, intensity, and geographic distribution of HABs have increased dramatically due to the influences of anthropogenic activities and climate change (7–11). The formation of HABs derived from specific species can occur periodically within a unified environment (2, 5, 12). However, to date, the underlying molecular mechanisms that drive bloom formation remain poorly understood.

Light availability, dissolved CO₂, and nutrient resources are the most important environmental factors that affect bloom progression owing to their essential roles in regulating cell growth and proliferation. Moreover, the ability to acquire light, CO₂, and nutrients heavily influence the bloom formation of different phytoplankton species. Light availability and nutrient composition, concentration, and ratio have been reported to trigger bloom occurrence and succession of dinoflagellate, diatom, green algae, and cyanobacteria in global estuaries (13–16). Carbon acquisition efficiency varies greatly among phytoplankton species, and the carbon starvation caused by rapid consumption of a high biomass partially affects the duration of bloom persistence (17, 18). In contrast to coexisting species, novel xanthorhodopsin-based light-harvesting systems, efficient carbon-concentrating mechanisms, and dissolved organic nutrient-acquiring mechanisms facilitate bloom occurrence of some dinoflagellates (19–24). These studies demonstrate the close causal relationship between bloom occurrence and the surrounding conditions. However, little is known about the cellular metabolic responses of specific species to ambient environmental changes during the bloom.

Molecular-level approaches, such as taxon-specific meta-omics have been increasingly applied to exploring the metabolic capacity of HAB-forming species (25, 26). Ecogenomic approaches reveal the genetic advantages that facilitate the bloom of *Aureococcus anophagefferens* in environments with high levels of organic matter, metal, and turbidity (27). Metatranscriptomic approaches provide new insights into the gene response and resource redistribution of cells during natural phytoplankton blooms and the blooms simulated by iron and deep seawater (24, 28–31). Comparative proteomics reveals that different light-harvesting abilities, nutrient assimilation mechanisms, and chemically mediated competition are partially responsible for the occurrence of nature and laboratory-simulated phytoplankton blooms (32–34). These studies shed light on the potential molecular mechanisms involved in the formation of HABs, and the discovery of the molecular behaviors of coexisting phytoplankton species within a unified environment have improved our understanding of the formation mechanisms of phytoplankton blooms.

The coastal East China Sea (ECS) is a highly eutrophic zone characterized by frequent occurrences of HABs in the past few decades (35). Long-term field investigations have revealed that *Prorocentrum donghaiense*, *Heterosigma akashiwo*, and other phytoplankton species cause extensive blooms from spring to early summer in this area (7, 36, 37). Similar bloom events and distinct ecological niches of these HAB species have also been reported in other coastal areas (38–41). However, the mechanisms that drive bloom occurrence of different phytoplankton species are unclear. Studies suggest that different ecological niches as well as nutrient utilization and light-harvesting abilities among phytoplankton species might play important roles in bloom formation. In this study, we applied a metaproteomic approach to investigate the global protein expression profiles of two coexisting phytoplankton species, *H. akashiwo* and *P. donghaiense*, during their bloom periods and characterized the differentially expressed proteins. Our results indicated that different light-harvesting ability and nutritional niche divergence in utilization of carbon, nitrogen, and phosphorus drove bloom occurrence of the two phytoplankton species under different ambient conditions.

RESULTS

Variations of chlorophyll *a* and nutrients during the bloom periods. The bloom processes of two coexisting phytoplankton species, *H. akashiwo* and *P. donghaiense*, were traced in the coastal ECS from 1 to 21 May 2014 (Fig. 1 and 2). In the first 2 days

FIG 1 Sampling locations. Each spot from the Za and Zb transects represents a sampling station. The nonbloom (NB) and bloom *H. akashiwo* (BHA) samples were collected at station Za3 (star) on 2 and 7 May, and the bloom *P. donghaiense* (BPD) sample was collected at station Zb7 (star) on 21 May. Map created with Ocean Data View software.

(1 May to 2 May), very low *H. akashiwo* and *P. donghaiense* cell densities were detected at each station of Za and Zb transects. *H. akashiwo* cell density increased at station Za3 from 2 May and then started to bloom radially and spread rapidly to the investigation area, covering transects Za and Zb. At station Za3, chlorophyll *a* (Chl *a*) concentration increased from 2.82 to 6.3 $\mu\text{g/liter}$ from 2 to 7 May, peaked around 9.67 $\mu\text{g/liter}$ on 9 May, and then dropped to 1.96 $\mu\text{g/liter}$ on 12 May. During the *H. akashiwo* bloom period, *P. donghaiense* cells were almost undetected. At the end of the *H. akashiwo* bloom, *P. donghaiense* cell density increased quickly from 13 May and began to bloom radially at station Zb7 (see Fig. S1 in the supplemental material). Chl *a* concentration increased from 2.54 to 6.2 $\mu\text{g/liter}$ from 13 to 21 May at station Zb7.

Blooming phytoplankton cells rapidly consumed nutrients of surface seawater, and a negative correlation between the concentrations of inorganic nutrients and Chl *a* was observed. Before the *H. akashiwo* bloom, the concentrations of nitrate, ammonia, and phosphate were 26.99, 5.3, and 1.34 μM , respectively, at station Za3 on 2 May and then

FIG 2 Pysiochemical conditions during the investigation. (A) Bloom process of *H. akashiwo* occurred at station Za3. (B) Bloom process of *P. donghaiense* occurred at station Zb7.

