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Transcriptomic and physiological analyses of the dinoflagellate *Karenia mikimotoi* reveal non-alkaline phosphatase-based molecular machinery of ATP utilisation

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Summary

The ability to utilize dissolved organic phosphorus (DOP) is important for phytoplankton to survive the scarcity of dissolved inorganic phosphorus (DIP), and alkaline phosphatase (AP) has been the major research focus as a facilitating mechanism. Here, we employed a unique molecular ecological approach and conducted a broader search for underpinning molecular mechanisms of adenosine triphosphate (ATP) utilisation. Cultures of the dinoflagellate *Karenia mikimotoi* were set up in L1 medium (+P), DIP-depleted L1 medium (–P) and ATP-replacing-DIP medium (ATP). Differential gene expression was profiled for ATP and +P cultures using suppression subtractive hybridisation (SSH) followed by 454 pyrosequencing, and RT-qPCR methods. We found that ATP supported a similar growth rate and cell yield as L1 medium and observed DIP release from ATP into the medium, suggesting that *K. mikimotoi* cells were expressing extracellular hydrolases to hydrolyse ATP. However, our SSH, qPCR and enzymatic activity assays indicated that 5′-nucleotidase (5NT), rather than AP, was responsible for ATP hydrolysis. Further gene expression analyses uncovered that intercellular purine metabolism was significantly changed following the utilisation of ATP. Our findings reveal a multi-faceted machinery regulating ATP utilisation and P metabolism in *K. mikimotoi*, and underscore AP activity is not the exclusive indicator of DOP utilisation.

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Introduction

Phosphorus (P) is a vital element required by all forms of life and its availability constrains the phytoplankton productivity in many marine ecosystems (Karl, 2014). The major dissolved P pools in aquatic environments consist of dissolved inorganic phosphorus (DIP) and dissolved organic phosphorus (DOP). DIP is the preferable form of P because it can be directly utilized by phytoplankton cells (Currie and Kalff, 1984), while the assimilation of DOP is completed with the assistance of different types of hydrolases produced by cells (Azam *et al.*, 1983; Cotner and Biddanda, 2002). Because DIP is rapidly consumed in the euphotic zone and replenishment from external sources is slow, it is often at growth-limiting concentrations in the ocean where DOP is relatively abundant (Paytan and McLaughlin, 2007; Lin *et al.*, 2016). For instance, the measured DIP concentration was 0.2–4 nM in surface waters of North Atlantic where DOP concentrations range from 40 to 300 nM (Wu *et al.*, 2000; Mather *et al.*, 2008). This gives a selection pressure to phytoplankton for the ability to utilize DOP. Therefore, DOP availability is a potential driver of phytoplankton species composition and may be of major importance to bloom initiation and maintenance of harmful algae (Dyhrman and Ruttenberg, 2006). Numerous physiological studies have shown that most dinoflagellates and other phytoplankters are able to assimilate various types of DOP when DIP is depleted (Oh *et al.*, 2002; Huang *et al.*, 2005; Richardson and Corcoran, 2015; Lin *et al.*, 2016). Alkaline phosphatase (AP), 5′-nucleotidase (5NT) and other enzymes can facilitate utilisation of DOP in eukaryotic phytoplankton and bacteria. In dinoflagellates, AP has

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been found to exist and respond to P stress in several species at both transcriptional and translational levels (Oh *et al.*, 2010; Lin *et al.*, 2011; 2012a,b), but 5NT activities seemed to mainly associate with the bacteria (Ammerman and Azam, 1985; Cotner and Wetzel, 1992). In general, AP has been the major focus of research and commonly used as an indicator of DIP depletion and DOP utilisation in phytoplankton in the past decades (Dyhrman and Palenik, 1999; Dignum *et al.*, 2004; Lomas *et al.*, 2004; Sylvan *et al.*, 2006; Rees *et al.*, 2009; Lin *et al.*, 2015a,b).

Nucleotides are an important source of DOP for microbes in oligotrophic oceans (Björkman and Karl, 2005). Adenosine-5′-triphosphate (ATP), an important form of DOP, occurs stably in seawater in significant concentration (Azam and Hodson, 1977). ATP utilisation as the P source had been documented in eukaryotic phytoplankton as well as bacteria (Oh *et al.*, 2002; Huang *et al.*, 2005; Krumhardt *et al.*, 2013). However, the efficiency of utilisation has rarely been investigated (Li *et al.*, 2015) and the regulating molecular mechanism remains unclear.

To address the gap of research, we chose to study ATP utilisation in the dinoflagellate *Karenia mikimotoi*, a cosmopolitan harmful algal bloom (HAB) forming species, which is able to use various organic phosphorus resources such as ATP and glycerophosphate when DIP is exhausted (Lei and Lu, 2011). In this study, differentially expressed genes were profiled for cultures grown under ATP and DIP conditions using the SSH (suppression subtractive hybridisation) combined with 454 pyrosequencing. For dinoflagellates, which have immense genomes and generally have limited transcriptional regulation, SSH has been proven to be more effective than whole transcriptome sequencing to identify targeted genes that are differentially expressed between different growth conditions (Diatchenko *et al.*, 1999, Zhang *et al.*, 2014). Combination with high throughput sequencing provides more comprehensive gene profiles compared to clone sequencing in the traditional SSH technique. Consequently, we identified a score of genes related to P utilisation, which provide insights into the metabolic shift between DIP and ATP utilisation in *K. mikimotoi*.

Results

Growth rate, DIP concentration and alkaline phosphatase activity

Starting from the same cell density of 5×10^3 cells ml^{-1}, the growth curve of *K. mikimotoi* in the ATP group and +P group shared similarly increasing trends. Both maintained exponential growth from Day 1 to Day 6 with the same average growth rates of 0.32 day^{-1}. After 6-day cultivation, the cell concentration reached 2.8×10^4 cells ml^{-1} (+P group) and 2.7×10^4 cells ml^{-1} (ATP group), roughly 5.5-fold of the initial concentration in both cultures, which indicate that *K. mikimotoi* could utilize ATP as efficiently as DIP. In contrast, the growth of *K. mikimotoi* in –P group was inhibited throughout the experiment (Fig. 1A).

DIP in the +P group was consumed rapidly and depleted on the sixth day, and that in the –P group was consistently scarce during the experiments. In contrast, DIP concentration in the ATP culture medium was undetectable at the beginning, but increased sharply to 11.81 μM on Day 3 and 21.41 μM on Day 4, exceeding concentration in the +P group (Fig. 1B). On Day 6, there was about 25 μM residual DIP in the ATP group, despite consumption to support the observed growth, indicating a large amount of DIP released from ATP.

Alkaline phosphatase activity (APA) was barely detectable in the first two days in all three cultures (Fig. 1C). On Day 3 and thereafter, APA increased rapidly in the –P group but remained essentially undetectable in the other two groups. This indicated that AP was expressed and functioning in P-stressed cells (in the –P group), but not in ATP-utilising cells (in the ATP group) or DIP-grown cells (in the +P group).

