Running title:

Plastid pyruvate carboxylase in *Emiliania*

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Enzymological Evidence for the Function of a Plastid-located Pyruvate Carboxylase in the Haptophyte alga *Emiliania huxleyi*: a Novel Pathway for the Production of C₄ Compounds

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Abbreviations

BC, biotin carboxylation
BCC, biotin carboxyl carrier
CBB, Coomassie Brilliant Blue
OAA, oxaloacetate
PEPC, phosphoenolpyruvate carboxylase
PHEM, acronym of PIPES, HEPES, EGTA, and MgCl₂
PYC, pyruvate carboxylase
TC, transcarboxylation
Abstract

Pyruvate carboxylase (PYC) catalyzes the β-carboxylation of pyruvate to yield oxaloacetate (OAA). We previously isolated a cDNA encoding putative PYC (EhPYC1) from the haptophyte alga *Emiliania huxleyi* and then proposed that EhPYC1 contributes to active anaplerotic β-carboxylation during photosynthesis although PYC activity was not detected in the cell extracts. Involvement of PYC in photosynthetic carbon metabolism is unique, since PYC generally functions in non-photosynthetic organisms. In the present study, we demonstrate that EhPYC1 is highly sensitive to endogenous proteases and therefore is easily degraded in cell extracts. By avoiding proteolytic degradation, PYC activity can be detected in the cell extracts of *E. huxleyi*. The activity of a recombinant His-tagged EhPYC1 expressed in *Streptomyces lividans* was inhibited by L-malate in a mixed non-competitive manner. Immunofluorescence labeling showed that EhPYC1 is located in the plastid. This result agrees with the prediction that a bipartite plastid-targeting signal is present that functions to deliver proteins into the four-membrane plastid of haptophyte algae. This is the first finding of a plastid-located PYC. These results indicate that *E. huxleyi* possesses a unique pathway to produce OAA catalyzed by PYC and the pathway may provide carbon skeletons for amino acid biosynthesis in the plastid. Database search indicate that *PYC* genes are widespread in green algae, diatoms, and brown algae, suggesting the crucial role of PYC in various aquatic phototrophs.

**[Key words]** anaplerotic reaction, β-Carboxylation, *Emiliania huxleyi* (Haptophyta), phosphoenolpyruvate carboxylase (PEPC), photosynthetic carbon metabolism, pyruvate carboxylase (PYC)
Introduction

Pyruvate carboxylase (PYC) (EC 6.4.1.1) catalyzes the ATP-dependent β-carboxylation of pyruvate to produce oxaloacetate (OAA). PYC belongs to a biotin enzyme family that is characterized by the possession of a covalently attached biotin as a cofactor. PYC from eukaryotes and most bacteria is a homo-tetramer (α₄ type) consisting of four identical subunits of 120-130 kDa (Jitrapakdee et al., 2008). The subunit of α₄ type PYC has three functional domains, namely the biotin carboxylation (BC) domain, the transcarboxylation (TC) domain, and the biotin carboxyl carrier (BCC) domain (Jitrapakdee et al. 2008). In eukaryotes, PYC has been known to be a cytosolic or a mitochondrial enzyme.

The reaction catalyzed by PYC serves as an anaplerotic pathway that replenishes TCA cycle intermediates depleted for biosynthesis (Jitrapakdee et al., 2008; Marin-Valencia et al. 2010). The physiological significance of PYC has been well documented in non-photosynthetic organisms such as bacteria, yeast, and mammals. In Saccharomyces cerevisiae, a mutant lacking PYC isozymes could not survive due to impairment of anaplerosis when glucose was supplied as the sole carbon source (Stucka et al. 1991). In mammalian cells, PYC is involved in gluconeogenesis and anaplerosis, and therefore the dysfunction of PYC causes several severe diseases in humans (Jitrapakdee et al., 2008; Marin-Valencia et al. 2010).