FIG 3 Protein information and taxonomic composition of the NB, BHA, and BPD samples. (A) Protein number of each phytoplankton group. (B) Percentage of each phytoplankton group based on protein abundances. (C) Percentage of each phytoplankton group based on 18S rDNA gene abundances.

changed to 19, 7.54, and 0.03 μM , respectively, on 7 May. In the dissipation phase of the *H. akashiwo* bloom, the concentrations of nitrate, ammonia, and phosphate were 7.59, 3.09, and 0.04 μM on 12 May. During the bloom period of *P. donghaiense*, the initial concentrations of nitrate, ammonia, and phosphate were 15.68, 5.8, and 0.11 μM on 13 May and then changed to 2.5, 3.47, and 0.21 μM on 21 May.

Overview of metaproteomics and phytoplankton community structure. Three phytoplankton samples representing the nonbloom (NB), blooming *H. akashiwo* (BHA), and blooming *P. donghaiense* (BPD) phases were selected for metaproteomic analysis; 310,084 $\pm$ 31,857, 327,175 $\pm$ 71,775, and 451,063 $\pm$ 26,774 tandem mass spectrometry (MS/MS) spectra were generated from the NB, BHA, and BPD samples, respectively. Using the combined sequence data set, 2.45 $\pm$ 0.23%, 8.6 $\pm$ 1.32%, and 5.22 $\pm$ 0.60% of the MS/MS spectra led to the identification of 9,446 high-confidence proteins (Table S1). Among them, 6,263, 6,707, and 7,566 proteins were detected in the NB, BHA, and BPD samples, respectively. Specifically, 1,542, 2,244, and 1,343 Ochrophyta proteins comprised 11.9%, 63.01%, and 1.75% of the total community protein abundance; 3,436, 3,278, and 5,073 Dinophyta proteins accounted for 15.74%, 20.85%, and 92.11% of the total abundance; 317, 301, and 252 Bacillariophyta proteins constituted 7.44%, 1.48%, and 0.96% of the total abundance; and 196, 145, and 169 Cryptophyta proteins contributed to 33.26%, 2.4%, and 1.35% of the total abundance. Other phytoplankton groups, such as Chlorophyta, Ciliophora, and Haptophyta, represented relatively small and stable proportions in the three samples (Fig. 3A and B).

The taxonomic composition of the 18S ribosomal DNA (18S rDNA) gene sequences also supported the metaproteomic results (Fig. 3C). Ochrophyta comprised 1.53%, 63.26%, and 0.74% of all community operational taxonomic unit (OTU) abundances in the NB, BHA, and BPD samples, respectively; Dinophyta comprised 64.48%, 33.24%, and 89.79% of the total abundance; Bacillariophyta comprised 3.40%, 0.18%, and 0.86% of the total abundance; and Cryptophyta comprised 4.22%, 0.23%, and 0.14% of the total abundance, followed by Chlorophyta, Ciliophora, and Haptophyta, which accounted for only 1.73%, 0.90%, and 1.82%, respectively.

Major biological processes in the blooming species. A total of 2,152 *H. akashiwo* proteins and 3,629 *P. donghaiense* proteins were detected in the three samples. In detail, the NB, BHA, and BPD samples contained 1,461, 2,150, and 1,274 *H. akashiwo* proteins and 2,350, 2,171, and 3,619 *P. donghaiense* proteins, respectively (Fig. 3A; Table S2). Regarding protein abundance, *H. akashiwo* contributed to 7.82%, 60.13%, and 1.35% in the NB, BHA, and BPD samples while *P. donghaiense* accounted for 4.74%, 2.19%, and 78.09% (Fig. 3B). Proteins related to cell growth and energy metabolism were highly expressed in the two blooming species (Fig. 4A; Table S3). For *H. akashiwo*, proteins related to carbon metabolism, ribosome, photosynthesis, photosynthesis antenna, oxidative phosphorylation, biosynthesis of amino acids, and glycolysis/gluconeogenesis were most abundant in the NB, BHA, and BPD samples. For *P. donghaiense*,

FIG 4 Heatmap of protein proportions at KEGG categories in the NB, BHA, and BPD samples. (A) Function classification of *H. akashiwo* and *P. donghaiense* in the three samples. Color gradient indicates the proportion of protein abundances mapped to each KEGG category of the total KEGG-annotated protein abundances in one species of each sample. (B) Abundance ratio of *H. akashiwo* to *P. donghaiense* at KEGG categories from each pair comparison. FC indicates fold change of *H. akashiwo* to *P. donghaiense*. NB/NB represents comparison between *H. akashiwo* in the NB sample and *P. donghaiense* in the NB sample, and BHA/BPD represents comparison between *H. akashiwo* in the BHA sample and *P. donghaiense* in the BPD sample.

proteins related to ribosome, spliceosome, photosynthesis, carbon metabolism, oxidative phosphorylation, protein processing in endoplasmic reticulum, amino acid biosynthesis, and photosynthesis antenna were most abundant in the NB, BHA, and BPD samples.

To minimize biomass and/or activity interferences among different bloom phases, the comparison of the two species within the NB sample and the comparison between *H. akashiwo* in the BHA sample and *P. donghaiense* in the BPD sample were performed (Fig. 4B; Table S3). As a result, proteins related to nucleotide excision repair, carbon metabolism, carbon fixation, carotenoid biosynthesis, vitamin B₆ metabolism, glutathione metabolism, photosynthesis, oxidative phosphorylation, and photosynthesis antenna from *H. akashiwo* occupied higher proportions than those from *P. donghaiense* in

the NB sample. Between the two blooming samples, proteins involved in carbon metabolism, carbon fixation, photosynthesis antenna, photosynthesis, nitrogen metabolism, oxidative phosphorylation, biosynthesis of *N*-glycan, carotenoid and fatty acid, glycolysis/gluconeogenesis, thiamine, and glycerolipid metabolism from *H. akashiwo* in the BHA sample accounted for greater proportions compared with those from *P. donghaiense* in the BPD sample. Correspondingly, proteins involved in ABC transporters, protein export, spliceosome, lysosome, phagosome, galactose metabolism, two-component systems, endocytosis, pyruvate metabolism, starch, and sucrose metabolism from *P. donghaiense* in the BPD sample constituted greater proportions than those from *H. akashiwo* in the BHA sample.