Utilisation of ATP

The similar growth rates in the +P group and the ATP group and the markedly increased DIP concentration in the ATP group indicated that copious Pi (24 μM residual concentration) was released from ATP. Taking into account the amount of phosphorus needed to support the observed cell growth, we estimated the consumption of P in the ATP group (C_{ATP}) using the following equation:

$$C_{ATP} = C_{L1} / M_{L1} \times M_{ATP} \quad (1)$$

$$M = (N_n + N_{n+1})/2 \quad (2)$$

Where C_{L1} is the consumption of P in L1 cultures, M is the average of cell numbers (N) on day n and day $n+1$, in the L1 cultures (M_{L1}) and ATP cultures (M_{ATP}) respectively. Here, we assumed that the DIP consumption by a single cell in the L1 culture cells was the same as that in the ATP culture cells according to the similar physiological status between both cultures. Hence, the daily consumption of P in the ATP cultures could be calculated from the amount of P consumed per cell in the L1 culture multiplied by the cell number in the ATP culture. By adding the residual DIP released into medium in the ATP cultures, we found that more than $>$ 45 μM had been supplied from ATP (Table 1), indicating that more than one phosphorus group were accessed by the species from each ATP molecule.

To examine the uptake of ATP by cells *in vivo*, an ATP analog, 2-APTP was amended into the P-depleted culture as described above. At the same time, the normal L1 culture was set up as the blank control (no analog added). The blue

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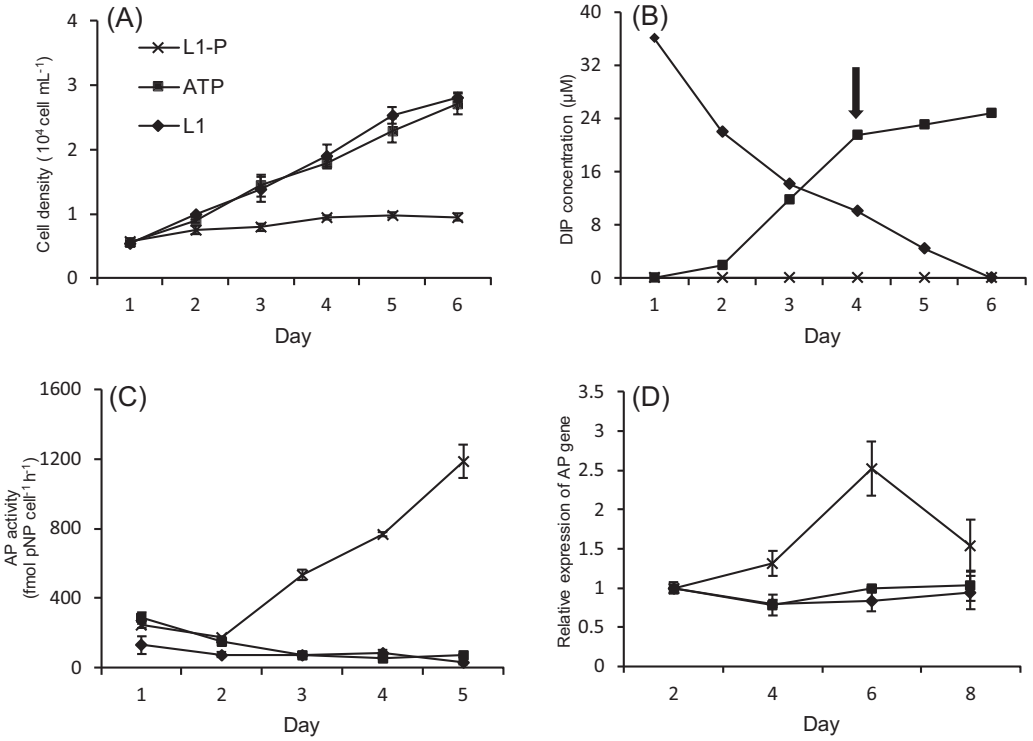

Fig. 1. Physiological characteristics of the P-depleted (–P), ATP and L1 (+P) cultures.
A. Growth curve.
B. Concentration of dissolved inorganic phosphate (DIP) in the culture medium. Arrow denotes the time when samples are collected for RNA extraction.
C. Activity of alkaline phosphatase (AP).
D. Relative expression of AP gene. Gene expression data were firstly normalized to GAPDH gene, then were secondarily normalized to Day 2 expression to allow direct comparison of fold change in different time point. Shown are means ± standard deviations (error bars) from the triplicate in each treatment group.

fluorescence was only observed in cells of the –P group added with 2-APTP (Fig. 2B), but absent in the +P group (Fig. 2A), indicating the nucleoside component (fluorescent group) of 2-APTP was imported into *K. mikimotoi* cells (not autofluorescence of the cells). Another experiment with this ATP analog added to L1 culture showed weaker fluorescence, indicating uptake of the ATP analog, albeit at a lower rate, under P-replete condition (Supporting Information Fig. S1). Evidently, *K. mikimotoi* cells were not only able to utilize the Pi released from ATP but also could take up its partial hydrolysis products (AMP or ADP).

Sequencing and assembly of SSH libraries

SSH forward and reverse libraries were constructed to contain those putatively upregulated genes in the ATP and

Table 1. The daily total release of DIP in the ATP cultures estimated from changes of DIP in the L1 (+P) cultures and in the ATP cultures.

Time	Consumption of DIP in L1 culture (μM)	Consumption of DIP in ATP culture (μM)[a]	Increment of DIP in ATP culture (μM)[b]	Total released DIP in ATP culture (μM)[c]
D1–D2	14.05	?	1.96	> 1.96
D2–D3	7.76	7.65	9.81	17.46
D3–D4	4.06	4.01	9.60	13.61
D4–D5	5.70	5.26	1.67	6.93
D5–D6	4.31	4.04	1.76	5.80
Total				> 45.76

a. Daily consumption of DIP from ATP in the ATP cultures calculated from consumption in the L1 cultures following formula 1 shown in the text.
b. Daily increment (i.e., release) of DIP in ATP culture.
c. Calculated as $a + b$.