In contrast to non-photosynthetic organisms, a different OAA-producing enzyme called phosphoenolpyruvate carboxylase (PEPC) (EC 4.1.1.31) functions in plants and algae instead of PYC. PEPC catalyzes the β-carboxylation reaction from phosphoenolpyruvate to OAA. PEPC is a major anaplerotic enzyme of photosynthetic organisms, since this enzyme is found throughout diverse photosynthetic organisms such as higher plants, cyanobacteria, and eukaryotic algae (Izui et al., 2004). In addition to its anaplerotic function, PEPC is employed as the primary carboxylation enzyme in C₄ photosynthesis and crassulacean acid metabolism (Jiao and Chollet,
1991). The importance of PEPC is well recognized in many photosynthetic organisms, and therefore, its reaction mechanism, allosteric regulation, and post-translational regulation are well characterized (Jiao and Chollet, 1991; Izui et al., 2004; Moellering et al. 2007).

Although PEPC has been investigated in various photosynthetic organisms, few reports have described PYC in photosynthetic organisms. PYC has been previously identified in dinoflagellates, which are a group of unicellular eukaryotic algae (Appleby et al., 1980). However, in other eukaryotic algae, such as Heterokontophyta (including diatoms and brown algae), Haptophyta, and Cryptophyta, no PYC activity was detected in crude cell extracts (Akagawa et al., 1972; Appleby et al., 1980; Descolas-Gros and Oriol, 1992, Tsuji et al., 2009). Additionally, the PYC gene has not been identified in higher plants or cyanobacteria, even though extensive genomic information on these organisms is available. Based on these studies, PEPC, not PYC, is considered to be the anaplerotic β-carboxylation enzyme in plants and algae, with one exception being the dinoflagellates. PYC is generally overlooked in studies on C₄ metabolism in photosynthetic organisms (Reinfelder et al., 2000; Cassar and Laws, 2007; Roberts et al., 2007).

In a previous study, we identified a cDNA encoding putative PYC (EhPYC1, GenBank Accession Number AB461363) in the unicellular marine haptophyte alga Emiliania huxleyi, even though PYC activity was not detected in the cell extracts (Tsuji et al., 2009). According to cDNA sequence analysis, E. huxleyi is assumed to possess multiple OAA-producing pathways involving either PEPC or PYC located in different organelles (Tsuji et al., 2009). The multiplicity of OAA-producing enzymes in E. huxleyi is unique since cytosolic PEPC is responsible for the production of OAA in higher plants (Siedow and Day, 2000; Izui et al., 2004).

Haptophyte algae acquired their plastid through the secondary endosymbiosis of eukaryotic red algae, while the plastid of higher plants is derived from cyanobacteria via primary endosymbiosis (Reyes-Prieto et al., 2007). Hence, haptophyte algae possess a so-called “complex
plastid” with four envelopes (Ishida, 2005; Reyes-Prieto et al., 2007). The storage polysaccharide of haptophytes is a soluble β-polyglucan (Vårum et al., 1986; Hirokawa et al., 2008), while that of higher plants is a crystallized α-glucan (starch). Such unique organization can also be inferred from genomic analyses of other secondary symbiotic algae such as diatoms (Kroth et al., 2008). Studies on the secondary endosymbiotic algae will provide novel insights into elucidating the diversity and evolution of photosynthetic carbon metabolism in eukaryotes.

In the present study, we experimentally demonstrated the presence of a functional PYC (EhPYC1) in the plastid of *E. huxleyi*. We also suggested a wide distribution of this enzyme in diverse eukaryotic algae following database analyses, indicating that this enzyme may play an important role in primary carbon metabolism of eukaryotic algae.

**Results**

**Expression of EhPYC1 in *E. huxleyi* cells**

In a previous study, we isolated a cDNA encoding putative PYC and designated its deduced protein as EhPYC (GenBank Accession Number BAH22705) (Tsuji et al., 2009). In the recent genome database of *E. huxleyi* (http://genome.jgi-psf.org/Emihu1/Emihu1.home.html), another putative *PYC* gene was identified. Therefore, we renamed the previous EhPYC as EhPYC1 (Table S1) and designated the newly identified putative PYC gene as EhPYC2 (Table S1, the protein ID in the JGI database is 198024). Both EhPYC1 and EhPYC2 are typical eukaryotic PYC consisting of a single polypeptide harboring the BC domain, the TC domain, and the BCC domain.