DISCUSSION

Much attention has been focused on phytoplankton blooms, and the biotic and abiotic factors stimulating the bloom formation have been studied extensively (5, 8, 9, 28, 30). However, the metabolic features of various coexisting phytoplankton species during the bloom period remain poorly understood. In this study, we quantitatively compared protein expression levels of two bloom-causing phytoplankton species, *H. akashiwo* and *P. donghaiense*, at their blooming phase. Our data revealed remarkable differences in metabolic features that likely promote bloom formation, especially in the metabolic processes related to light harvesting and nutrient utilization.

Light utilization. Marine phytoplankton rely on light to fix CO₂ into organic carbon, and light availability heavily affects the photosynthetic efficiency of phytoplankton. Therefore, light-harvesting ability is an important determinant of phytoplankton growth and proliferation in the ocean. Light-harvesting complex (LHC) proteins bind antenna chlorophyll and carotenoid pigments that augment the light-capturing capacity of phytoplankton (42). A greater number of gene copies of LHCs in a HAB species is considered to be a genetic advantage that facilitates its dominance among the coexisting species under a highly turbid estuary (27). Higher abundances of light-harvesting complex I chlorophyll *a/b* binding protein 1 (LHCA1), LHCA4, light-harvesting complex II chlorophyll *a/b* binding protein 3 (LHCB3), and LHCB5 from *H. akashiwo* were detected than those from *P. donghaiense* during the bloom period, especially in the NB sample (Fig. 5A). This finding indicated the stronger light-harvesting ability of *H. akashiwo*. The coastal ECS is characterized by high turbidity, and the intensity of photosynthetically active radiation attenuates rapidly with increasing water depth (43). The high expression level of LHC proteins in *H. akashiwo* may be an adaptive response to the high turbidity of the coastal ECS, enabling this species to outcompete other coexisting phytoplankton species and, thus, facilitating its bloom under conditions of high turbidity. Correspondingly, *H. akashiwo* exhibits an optimal growth rate over a wide light intensity range of 100 to 600 $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ (44). Moreover, it has been reported that the cellular chloroplast number of *H. akashiwo* can be adjusted for adaptation to varying light intensities (45).

The high cell biomass during phytoplankton blooms significantly attenuates light intensity in the water column. The *Prorocentrum minimum* bloom causes a >6-fold increase of the light diffuse attenuation coefficient and limits the average growth depth of phytoplankton from 1 to 0.5 m (46). In our study, both *H. akashiwo* and *P. donghaiense* bloom cells highly expressed LHC proteins for maximum light acquisition to cope with decreasing light availability (Fig. 5). Higher expression of LHC genes in the *H. akashiwo* and *P. donghaiense* bloom cells relative to nonbloom cells was also detected (24–37). LHC protein abundance from the *in situ* early-blooming cells of dinoflagellate *Scrippsiella acuminata* is higher than that from the late-blooming cells, which is mainly caused by the lower division rate of the late-blooming cells under severe nutrient-starved conditions (34). Degradation of LHCs is a vital responsive strategy to adapt to nutrient starvation, which provides a nutrient source for other essential metabolic processes to maintain growth (47, 48). Taken together, *H.*

FIG 5 Comparisons of protein abundances between the species *H. akashiwo* and *P. donghaiense* in the NB, BHA, and BPD samples. (A) Light-harvesting proteins. (B) Inorganic carbon assimilation proteins. (C) Carbon fixation proteins. CA, carbonic anhydrase; LHCA1, light-harvesting complex I chlorophyll *a/b* binding protein 1; LHCA4, light-harvesting complex I chlorophyll *a/b* binding protein 4; LHCB3, light-harvesting complex II chlorophyll *a/b* binding protein 3; LHCB5, light-harvesting complex II chlorophyll *a/b* binding protein 5; RBCII, ribulose 1,5-bisphosphate carboxylase oxygenase, form II; RBCL, ribulose 1,5-bisphosphate carboxylase oxygenase, large subunit; RBCS, ribulose 1,5-bisphosphate carboxylase oxygenase, small subunit; SLC4A10, solute carrier family 4 (sodium bicarbonate cotransporter), member 10.

akashiwo and *P. donghaiense* possessed strong abilities to adjust expression of LHC proteins to adapt to varying ambient conditions during the bloom period.

Carbon acquisition and fixation. The concentration of dissolved inorganic carbon (DIC) is a vital yet undervalued factor affecting the growth of phytoplankton in marine

environments. DIC availability has been reported to influence the formation and distribution of phytoplankton species (17, 49). The DIC in seawater comprises the sum of the relatively constant concentrations of HCO_3^- and CO_3^{2-} and the variable concentration of CO_2 . The CO_2 in surface seawater is partially starved due to its subsaturating concentration for phytoplankton RuBisCO affinity (50). This limitation is exacerbated by the oxygenation activity of RuBisCO that competes with carboxylation, leading to the dissipation of fixed carbon (51).