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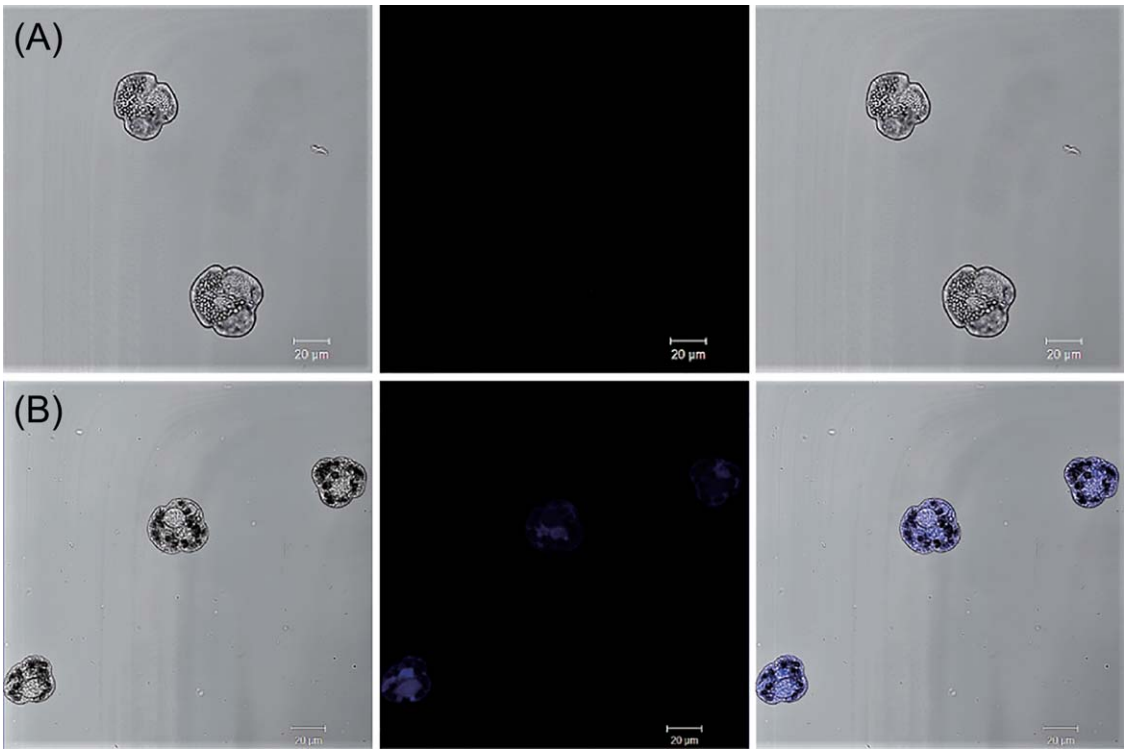

Fig. 2. Microscopic observation of 2-Aminopurine-riboside-5′-triphosphate (2-APTP) uptake. From left to right are bright-field, fluorescence and superposition of bright-field with fluorescence images respectively.
A. L1 culture as a blank control.
B. P-depleted culture amended with 2-APTP, a fluorescent analog of ATP.

+P groups respectively. 454-pyrosequencing generated 58 242 raw reads (average length 480 bp, 27.96 Mbp in total) from the forward library and 46 301 raw reads (average length 470 bp, 21.76 Mbp in total) from the reverse library (Supporting Information Table S1). After the removal of poor-quality sequences, assembly of high-quality reads yielded 4842 unigenes in the forward library and 4394 unigenes in the reverse library respectively. Rarefaction curves plotted by number of unigenes vs. the number of reads resulted in steady increase instead of a plateau indicating there were more unigenes remaining to be recovered in both libraries despite the large number of reads obtained (Supporting Information Fig. S2). In some previous transcriptomic studies of dinoflagellates, similar sequencing depths were found to be well below saturation threshold (Yang *et al.*, 2010; Zhuang *et al.*, 2015).

Gene annotation and functional categorisation

BLASTX against NCBI nr database and Swiss-port protein database resulted in the annotation of 3320 (66.5%) and 2917 (66.4%) unigenes from the forward and reverse libraries respectively. The remaining unigenes of forward library (33.5%) and reverse library (33.6%) had no homologs in the databases, indicating these unigenes encoded proteins with novel functions or with sequences highly diverging from homologs of other organisms.

Of the annotated unigenes 143 from the forward library were either found exclusively or in markedly higher abundance (difference $>$ 1.5-fold after calibrating for sequencing scale) in the forward library than in the reverse library, representing upregulated genes in the ATP group. Meanwhile, 81 unigenes were identified in the reverse library exclusively or in markedly higher abundances (difference $>$ 1.5-fold after calibrating for sequencing scale) in the reverse library, representing upregulated gene profiles in the +P group. These strongly differentially expressed genes were functionally categorized by mapping to KEGG orthology and pathways. Genes with the predicted function of 'Metabolism' (39.2%), 'Genetic information processing' (20.3%) and 'Environmental information processing' (18.2%) accounted for the majority of the forward library (Fig. 3A), and the rest was categorized as 'Cellular processes' (6.3%) and 'Organismal systems' (0.7%). A similar pattern of function categorisation was observed in the reverse library, about 39.5%, 18.5% and 13.6% of unigenes with the corresponding functions in 'Metabolism', 'Genetic information processing' and 'Environmental information processing', respectively, while 'Cellular processes' and 'Organismal systems' contributed less than 5% (Fig.

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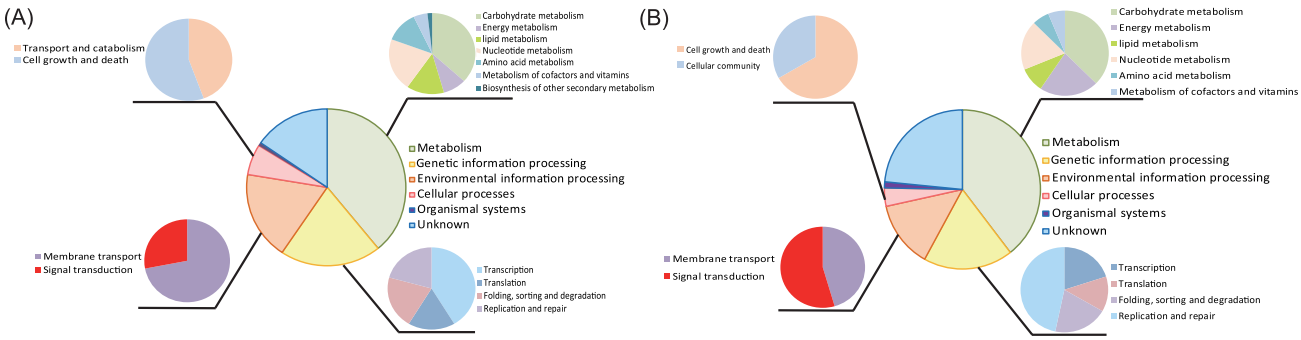

Fig. 3. Functional distributions of genes identified from the SSH forward library (A) and reverse library (B).

3B). In both libraries, unigenes involved with metabolism of carbohydrates was most enriched. In addition, categories of nucleotide metabolism, membrane transport and transcription were also the dominant processes in the forward library.

Among the genes identified in the SSH forward library, 5′-nucleotidase (NT5) was most likely related to ATP hydrolysis based on the functional annotation of all the genes. Surprisingly, AP, the most expected gene in the ATP culture-derived transcriptome, was not detected in the SSH libraries. This was consistent with the very limited AP activity detected in both the +P and ATP groups (Fig. 1C).