In the previously cited study, we did not detect PYC activity in the cell extracts of *E. huxleyi*. Therefore, we initially performed a Western blot analysis using anti-EhPYC1 antibody to examine the expression of EhPYC1 protein in *E. huxleyi* cells (see Materials and Methods). To
prepare crude extracts, we disrupted cells using either a French pressure cell or a bath-type sonicator. When we used the French pressure cell, we observed several immune-reactive bands including a 135-kDa major band (expected as EhPYC1) immediately after preparation (Fig. 1A). The major band degraded to smaller bands after a 1-h incubation, but this degradation was partially suppressed by the addition of protease inhibitors (Fig. 1A). In contrast, when a bath-type sonicator was employed, we did not observe any degradation of EhPYC1, even in the absence of protease inhibitors (Fig. 1B). These data show that EhPYC1 is highly sensitive to endogenous proteases in cell extracts prepared using a French pressure cell. By using the bath-type sonicator to prepare the cell extract, we were able to detect pyruvate-dependent $\text{H}^{13}\text{CO}_3^-$ fixation activity in the cell extracts (Table 1). This activity required ATP and MgCl$_2$, but was inhibited by avidin, an inhibitor of biotin carboxylases including PYC, demonstrating that it is actually derived from PYC (Table 1). The specific activity of PYC in the crude extracts [6.9 nmol (mg protein)$^{-1}$ min$^{-1}$] was comparable to that of another β-carboxylation enzyme, PEPC [8.0 nmol (mg protein)$^{-1}$ min$^{-1}$] (Tsuji et al., 2009), and the C$_3$ cycle enzyme NADPH-dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) [27 nmol (mg protein)$^{-1}$ min$^{-1}$] (Maberly et al., 2010). Since both PEPC and NADPH-dependent GAPDH are key enzymes in primary carbon metabolism, PYC activity comparable to PEPC and GAPDH implies that metabolic flux through PYC plays a significant role in E. huxleyi. An additional putative PYC (EhPYC2) was identified in the genome of E. huxleyi (Table S1). To confirm that the anti-EhPYC1 antibody specifically recognizes EhPYC1, but not EhPYC2, we performed a Western blot analysis of biotin enzymes purified from E. huxleyi (Fig. 2). Since all biotin enzymes have a covalently attached biotin molecule, biotin enzymes, including PYC, are easily purified by exploiting the binding ability of avidin to biotin. Here, we used avidin conjugated magnetic beads for affinity purification of biotin enzymes from the crude extracts (see Materials and Methods). In the Western blot analysis of the purified biotin
enzyme fraction, only a band at 135 kDa reacted with the anti-EhPYC1 antibody (Fig. 2, white arrow). This band was subjected to N-terminal sequencing and shown to be EhPYC1. The identified N-terminal amino acid sequence was VAPVEAP, which corresponds to V47–P53 of the deduced EhPYC1. The first 46 residues of EhPYC1 are expected to be the organelle-targeting signal and most plausibly are the bipartite plastid-targeting signal (Tsuji et al., 2009); the residues should be cleaved off immediately after translocation into the organelle. A band thought to be EhPYC2 (Fig. 2, black arrow) was not recognized by the anti-EhPYC1 antibody.

To analyze the function of EhPYC1, such as its relation to photosynthesis, we examined the effect of light on its abundance. The results clearly showed no significant differences in the abundance of EhPYC1 between light-grown cells and dark-adapted cells (Fig. 3). Since post-translational modification of PYC has not been reported, EhPYC1 seems to function constitutively in vivo.

Production and Characterization of Recombinant His-tagged EhPYC1

To demonstrate that EhPYC1 is a functional protein, we attempted to produce a recombinant His-tagged EhPYC1 (His-EhPYC1). Although Escherichia coli is generally used as a host organism for the production of recombinant proteins, our attempts with E. coli were not successful for unknown reasons (data not shown). E. coli may be unsuitable for the expression of PYC, as reported in previous studies (Jitrapakdee, et al. 1999, Lai et al. 2006). In addition, rare codons for E. coli are contained in EhPYC1 cDNA at a high frequency. We thought that the biased codon usage in EhPYC1 might be inhibiting its translation in E. coli. Therefore, we used another bacterium, Streptomyces lividans, to express EhPYC1 since the codon usage of S. lividans is similar to that of E. huxleyi.