Almost all marine phytoplankton species have evolved carbon-concentrating mechanisms (CCMs) to enrich CO_2 at the RuBisCO catalytic site (52). As the key enzyme in CCMs, carbonic anhydrase (CA) facilitates the extra- and intercellular conversion between HCO_3^- and CO_2 (53). The existence of CCMs in *H. akashiwo* has been questioned, as previous studies failed to detect CA activity under different DIC limitation conditions (54, 55). In the present study, higher proportions of intercellular CA, α -CA, β -CA, and γ -CA from *H. akashiwo* were detected in the BHA sample than in the NB and BPD samples (Fig. 5B), indicating that *H. akashiwo* possesses CCMs that allow adaptation to ambient DIC changes. Multiple types of CA have also been reported in a metatranscriptome study (37). Compared with those from *H. akashiwo*, only very small proportions of CA and δ -CA from *P. donghaiense* were detected in the three samples. Moreover, CA and δ -CA from *P. donghaiense* were more highly expressed in the BHA and BPD samples than in the NB sample (Fig. 5B). A previous metatranscriptomic study reveals a significant increase of two δ -CA genes in a dinoflagellate, *Alexandrium fundyense*, in natural bloom conditions relative to laboratory culture conditions (56). Higher abundance and enzyme activity of intercellular CA protein from a dinoflagellate, *Protoceratium reticulatum*, sustain normal photosynthetic rates under low CO_2 conditions (21). All of these results indicated the vital role of CAs to adapt to low CO_2 conditions during the blooming phases of *H. akashiwo* and *P. donghaiense*. Strikingly, cell membrane protein of solute carrier family 4, member 10 (SLC4A10) from *H. akashiwo*, responsible for transmembrane bicarbonate transport, was detected in the NB and BHA samples. A substantial contribution of SLC4 to CCMs under low CO_2 conditions has been documented in diatoms (57, 58). In the phytoplankton blooming phases, the rapid consumption of CO_2 and high alkaline carbonate chemistry of seawater generated by high biomass lead to severe CO_2 limitation (59). Therefore, *H. akashiwo* cells expressed an efficient HCO_3^- transport system and highly abundant CA to acquire sufficient CO_2 for carbon fixation during the bloom period.

The slow carboxylation reaction of RuBisCO is a critical rate-limiting step for carbon fixation and cell growth. Apart from the elevation of cellular CO_2 concentration, increased RuBisCO abundance represents another vital adaptive strategy to enhance the CO_2 fixation efficiency in phytoplankton (52). A higher proportion of RuBisCO from *H. akashiwo* was observed compared with that from *P. donghaiense* during the bloom periods (Fig. 5C). Among six distinct phytoplankton taxa, dinoflagellates exhibit the lowest substrate specificity factor for RuBisCO (50). The poor kinetics of RuBisCO and the consequential low photosynthetic efficiency have been proposed to be responsible for the slow growth rate of dinoflagellates (49). Combined with enzyme kinetics studies, a higher abundance of RuBisCO from *H. akashiwo* reflected its higher carbon fixation efficiency than that from *P. donghaiense* during their bloom periods. *H. akashiwo* cells are also characterized by the presence of a great number of chloroplasts where carbon fixation occurs (60), consistent with our finding that *H. akashiwo* possessed high abundances of CA and RuBisCO. Taken together, the inorganic carbon transportation and fixation capabilities of *H. akashiwo* were more powerful than those of *P. donghaiense*, likely providing a genetic advantage that explains the earlier onset of the *H. akashiwo* bloom.

Phosphorus uptake and metabolism. Nutrient availability determines cell growth rates and partially influences the niche partition of phytoplankton in the marine environment (61, 62). Phosphorus (P) is a limiting nutrient for growth and productivity of phytoplankton in the ocean (63). Phytoplankton cells are known to be capable of

balancing P uptake, metabolism, and storage to maintain the bioavailability of inorganic phosphate (Pi) and dissolved organic phosphorus (DOP). As documented, *H. akashiwo* is less tolerant of P limitation than *P. minimum*, and low concentrations of ambient Pi eventually lead to the dissipation of the *H. akashiwo* bloom (54). In the present study, the dissolved inorganic N/P ratio varied between 21.72 and 822.89 (higher than the Redfield ratio of 16:1), indicating that Pi limitation occurred during the bloom periods. At the end of the *H. akashiwo* bloom, the concentration of Pi was 0.03 μM , whereas the concentration of Pi was relatively high during the bloom period of *P. donghaiense*, ranging from 0.11 μM to 0.21 μM . Thus, it is postulated that the low concentration of ambient Pi was a crucial factor responsible for the dissipation of the *H. akashiwo* bloom.

Solute carrier family 37 (glycerol-3-phosphate transporter) member 3 (SLC37A3) from *H. akashiwo* was detected in the BHA and BPD samples, while only a low-affinity inorganic phosphate transporter (PHO84) from *P. donghaiense* was detected in the three samples (Fig. 6A). SLC37A3, also known as sugar phosphate exchanger 3, is a sugar-phosphate antiporter that transports phosphate into the cell (64). In addition, vacuolar transporter chaperone 4 (VTC4), which is involved in the metabolism of polyphosphate, was detected only from *H. akashiwo* in the BHA sample (Fig. 6A). Polyphosphate serves as both a major cellular phosphate reservoir and an energy storage pool that can be used as a source of ATP (65). Some phytoplankton species can acquire Pi to synthesize polyphosphate under Pi-sufficient conditions and degrade polyphosphate to release Pi through upregulation of VTC4 under Pi-deficient conditions (66, 67). These results indicated that *H. akashiwo* initiates external phosphate transport and internal storage systems to adapt to low-Pi environments, thus supporting its bloom prior to that of *P. donghaiense*.