Gene expression quantified by RT-qPCR

Based on the SSH screening and the BLASTX result of the sequences obtained, a total of 13 genes related to P utilisation, N transport as well as nucleotide metabolism were selected for verifying expression patterns using RT-qPCR. Seven genes (87.5%) from the SSH forward and four genes (80.0%) from reverse libraries showed differential expression patterns between ATP and +P cultures consistent with corresponding SSH results. Among genes identified from the forward library, two kinds of nitrogen transporter genes, nitrate transporter (NRT) and high affinity nitrate transporter (HANRT), were upregulated by 1.5-fold and 1.3-fold in the ATP group, respectively, based on their RT-qPCR results. However, nitrite transporter (NirC) hardly showed difference in expression between the ATP and the +P groups (Fig. 4). Phosphonate ABC transporter phosphate-binding periplasmic component (PhnD), NT5 and phosphate transporter family protein (Pit) are related to phosphorus transport and assimilation. RT-qPCR showed that expression of PhnD in the ATP group was 2.3-fold higher than that in the +P group. RT-qPCR of NT5 did not display significant difference between ATP and +P group. Among the few cases in which RT-qPCR results were not consistent with SSH results, Pit selected from SSH-reverse library showed upregulation in RT-qPCR by more than 1.5-fold in the ATP group. Three of the genes obtained from the forward library, including Canalicular multispecific organic anion transporter (cMOAT), Nucleoside triphosphatase (NTPCR) and AMP deaminase (AMPD), and four obtained from the reverse library, including cAMP-specific 3′5′-cyclic phosphodiesterase (PDE4), 3′5′-cyclic nucleotide phosphodiesterase (PDE1), 2′3′-cyclic-nucleotide 2′-phosphodiesterase (CpdB) and nucleoside diphosphate kinase (NME), are mainly involved

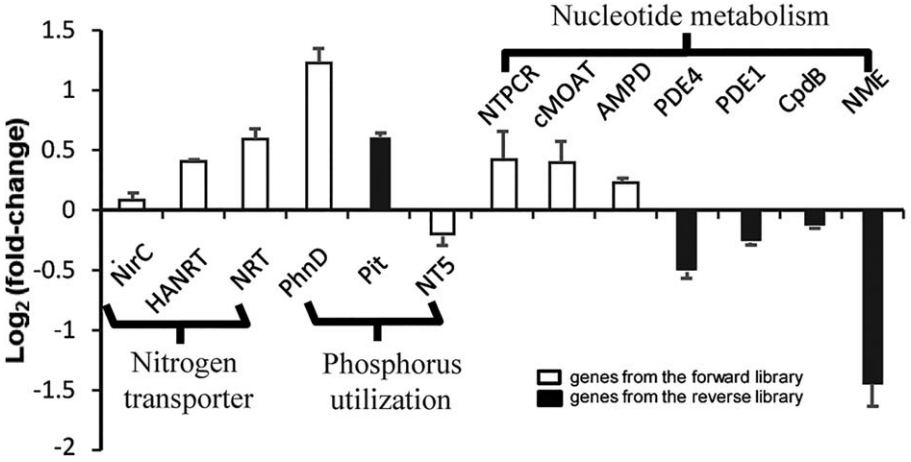

Fig. 4. Relative expression of 13 selected genes analysed using RT-qPCR. Relative gene expression was calculated as $\log_2$ (the expression level of gene in the ATP cultures divided by the expression level of gene in the +P cultures). The positive (negative) value indicates genes upregulated (downregulated) in the ATP cultures. Error bars indicate standard error.

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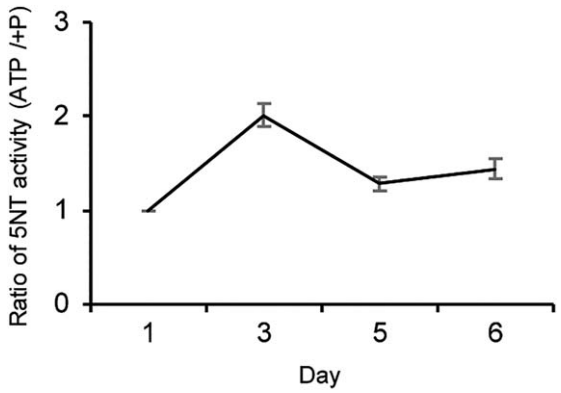

Fig. 5. 5′-nucleotidase (NT5) enzymatic activity in the ATP group relative to that in the +P group measured over several days. Error bars indicate standard error.

in nucleotide transport and metabolism. Among them, cMOAT and NTPCR were consistently upregulated by 1.3-fold in the ATP group. Expression of PDE4 and NME in the +P group was about 1.4-fold and 2.8-fold higher than that in the ATP group respectively. AMPD, PDE1 and CpdB did not exhibit significant differential expression between the ATP and the +P groups.

In addition, albeit not detected in the SSH libraries, AP gene was included for RT-qPCR analysis because it has been widely used as an indicator of DIP-limitation and DOP utilisation. RT-qPCR results showed that relative expression of AP gene increased gradually in the –P group over time reaching a 2.5-fold higher level on Day 6 than that in the +P group. In contrast, expression of AP gene in the ATP group was at baseline, the same as that in the +P group (Fig. 1D).

5NT enzyme activity

5NT enzyme activity was undetectable on Day 1 in either the +P group or the ATP group, probably because of short treatment time (1 h) and low cell density. On Day 3, 5NT enzyme activity was nearly 2-fold higher in the ATP group than that in the +P group, then decreased to 1.3-fold higher on Day 5 and remained at a relatively stable level (1.4-fold) on Day 6 (Fig. 5).

Effects of ATP utilisation on cellular metabolic network

Our physiological experiments indicated that not only some of the phosphorus atoms in ATP were hydrolytically released to the culture to support algal growth, but the remaining hydrolysis nucleotide products (ADP/AMP) can also be transported into *K. mikimotoi* cells. Accordingly, we noticed significant shifts in the purine metabolism in the ATP culture, as expression of series of genes associated with ADP/AMP synthesis and decomposition were downregulated and upregulated respectively (Fig. 6). Additionally, we also observed changes in TCA cycle and glycolysis (Fig. 7), likely a result of the change of purine metabolism during ATP utilisation.

Discussion

Molecular mechanism of ATP uptake and utilisation

ATP functions in the energy transfer for anabolic and catabolic cellular processes and, together with ADP and AMP, controls the energy status of the cell (Karl and Craven, 1980). Besides, ATP is also an organic phosphorus source and has been reported to occur in a wide variety of aquatic habitats ranging from eutrophic freshwater lakes to temperate marine coastal regions and Antarctic seawaters (Azam and Hodson, 1977; Riemann, 1979; Hodson *et al*., 1981; McGrath and Sullivan, 1981; Maki *et al*., 1983). In general, utilisation of phosphorus esters facilitated by AP is believed to be the most common way of DOP utilisation and has been the major focus of research pertaining to DOP utilisation (Hoppe, 2003; Huang *et al*., 2005; Labry *et al*., 2005; Nicholson *et al*., 2006). Previous studies have also attributed the hydrolysis of ATP in aquatic ecosystems mainly to AP (Dyhrman and Ruttenberg, 2006; Van Wambeke *et al*., 2008; Duhamel *et al*., 2010), although the role of ectoenzyme 5NT has also been reported (Ammerman and Azam, 1985; Krumhardt *et al*., 2013).

Our results showed that ATP was utilized to support the growth of *K. mikimotoi* (bacteria excluded by the antibiotic treatment) as efficiently as DIP. It is clear that ATP was hydrolysed because an increasing concentration of DIP was observed in the medium. The release of DIP in the culture medium is consistent with the notion of ectoenzymatic hydrolysis of ATP. However, in the present study, we found that *K. mikimotoi* did not express and employ AP for ATP hydrolysis. Furthermore, our detection of increased DIP concentration in the medium simultaneous with *K. mikimotoi* growing at a comparable rate as in L1 medium suggested that more than one P molecule of ATP were utilized by *K. mikimotoi*. This is because ATP was provided to the ATP cultures at the same molar concentration as DIP to the L1 cultures (36 μM). Interestingly, a similar mode of ATP utilisation has been reported in *Prochlorococcus* MED4 where this was attributed to a 5NT-like enzyme (Krumhardt *et al*., 2013). Although the genes encoding this enzyme are not well characterized in phytoplankton, putative 5NT has been detected in the genomes of the cyanobacteria and some of the eukaryotic algae examined to date such as the diatom *Thalassiosira pseudonana* (Armbrust *et al*., 2004) and the dinoflagellate *Symbiodinium kawagutii* (Lin *et al*., 2015a,b). 5NT activity to hydrolyse nucleotides has been shown to be induced in *T. pseudonana* (Dyhrman *et al*., 2012) and the coccolithophorid *Emiliania huxleyi* (Dyhrman and Palenik, 2003).

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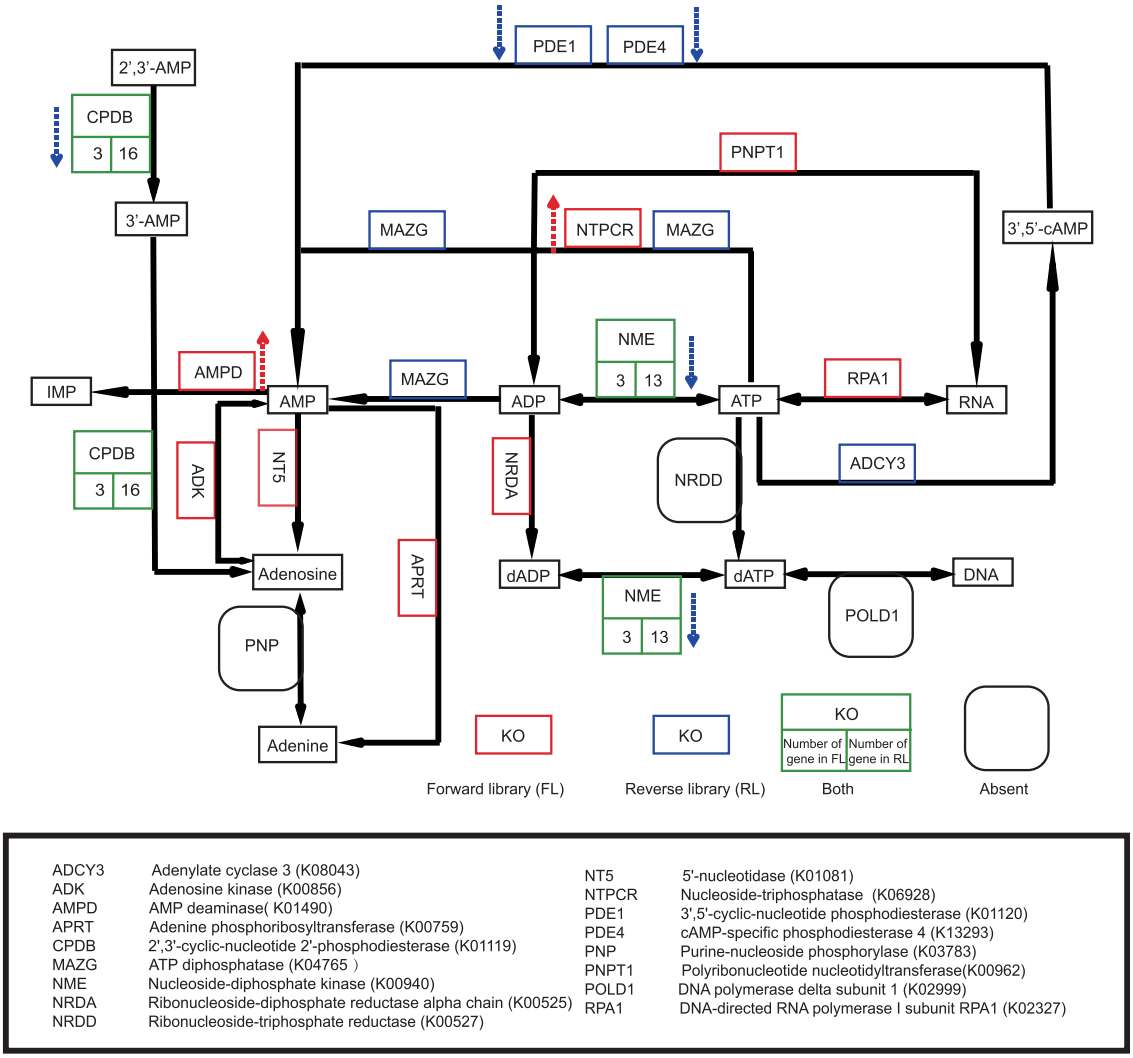

ADCY3	Adenylate cyclase 3 (K08043)	NT5	5'-nucleotidase (K01081)
ADK	Adenosine kinase (K00856)	NTPCR	Nucleoside-triphosphatase (K06928)
AMPD	AMP deaminase(K01490)	PDE1	3',5'-cyclic-nucleotide phosphodiesterase (K01120)
APRT	Adenine phosphoribosyltransferase (K00759)	PDE4	cAMP-specific phosphodiesterase 4 (K13293)
CPDB	2',3'-cyclic-nucleotide 2'-phosphodiesterase (K01119)	PNP	Purine-nucleoside phosphorylase (K03783)
MAZG	ATP diphosphatase (K04765)	PNPT1	Polyribonucleotide nucleotidyltransferase(K00962)
NME	Nucleoside-diphosphate kinase (K00940)	POLD1	DNA polymerase delta subunit 1 (K02999)
NRDA	Ribonucleoside-diphosphate reductase alpha chain (K00525)	RPA1	DNA-directed RNA polymerase I subunit RPA1 (K02327)
NRDD	Ribonucleoside-triphosphate reductase (K00527)		

Fig. 6. Genes from SSH libraries associated with purine metabolism pathway. The red and blue dashed arrows indicate upregulation (present in forward library) and downregulation (present in the reverse library).