DOP serves as an important P source for phytoplankton under low-Pi conditions, but most DOP must be converted to Pi by cell surface alkaline phosphatases (APs) before use (63, 64). An alkaline phosphatase D (*phoD*) belonging to the AP family was detected from *H. akashiwo* in the NB and BHA samples (Fig. 6A), indicating that the cells utilized extracellular DOP in the low-Pi environment. However, we did not identify AP from *P. donghaiense* despite the existence of its sequences in the database, suggesting that AP might be present at low abundance or was not expressed. Interestingly, 5'-nucleotidase from *P. donghaiense* was detected in the NB and BPD samples (Fig. 6A). Recently, 5'-nucleotidase was reported to take function at extracellular ATP hydrolysis to maintain growth in dinoflagellate *Karenia mikimotoi* (68). Similarly, the *in situ* *P. donghaiense* cells may rely on 5'-nucleotidase rather than AP to utilize extracellular ATP as a P source during the bloom period. Utilization of the intracellular DOP represents another important adaptive strategy to manage low-Pi stress. For *H. akashiwo*, protein phosphatase in the BHA sample, phospholipase in the NB and BHA samples, and phosphatidylinositol phospholipase C and 3'(2'),5'-bisphosphate nucleotidase in the three samples were detected, while for *P. donghaiense*, acid phosphatase in the BPD sample and protein phosphatase and phosphatase 2C in the three samples were identified (Fig. 6A). Protein phosphatase, acid phosphatase, and 3'(2'),5'-bisphosphate nucleotidase hydrolyze phosphoric esters, while phospholipase and phosphatidylinositol phospholipase C hydrolyze structural phospholipids to release phosphate (69–71). These enzymes and their homologs involved in DOP reutilization are found to be highly expressed under P-deficient conditions (66–68). The multiple but varied DOP utilization strategies enable *H. akashiwo* and *P. donghaiense* to adapt to low-Pi concentrations during their blooming periods.

Nitrogen uptake and metabolism. Nitrogen is an essential nutrient for phytoplankton growth. In our study, concentrations of nitrogen decreased sharply during the bloom processes from *H. akashiwo* to *P. donghaiense*. Ammonium transporter and nitrite transporter Nar1 from *H. akashiwo* were detected in the three samples, while no inorganic nitrogen transporter from *P. donghaiense* was detected (Fig. 6B). Compared with those of *P. donghaiense*, higher abundances of substrate-specific transporters in *H.*

FIG 6 Comparisons of protein abundances between the bloom species *H. akashiwo* and *P. donghaiense* in the NB, BHA, and BPD samples. (A) Phosphorus metabolism-related proteins. (B) Nitrogen metabolism-related proteins. (C) Hydrolytic enzymes. 5'-nucleotidase; ABC.PA.A, polar amino acid transport system ATP-binding protein; ABC.PA.S, polar amino acid transport system substrate-binding protein; ACP, acid phosphatase; AMT, ammonium transporter; ARSA, arylsulfatase A; ARSB, arylsulfatase B; ARS_I_J, arylsulfatase I_J; AsL, argininosuccinate lyase; AsuS, argininosuccinate synthase; BPNT1, 3'(2'),5'-bisphosphate nucleotidase; CPB, cysteine peptidase B; CPS, carbamoyl-phosphate synthase; CTS, cathepsin; GNS, *N*-acetylglucosamine-6-sulfatase; GOGAT1, glutamate synthase (NADPH/NADH); GOGAT2, glutamate synthase (ferredoxin); GS, glutamine synthetase; GUSB, beta-glucuronidase; IDS, iduronate 2-sulfatase; NarI, nitrite transporter NirC; NiR, nitrite reductase; NR, nitrate reductase; OTC, ornithine carbamoyltransferase; PHO84, MFS transporter, PHS family, (Continued on next page)

akashiiwo indicated the stronger competitive ability for ammonium and nitrite of *H. akashiwo*. A previous field study also revealed that the bloom of *H. akashiwo* occurs after an increase of Pi and dissolved inorganic nitrogen concentrations while addition of phosphate and nitrate promotes its earlier bloom (72).

Interestingly, polar amino acid transport system ATP-binding protein (ABC.PA.A) and substrate-binding protein (ABC.PA.S) from *P. donghaiense* were identified in the three samples (Fig. 6B; see also Table S2 in the supplemental material). Although we did not measure the concentrations of amino acids, a significant decrease of dissolved amino acids coupled with the spring bloom succession from diatom *Skeletonema costatum* to *P. donghaiense* is observed in the same investigation area (22). Increasing evidence suggests that certain dinoflagellate species prefer dissolved organic nutrients to inorganic nutrients (19, 73). Taken together, after the dissipation of the *H. akashiwo* bloom, *P. donghaiense* activated the amino acid uptake system to maintain cell growth in an environment of low inorganic and high organic nitrogen contents. Moreover, a strong capability of amino acid acquisition promotes its bloom formation and maintenance in the presence of other coexisting phytoplankton species.