5NT has been shown to be able to sequentially hydrolyse all the three phosphate groups of ATP (Bengis-Garber and Kushner, 1982). Our detection of 5NT in the SSH forward library is consistent with those reports. In accordance, we measured a higher 5NT enzyme activity in the ATP group than that in the +P group. The failure of our RT-qPCR to show significant differential expression of 5NT between the ATP and the +P groups was likely because this gene is mainly regulated at the posttranscriptional level. Taken together, our results clearly indicate that *K. mikimotoi* cleaves PO_4^{3-} from ATP using a different enzyme than AP, most likely 5NT.

The utilisation of ATP does not seem to stop at the putative NT5-based hydrolysis and phosphate transport. Using the fluorescent ATP analog 2-APTP, we showed that *K. mikimotoi* cells absorbed the fluorescent compound, indicating that this species can also take up hydrolysis products of ATP (ADP, AMP, or adenosine). In further support of this possibility, we identified an ATP-binding cassette transporter, cMOAT. Previous studies have shown that cMOAT can transport nucleotide analogs (Miller, 2001). In accordance, our RT-qPCR result showed that cMOAT was upregulated (by ~1.5-fold) in the ATP group relative to the +P group. So far, this protein has been mainly reported in mammals for its role in drug resistance in cancer cells, and our finding is the first documentation of this protein in dinoflagellates. By BLASTN search against NCBI Sequence Read Archive (SRA) databases, we also detected the presence of this gene in *Symbiodinium* sp. (SRX076709), with nearly 70% identities to that in *K. mikimotoi*. Furthermore, we performed the BLASTX searches against the database produced in the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) and found cMOAT gene in 19 dinoflagellate

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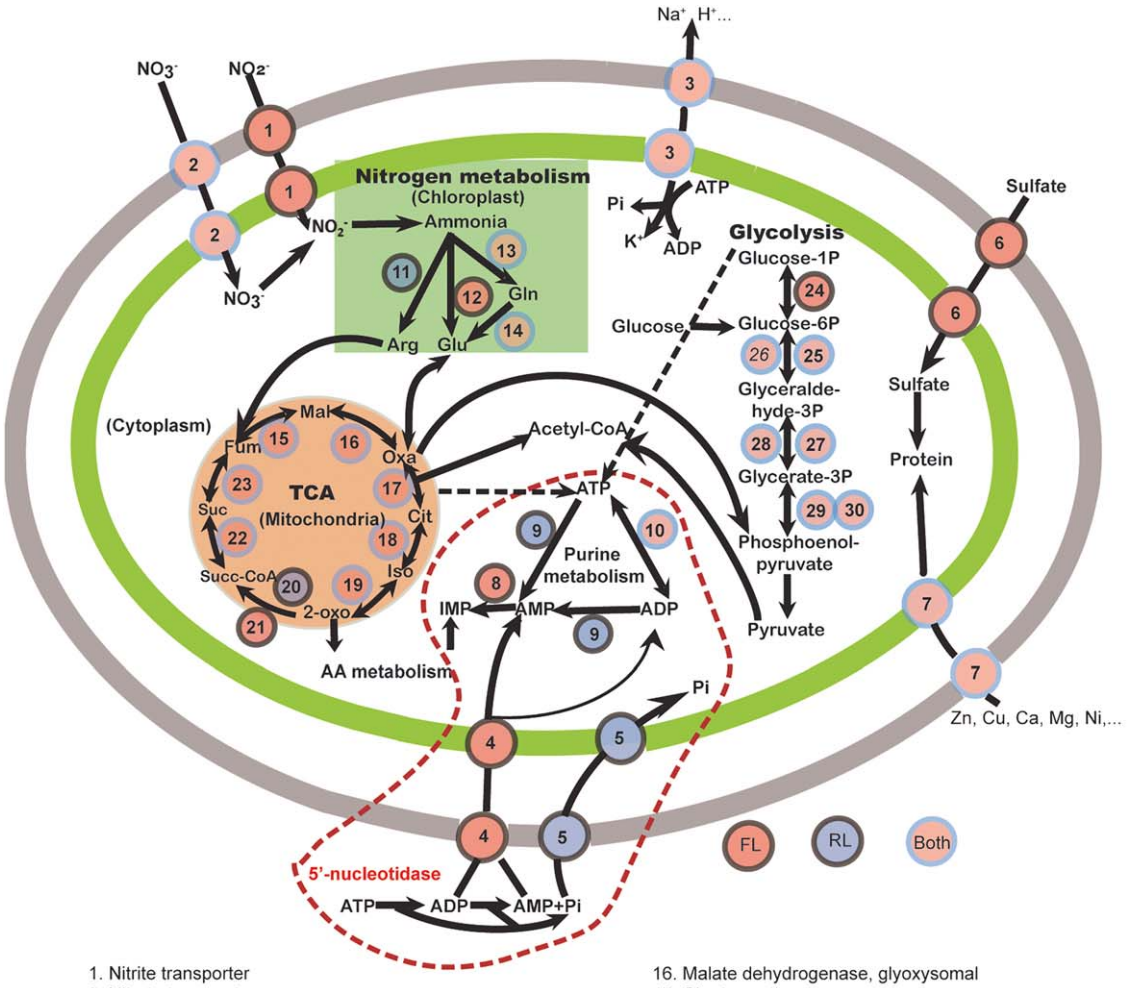

1. Nitrite transporter
2. Nitrate transporter
3. ATPase
4. Canalicular multispecific organic anion transporter
5. Phosphate transporter
6. Sulfate transporter
7. Metal ion transporter
8. AMP deaminase
9. ATP diphosphatase
10. Nucleoside-diphosphate kinaseCarbamoyl-phosphate synthase
11. Carbamoyl-phosphate synthase
12. Glutamate synthase 1 [NADH]
13. Glutamine synthetase
14. Ferredoxin-dependent glutamate synthase 2
15. Fumarate hydratase
16. Malate dehydrogenase, glyoxysomal
17. Citrate synthase
18. Aconitate hydratase
19. Isocitrate dehydrogenase [NADP]
20. 2-oxoglutarate dehydrogenase E1/E2 component
21. 2-oxoglutarate ferredoxin oxidoreductase subunit alpha
22. Succinyl-CoA ligase
23. Fumarate reductase 2
24. Phosphoglucomutase, cytoplasmic 1
25. Fructose-1,6-bisphosphatase
26. Fructose-bisphosphate aldolase
27. Glyceraldehyde-3-phosphate dehydrogenase
28. Phosphoglycerate kinase
29. Phosphoglycerate mutase
30. Enolase

Fig. 7. Schematic cell model depicting genes whose expression seems to be modulated by ATP utilisation, including those involved in nutrient transport and primary metabolism in *K. mikimotoi*.

species (Supporting Information Table S2) such as *Karenia brevis* (CAMPEP_0114848910) and *Alexandrium tamarense* (CAMPEP_0116318926), suggesting that cMOAT may be a common mechanism in dinoflagellates to transport nucleotides.

It is worth noting that another HAB dinoflagellate, *Prorocentrum donghaiense*, can also efficiently utilize ATP, but has shown no evidence of DIP release into the medium (Li *et al.*, 2015). One possibility is that extracellular ATP hydrolysis took place slowly and was coupled tightly by uptake, leaving no residual DIP. The second possibility is that ATP was absorbed directly without extracellular hydrolysis. Yet another possibility is that both extracellular ATP hydrolysis and uptake of ATP or its hydrolysis products occurred, as evident in the present study on *K. mikimotoi*. These contrasting results suggest that the mechanism underlying the

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utilisation of ATP can be different in different species of dinoflagellates, and likely other groups of phytoplankton as well.

Modulation of metabolic pathways by ATP

The more active expression of phosphate transporter genes observed in the ATP group than in the +P group was indicative of more active DIP uptake in the ATP cultures. It is not surprising given that the release of DIP from ATP hydrolysis caused DIP concentration to go higher in the ATP group than in the +P group (especially when samples were collected for SSH analysis; Fig. 1). Meanwhile, nitrate transporter was upregulated more than 1.5-fold in the ATP group (Fig. 4), which likely has resulted from the decline of the N: P ratio due to higher P concentration in the ATP group. Similar results have been observed in other dinoflagellates, such as *K. brevis* (Morey *et al.*, 2011) and *Alexandrium fundyense* (Zhuang *et al.*, 2015). Compared to the cells in the +P group, the greater uptake of N, P and nucleotide such as ADP and AMP in the ATP group might affect the assimilation of other nutrients, even several primary metabolisms in *K. mikimotoi* (Fig. 7).

As ADP and AMP are transported into cells, as discussed earlier, their concentrations in cytoplasm will rapidly increase, which would have profound impacts on cellular metabolisms, especially purine metabolism. Indeed, we found that most of the genes upregulated in the ATP group (recovered from the SSH forward library) were related to the catabolism of AMP and ADP. For example, AMPD and Adenine phosphoribosyltransferase (APRT) can react with AMP to produce inosine monophosphate (IMP), Adenosine and Adenine respectively. IMP is not only the first nucleotide formed during the synthesis of purine, but is also related to amino acid metabolism. Ribonucleoside-diphosphate reductase alpha chain (NRDA) catalyses the deoxygenation of ADP to form dADP, one of the substrates for synthesis of DNA. Conversely, most enzymes of purine metabolism identified from the reverse library catalyse the anabolism of AMP. These enzymes, including 3′5′-cyclic nucleotide phosphodiesterase (PDE1) and cAMP-specific phosphodiesterase (PDE4), are critical enzymes that catalyse the reaction from cAMP to AMP. Although nucleoside-diphosphate kinase (NME) and 2′3′-cyclic-nucleotide 2′-phosphodiesterase (CpdB) found in both SSH libraries (which was likely due to high expression levels), RT-qPCR results showed that they were upregulated in the +P group, especially in the case of NME (> 2.8-fold). The expression of purine metabolism genes in *K. mikimotoi* showed the most consistent pattern with SSH sequencing and the fluorescent ATP analog experiment results. These indicated accelerated nucleotide metabolism in the ATP group.

ADP and AMP uptake by the cells can also have impacts on glycolysis, the metabolic pathway that converts glucose into pyruvate, generating relatively less ATP. For instance, ADP and AMP can allosterically activate the conversion of fructose-6-phosphate and ATP to fructose-1,6-bisphosphate and ADP (Martínez-Costa *et al.*, 2004), a key step in glycolysis. We detected a higher transcript abundance of fructose-1,6-bisphosphatase (FBP) in the reverse library. Because FBP catalyses the reverse of the above-mentioned reaction (Marcus and Harrsch, 1990), its upregulation is expected to promote glycolysis in the ATP group, hence fuelling the TCA cycle.

Conclusions

This study is the first attempt to combine the SSH technology and 454 transcriptome sequencing for the analysis of differential expression gene in dinoflagellates under different P conditions. Results allow us to interrogate the process and molecular mechanism regulating ATP uptake by *K. mikimotoi*, and shed light on the effects of ATP utilisation on nutrients transport and cellular metabolism. Importantly, our results indicate that AP activity was an indicator of P stress, but not exclusive indicator of DOP utilisation, and 5′-nucleotidase (NT5) is most likely responsible for ATP hydrolysis. In addition, cMOAT, a previously undocumented transporter in dinoflagellate species, was identified and its upregulation in the ATP cultures suggests its involvement in the transport of ATP or its hydrolysis products. The estimated amount of consumed Pi in the ATP cultures also suggests that more than one Pi was utilized from each ATP molecule. Our results underscore that NT5 should be studied further, and a broad-based approach like the one used here is needed in future effort, to pinpoint the mechanism of ATP (and other DOP) utilisation by dinoflagellates and other phytoplankters in the ocean.

Experimental procedures

Cultures and sample collection under contrasting P-nutrient conditions

K. mikimotoi (strain C32-HK, kindly provided by Dr. Songhui Lu, Jinan University, Guangzhou, China) was maintained in L1 medium (Guillard and Hargraves 1993) prepared with sterilized seawater (30 PSU). An antibiotic cocktail containing Ampicillin (final concentration at 200 μg/ml), Kanamycin (final concentration at 100 μg/ml) and Streptomycin (final concentration at 100 μg/ml) was added into the medium to inhibit the growth of bacteria, which was confirmed by microscopic observation. Cultures were incubated at 25°C under a light: dark cycle of 14:10 with a photon flux of 125 $\mu E \cdot m^{-2} \cdot s^{-1}$. At exponential growth phase, cells were inoculated to fresh L1 medium without phosphate for several days. When DIP concentration in the culture was below the detection limit of 0.2 μM, it was used to set up experimental cultures in three

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groups as following: control group (+P group) grown in regular L1 medium that contained 36 μM DIP, ATP group grown in the L1 medium with the replacement of DIP to 36 μM ATP (ATP group), and P-depleted group (–P group) treated with L1 medium missing phosphate, each group in triplicate respectively.