In marine phytoplankton, nitrate reductase (NR) and nitrite reductase (NiR) catalyze the reduction of NO_3^- to NH_4^+ . The cellular reduced and extracellular imported NH_4^+ is assimilated into glutamate through the GS-GOGAT pathway, which is catalyzed by glutamine synthetase (GS) and glutamate synthase (GOGAT) (74). The ornithine-urea cycle (OUC) converts NH_4^+ into amino acids and links the amino acid metabolism, TCA cycle, and GS-GOGAT pathway (75, 76). All of these intracellular nitrogen metabolizing enzymes and some OUC enzymes were detected from *H. akashiwo* and *P. donghaiense* in the three samples. Meanwhile, higher proportions of GS, GOGAT, and four OUC enzymes (argininosuccinate synthase, argininosuccinate lyase, carbamoyl-phosphate synthase, and ornithine carbamoyltransferase) and lower proportions of NR and NiR from *H. akashiwo* in the NB and BHA samples were detected, compared with those from *P. donghaiense* in the NB and BPD samples (Fig. 6B). As the GS-GOGAT and OUC pathways catalyze incorporation of NH_4^+ into organic molecules, the higher expression of related proteins further validated the significant contribution of ambient ammonium to the bloom formation of *H. akashiwo*. As documented, *H. akashiwo* prefers to acquire ammonium under both nitrogen sufficient and subsufficient conditions (77). It is predictable that *H. akashiwo* had a strong capability of utilizing various ambient sources of inorganic nitrogen, such as ammonium, nitrite, and nitrate, whereas *P. donghaiense* relied on nitrate/nitrite and amino acids as nitrogen resources. The different nutritional niches of nitrogen resources partially facilitated the subsequent bloom occurrence from *H. akashiwo* to *P. donghaiense* in the coastal ECS.

Hydrolysis of organic matter. Lysosomes are spherical vesicles that contain hydrolytic enzymes for the breakdown of various biomolecules from extracellular environments and cellular obsolete components (78). Functions of lysosomes are well-studied in animals, and lysosome-like vacuoles have been found in some plant and phytoplankton species (79, 80). Higher proportions of lysosome-like proteins from *P. donghaiense* were detected than those from *H. akashiwo* in the three samples (Fig. 4B and 6C; Table S3). Several subunits of cathepsin and tripeptidyl-peptidase involved in polypeptide hydrolysis were detected in both species (81). Beta-glucuronidase from *P. donghaiense*, catalyzing the hydrolysis of complex carbohydrates (82), was detected in the three samples. In addition, extracellular sulfatase Sulf, arylsulfatase subunit B, iduronate-2-sulfatase, and *N*-acetylglucosamine-6-sulfatase from *P. donghaiense* were detected in the three samples. All of these proteins catalyze the hydrolytic breakdown of complex sulfuric esters to release sulfate (83, 84). It has been reported that dissolved organic carbon and nitrogen accumulate substantially during the bloom period of *H. akashiwo* and peak at

FIG 6 Legend (Continued)

inorganic phosphate transporter; *phoD*, alkaline phosphatase D; PLCD, phosphatidylinositol phospholipase C; PLD, phospholipase; PP, protein phosphatase; PP2C, protein phosphatase 2C; SLC37A3, solute carrier family 37 (glycerol-3-phosphate transporter), member3; SULF, extracellular sulfatase Sulf; TPP1, tripeptidyl-peptidase I; TPP2, tripeptidyl-peptidase II; VTC4, vacuolar transporter chaperone 4.

the postbloom stage (85). The utilization of dissolved organic matter by mixotrophic species, for example, *P. donghaiense*, proves advantageous for the geographical distribution of HABs (86, 87). *H. akashiwo* released a large amount of dissolved organic matter into seawaters at the dissipation stage, thus providing carbon, nitrogen, phosphorus, and other nutrient sources for the cell growth and proliferation of *P. donghaiense*. Meanwhile, the high expression levels of various hydrolases utilizing organic matter in *P. donghaiense* supported this postulation to a certain degree.

Database construction for protein identification. For metaproteomic study, a suitable protein searching database is critical for achieving accurate functional and taxonomic characterization. The following two main approaches have been developed to construct protein searching databases: combining the public sequence data or conducting simultaneous metagenomic analysis (88, 89). Growing evidence suggests that protein sequences from the public database relative to the simultaneous metagenome will cause statistical bias on protein identification and then lead to different biological conclusions (89, 90). Sequencing simultaneous metagenome or metatranscriptome is therefore an ideal choice for metaproteomic study. When metagenome and/or metatranscriptome are not available, constructing the most suitable database to reflect the real environmental community structure is an alternative choice. In our study, we constructed a database consisting of phytoplankton sequences from two public databases, a transcriptome of bloom-causing species and an *in situ* metatranscriptome (see Table S4 in the supplemental material), to compensate for the lack of metagenome. Even though the database had only 5.7% of the protein sequences attributed to Ochrophyta (*H. akashiwo* belongs to this group), Ochrophyta proteins accounted for 24.6%, 33.5%, and 17.8% of the total proteins in the NB, BHA, and BPD samples. Moreover, a high degree of similarity of taxonomic composition inferred from the protein and 18S rDNA gene data were also observed in the three samples. These results indicated that the combined database largely contained the potential species in the investigation area, and it was suitable to unveil metabolic activities of each phytoplankton species during the bloom period, especially the two bloom-causing species.

In conclusion, our metaproteomic study revealed the different molecular behaviors of two coexisting phytoplankton species, *H. akashiwo* and *P. donghaiense*, during their bloom periods (Fig. 7). *H. akashiwo* exhibited strong capabilities of light harvesting, as well as acquisition and metabolism of inorganic carbon, nitrogen, and phosphorus, thus facilitating its earlier bloom under the conditions of high turbidity and inorganic nutrient concentrations. In the bloom phase, *H. akashiwo* cells highly expressed low-affinity phosphate transporter and activated intra- and extracellular organic phosphorus utilization to adapt to low-Pi stress. However, the low concentration of ambient Pi eventually led to the termination of the *H. akashiwo* bloom. *P. donghaiense* cells exhibited strong capabilities of acquisition and hydrolytic breakdown of extra- and intracellular organic matter in its bloom phase. Therefore, the different light-harvesting capability and nutritional niche divergence of the two coexisting phytoplankton species might drive the bloom occurrence under different ambient conditions. Our study sheds light on the molecular mechanisms of different phytoplankton blooms. Future efforts should be devoted to metaproteomic studies of blooms involving different phytoplankton species, and the results will help us more comprehensively understand the mechanisms of phytoplankton bloom formation in the ocean.