Cell concentration, DIP concentration and AP activity (APA) in the medium were determined daily during the experiments. Cell density was monitored by cell count using Sedgewick–Rafter counting chamber (Phycotech, St. Joesph, MI, USA). Phosphate concentration was determined using molybdenum blue method (Parsons *et al.*, 1984). APA was measured using 4-Nitrophenyl phosphate (pNPP) as substrate following the method described previously (Lin *et al.*, 2011). Cell samples of each group were collected daily by centrifugation at 4000 g, 20°C for 15 min to harvest about 2.5×10^5–1×10^6 cells per sample. Cell pellets were resuspended in Trizol (Invitrogen, Carlsbad, CA, USA) and stored at −80°C until RNA extraction.

RNA isolation and SSH library construction

Total RNA was extracted as previously reported (Zhang *et al.*, 2007) and quantified using a NanoDrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). The samples collected on Day 4 (exponential growth stage) were used for SSH analysis, which was carried out essentially following a previously reported protocol (Zhang *et al.*, 2014) except for the high-throughput sequencing used in this study. Briefly, equal amounts of RNA aliquots from the triplicate samples of each group were pooled, and 1 μg of total RNA from +P group and ATP-group, respectively, was used to conduct cDNA synthesis and amplification using SMARTer PCR cDNA Synthesis Kit according to the manufacturer's protocol (Clontech, Takara Biotechnology [Dalian] Co., Ltd., Dalian, China). Forward (ATP-group RNA as tester, +P-group RNA as driver) and reverse libraries (+P-group RNA as tester, ATP-group RNA as driver) were constructed by SSH following the manufacturer's instructions of PCR-Select cDNA subtraction kit (Clontech, Takara Biotechnology [Dalian] Co., Ltd., Dalian, China).

454-Pyrosequencing of SSH libraries

PCR products acquired from both forward and reverse libraries were subject to 454 sequencing. First, double-stranded cDNAs of each library were amplified by using 454AL41-nestP1 and 454AL42-nestP1, respectively, as the forward primer paired with 454BL-nest2R as the reverse primer under the PCR condition as follows, 94°C for 10 s, 68°C for 30 s, 72°C for 1.5 min for 10 cycles. The amplicons were purified using Agencourt AMPure XP beads (Beckman, CA, USA) to remove those products shorter than 200 bp. The purified resultant amplicon library was examined on Bioanalyzer DNA 7500 LabChip (Agilent technologies) to confirm the complete removal of short fragments. Size-selected cDNA amplicons (> 200 bp) of each library were subjected to pyrosequencing on Roche/454 GS FLX Titanium System at State Key Laboratory of Marine Environmental Science, Xiamen University, China, following the manufacturer's protocols.

Bioinformatics analysis

Raw 454 sequencing reads were picked according to their barcode (0 mismatch) and specific primers (< 1 bp mismatch). The quality of acquired sequences was screened by the following criteria: in windows of 50 bp, sequences with an average phred score less than 25 bp were trimmed. The resulting reads were filtered again to remove sequences shorter than 200 bp and longer than 1000 bp, which was carried out on QIIME (Caporaso *et al.*, 2010). Poor-quality and nonsense sequences were filtered off as outliers. High-quality sequences of each library sharing ≥ 90% similarity were clustered into a contig. The resulting nonredundant sequences were annotated using BLASTX against the nonredundant (nr) protein database at NCBI as well as Swiss-port database, and the functional category of protein matches were determined by comparing sequence reads against the Kyoto Encyclopedia for Genes and Genomes (KEGG) database.

Reverse transcription quantitative PCR (RT-qPCR)

Based on the annotation results, genes identified from the sequenced SSH libraries that are involved in purine metabolism, nitrogen metabolism and especially in P uptake were selected for further verification using RT-qPCR. Specific primers (Supporting Information Table S3) were designed based on the unigene sequences obtained from 454 sequencing and tested for specificity and efficiency of PCR amplification. Although AP gene was not detected in the SSH libraries, this gene is of interest because it has been widely used as an indicator of DIP-limitation and DOP utilisation. Based on the AP sequence of *K. mikimotoi* described by Lin *et al.* (2015a,b), specific primers (Supporting Information Table S3) was designed to assess the differential expression of AP gene under different P conditions. Given its relative stable expression level between L1 and ATP culture conditions, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was chosen as reference gene to normalize the expression of the target genes. A relative quantification approach was adopted in RT-qPCR (Hou *et al.*, 2010) and performed on CFX-96 Real Time System (Bio-Rad Laboratories, Inc., Shanghai, China) using Bio-Rad iQ SYBR Green Supermix kit (Bio-Rad), following the methods reported by Zhang *et al.* (2014). Initial data analysis was carried out using the Bio-Rad CFX-96 manager software and the relative transcription level of target genes were calculated based on the efficiency corrected mathematical model described in Pfaffl (2001).

Uptake of ATP analog

To directly observe the uptake of ATP by *K. mikimotoi* cells, we examined the uptake of 2-Aminopurine-riboside-5′-triphosphate (2-APTP; Jena Bioscience, Germany), a fluorescent ATP analog that consists of three P groups and one nucleoside analog with a fluorescent group. This was added into the P-depleted medium at a concentration of 36 μM. After one-day incubation under the same temperature and light conditions as described above, cell samples were observed under a Laser Scanning Confocal Microscope (LSM 780 NLO, ZEISS, Jena, German) with excitation by a 405 nm argon laser. The

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range of wavelength of the detection windows are 394–471 nm.

5NT activity measurement

The extracellular 5NT activity of the cultures was measured following the protocol of 5′-Nucleotidase Activity Assay Kit (Biovision, CA, USA). Briefly, 2 μl of 5NT Converter and 10 μl of 5NT Substrate were added into 1 ml culture sample. The reaction was carried out in sterile microcentrifuge tubes, and the tubes were incubated at 20°C for 2 h. The sample was centrifuged at 10 000 g for 2 min (Eppendorf, Hauppauge, NY, USA). Then, 100 μl of supernatant was transferred to a clean 96-well plate with flat bottom, and 4 μl of stop solution was then added into each well followed by 80 μl of 5NT Developer I and 40 μl of Developer II. The 96-well plate was incubated at RT for 20 min and used for OD measurement at 670 nm on a SpectraMax® Paradigm® (Molecular Devices, CA, USA).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Left panel: merger of confocal fluorescence image (middle panel) and bright-field image (right panel) for –P+ATP analog culture (A) and L1+ATP analog culture (B).

Fig. S2. Rarefaction curve of SSH forward and reverse library datasets. The continued upward trends indicate that there were more genes in both libraries that were not recovered by our sequencing depth.

Table S1. Summary of the sequencing and annotation.

Table S2. cMOAT genes identified in dinoflagellates by BLASTX search against the Marine Microbial Eukaryote Transcriptome Sequencing Project (MMETSP) database.

Table S3. Primers designed for 454 sequencing and RT-qPCR analysis.

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