MATERIALS AND METHODS

Field survey and sampling. Field investigation of the spring phytoplankton bloom in the coastal ECS was conducted through daily surveying of each station along the Za and Zb transects from 1 to 21 May 2014 (Fig. 1). During the investigation, the physical oceanographic parameters were monitored by an onboard CTD (Sea-Bird, Bellevue, WA, USA). At each sampling station, three 50-ml surface seawater samples (1 m) were collected and fixed with Lugol's solution for microscopic examination. To avoid diel interference, all samples for Chl *a*, 18S rDNA, and metaproteomic analysis were collected with the same procedure between 11:00 and 14:00 each day. The surface seawater samples (1 m) were first filtered through a 200- μ m nylon net, and then through a 1.6- μ m GF/A membrane (Whatman, GE Healthcare Life Science). Three 200-ml surface seawater samples at each sampling station were collected for Chl *a* and nutrient measurements. The filtration membranes were kept at -20°C for Chl *a* analysis, and the filtrates

FIG 7 Summary of the key molecular events occurring at the *H. akashiwo* and *P. donghaiense* cells during their bloom periods. (A) *H. akashiwo* of the NB sample. (B) *P. donghaiense* of the NB sample. (C) *H. akashiwo* of the BHA sample. (D) *P. donghaiense* of the BPD sample. The red and green colors represent higher and lower proportions, respectively, of metabolic processes and enzymes of one species relative to those of the other species in the same blooming phase, and the gray color indicates no detection. The arrow represents the sample point. Protein abbreviations are annotated in Fig. 5 and 6.

were kept frozen for nutrient analysis. Two 1-liter surface seawater samples at each sampling station were collected, and the filtration membranes were stored at -20°C for 18S rDNA analysis. For metaproteomic analysis, two 30- to 60-liter surface seawater samples were collected, and the filtrates were frozen in liquid nitrogen immediately and then transferred for storage at -80°C .

Chl *a* and nutrient measurements. Chl *a* was extracted with 90% acetone and then analyzed using an Agilent series 1100 high-pressure liquid chromatography (HPLC) system fitted with a 3.5- μm Eclipse XDB C_{18} column (100 by 4.6 mm; Agilent Technologies) with a modified procedure (91). Nutrients were analyzed photometrically using an autoanalyzer (Skalar SANplus). The analytical precisions of NO_3^- , NH_4^+ , and PO_4^{3-} were 0.1 μM , 0.1 μM , and 0.05 μM , respectively.

DNA extraction and 18S rDNA sequencing. Before DNA extraction, the filtration membrane was suspended in DNA lysis buffer (10 mM Tris, pH 8.0; 100 mM EDTA, pH 8.0; 0.5% SDS; 100 $\mu\text{g}/\text{ml}$ proteinase K) and incubated at 55°C for 2 days. Then, for each sample, DNAs with two biological replicates were extracted following a previous protocol (92). The V4 to V5 hypervariable region of eukaryotic 18S rDNA was amplified with the 528F and 706R primers (93). PCR amplification was carried out in 30- μl reactions with 15 μl of Phusion high-fidelity PCR master mix (New England Biolabs), 0.2 μM forward and reverse primers, and about 10 ng of template DNA. Thermal cycling consisted of initial denaturation at 98°C for 1 min, followed by 30 cycles of denaturation at 98°C for 10 s, annealing at 50°C for 30 s, and elongation at 72°C for 60 s. The final elongation was allowed to proceed at 72°C for 5 min. A negative PCR control without template DNA was included. All amplicons were then sequenced on a single run using the Illumina MiSeq platform (2×250 bp). Paired-end reads from the original DNA fragments were merged using FLASH (94) and then assigned to each sample according to the unique barcodes. Sequences analysis was performed with the UPARSE software package (95) using the UPARSE-OTU and UPARSE-OTUref algorithms. Sequences with $\geq 97\%$ similarity were assigned to the same operational taxonomic

units (OTUs). Representative sequences were picked for each OTU using the RDP classifier (96) to annotate taxonomic information for each representative sequence against Silva release 119 (97).

Protein extraction, separation, and liquid chromatography-electrospray ionization-mass spectrometry analysis. Proteins from two biological replicates of each sampling site were extracted following a modified protocol (33). Briefly, the filtration membrane with 10 ml TRIzol reagent was sonicated in ice for 5 min. Subsequently, we added 2 ml chloroform to the cell lysate and held at room temperature for 5 min after being vortexed for 15 s, centrifuged at $12,000 \times g$ for 15 min at 4°C, removed the top pale-yellow or colorless layer, and added 3 ml ethanol to resuspend the reddish bottom layer. The mixture was vortexed and centrifuged at $2,000 \times g$ for 5 min at 4°C, and then the supernatant was transferred to a new tube and 10 ml isopropanol was added. The mixture was stored at -20°C for at least 2 h for protein precipitation, and then centrifuged at $14,000 \times g$ for 30 min at 4°C. After washing with 5 ml of 95% ethanol, the pellet obtained was dissolved in 0.5 M tetraethylammonium tetrahydroborate (TEAB) (Applied Biosystems, Milan, Italy). After centrifuging at $30,000 \times g$ at 4°C, an aliquot of the supernatant was taken for further analysis. Protein quantification was performed using a two-dimensional (2D) Quant kit (GE Healthcare, San Francisco, CA).

After adjusting the pH to 8.5 with 1 M ammonium bicarbonate, total protein (100 µg) from each sample was reduced with dithiothreitol (DTT) for 1 h at 60°C and carboxyamidomethylated with iodoacetamide for 45 min at room temperature in the dark. Each sample was digested twice using Trypsin Gold (Promega, Madison, WI, USA) with a protein/trypsin ratio of 30:1 at 37°C for 16 h. After desalting on a Strata-X C₁₈ solid-phase extraction column (Phenomenex), the trypsin-digested samples were evaporated and reconstituted in 0.5 M TEAB. Fractionation of peptide samples was performed by strong cation exchange (SCX) chromatography using a LC-20AB HPLC pump system (Shimadzu, Kyoto, Japan). The predried peptide samples were redissolved in 4 ml buffer (25 mM NaH₂PO₄ in 25% acetonitrile, pH 2.7) and loaded onto a 4.6 by 250 mm Ultremex SCX column containing 5-µg particles (Phenomenex). The eluted peptides were separated into 20 fractions, desalted with a Strata-X C₁₈ column (Phenomenex) and vacuum dried. Each fraction was redissolved in buffer A (5% acetonitrile, 0.1% formic acid) and injected into a 2-cm C₁₈ trap column of LC-20AD nanoHPLC (Shimadzu, Kyoto, Japan). Peptides were eluted from the trap column and separated on an analytical C₁₈ column (75 µm by 100 mm) with a 35-min linear gradient at $300 \mu\text{l min}^{-1}$ from 2% to 35% buffer B (95% acetonitrile, 0.1% formic acid), followed by a 5-min linear gradient to 60%, a 2-min linear gradient to 80%, and maintenance at 80% for 4 min. Upon completion of the gradients, the column was washed with 90% buffer B and reequilibrated with buffer A. Mass spectra acquisition was performed with a TripleTOF 5600 system (AB SCIEX, Concord, ON) fitted with a NanoSpray III source (AB SCIEX, Concord, ON) and a pulled quartz tip as the emitter (New Objectives, Woburn, MA). Data were acquired using ion spray voltage of 2.5 kV, curtain gas of 30 lb/in², nebulizer gas of 15 lb/in², and a temperature of 150°C. For information-dependent acquisition (IDA) scanning, the mass range was from 350 to 1,500 *m/z*, survey scans were acquired of 100 ms, and the top 40 product ion scans were collected with a threshold of 150 counts per s (cps) and with a 2+ to 5+ charge state of a total cycle time of 2.8 s. Four time bins were summed for each four-anode channel scan at a pulse frequency value of 11 kHz and a monitor frequency value of 40 GHz. Dynamic exclusion was set for 1/2 of peak width (15 s), and then the precursor was refreshed off the exclusion list.

Protein identification and bioinformatics analysis. Raw peptide data (.wiff) were converted to the Mascot generic file format (.mgf) using the SCIEX MS data converter (version 1.3 beta). The MS/MS peak lists were searched against the phytoplankton database, which was combined from four sources (see Table S4 in the supplemental material). Two of them were downloaded from the websites of the National Center for Biotechnology Information (NCBI) and Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP). The other two sequence databases included the transcriptomes of *P. donghaiense* grown under the phosphorus-replete and -starved conditions and the metatranscriptome of nonbloom samples collected in the investigation area on 5 November 2013 (4,665,287 + 245 (contaminant) sequences, 2.2 GB). Protein identification and quantification were performed using the MetaPro-IQ approach (98). Briefly, a database search against the combined phytoplankton database was firstly performed to generate a reduced database that contains all possible proteins derived from peptide-spectrum matches (PSMs) for all samples using X! Tandem software (version 2017.2.1) without any criterions. The reduced database containing the resulting protein lists was then imported into MaxQuant software (version 1.6.1.0) for protein quantification (99). The following parameters were selected: trypsin specificity with allowance for one missed cleavage; fixed modifications of carbamidomethyl (C); variable modifications consisting of Gln->pyro-Glu (N-term Q), deamidation (N, Q), and oxidation (M); peptide charge of 2+, 3+, and 4+; 20 ppm of peptide mass tolerance; and 0.05 Da of fragment mass tolerance. To reduce the probability of false peptide identification, the false discovery rate (FDR) was set to less than 1% at both PSM and protein levels. High-confidence proteins matching at least two peptides and one unique peptide were selected for further analysis.

Proteins identified by the same set or a subset of peptides were grouped together as one protein group. Leading proteins (defined as the top rank protein in a group; ranking is based on the number of peptide sequences, the number of PSMs, and the sequence coverage) of the protein group were chosen for further taxonomic and functional analyses (98). For taxonomic annotation, the matched sequences from NCBI, MMETSP, and *P. donghaiense* transcriptome were species-specific, and sequences from metatranscriptome were annotated against the NCBI nonredundant (NCBInr) protein database for species assignment. For functional analysis, the matched protein sequences were annotated against the NCBInr and Kyoto Encyclopedia of Genes and Genomes (KEGG) database. For proteome-based community structure analysis, relative abundances of each organismal group were calculated by summing their total protein abundances and then divided by the sum of all protein abundances in a sample

(community-level analysis). For function comparisons, the relative abundance of each protein and metabolic KEGG pathway was calculated by summing the related protein abundances and then dividing by the sum of all protein abundances in a species (species-level analysis).

Data availability. The mass spectrometry proteomic data has been submitted to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD014327.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/AEM.01425-19>.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

SUPPLEMENTAL FILE 2, XLSX file, 4.9 MB.

SUPPLEMENTAL FILE 3, XLSX file, 4.2 MB.

SUPPLEMENTAL FILE 4, XLSX file, 0.03 MB.

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We declare no competing interests.

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