By using S. lividans as the host organism, we succeeded in making a functional His-EhPYC1.
This is the first successful case of the production of eukaryotic PYC using a bacterial expression system. His-EhPYC1 was purified to a single band using SDS-PAGE on a Ni–Sepharose column (Fig. 4A). The purified His-EhPYC1 was used for kinetic analyses to determine the $K_m$ values for various substrates (Table 2). According to the generated Lineweaver–Burk plots, the $K_m$ values for ATP, pyruvate, and $\text{HCO}_3^-$ were 0.048, 0.88, and 0.47 mM, respectively (Table 2). The calculated $V_{\text{max}}$ of His-EhPYC1 was 2.1 µmol (mg protein)$^{-1}$ min$^{-1}$. Comparison of $K_m$ values among various organisms indicated that EhPYC1 has relatively high affinity for bicarbonate while its affinity for pyruvate was slightly lower in comparison with yeast and mammalian PYCs (Table 2).

We tested for the effect of various compounds on His-EhPYC1. Although acetyl-CoA is known to enhance the activity of PYC in some organisms (Jitrapakdee et al., 2008), it did not affect the activity of His-EhPYC1 (Table 3). The addition of avidin, a known inhibitor of biotin carboxylases including PYC, completely inhibited the His-EhPYC1, while other compounds, such as Asp, Asn, Gln, 3-phosphoglycerate, glucose-6-phosphate, and fructose-6-phosphate, were not effective (Table 3). L-malate (4 mM) inhibited ca. 30% of the activity and Glu and 2-oxoglutarate had a weak inhibitory effect (Table 3). Asp is known to be an allosteric inhibitor of yeast PYC (Jitrapakdee et al., 2008).

The inhibition of PYC by L-malate was not previously reported, although the inhibition by Glu and 2-oxoglutarate was already characterized in other eukaryotes (Libor et al., 1978). We further tested the effects of L-malate on His-EhPYC1. L-malate has a stronger inhibitory effect than Asp, as a reference (Fig 4B). His-EhPYC1 activity was inhibited 70% by 10 mM L-malate while the same concentration of Asp inhibited the activity only 30%. The concentration for half-inhibition ($I_{50}$) was calculated as 6 mM for L-malate (Fig. 4B). The sigmoidal inhibition curve observed in
Fig. 4B indicated that inhibition by L-malate is cooperative. Kinetic analysis showed that the inhibition pattern of His-EhPYC1 by L-malate is a mixed non-competitive inhibition with a change in both $K_m$ and $V_{max}$ (Fig. 4 C, D). The $K_m$ was increased 4-fold from 0.51 to 2.08 mM while the $V_{max}$ was decreased 2-fold from 2.30 to 1.04 µmol (mg protein)$^{-1}$ min$^{-1}$ in the presence of 10 mM L-malate (Fig 4D). The mixed-noncompetitive type inhibition is commonly considered evidence for the occurrence of allosteric inhibition. Therefore, L-malate is recognized as an allosteric inhibitor of His-EhPYC1.

Subcellular Localization of EhPYC1

We predicted EhPYC1 to be located in the plastid based on the prediction of the N-terminal targeting signal (Tsuji et al., 2009). Although weak PYC activity was detected in the roughly isolated plastid fraction, we could not exclude the possibility that the activity is derived from contaminated cytosol or mitochondrion (Tsuji et al. 2009). Thus we tried to show the subcellular localization of EhPYC1 by using an indirect immunofluorescence labeling assay. In this experiment we used anti-EhPYC1 antiserum and FITC-conjugated anti-rabbit IgG to visualize EhPYC1 in paraformaldehyde-fixed *E. huxleyi* cells. FITC fluorescence of fixed cells gave a cup-shaped signal (Fig. 5A) that was very similar to chlorophyll fluorescence of intact cells (Fig. 5C). No FITC signal was observed in negative control experiments in which anti-EhPYC1 serum was omitted (Fig. 5B). Based on these results we concluded that EhPYC1 is located in the plastid. In this experiment, co-observation of the FITC signal and chlorophyll fluorescence in the same preparation was impossible since the permeabilization treatment removed chlorophylls from the fixed cells. Thus, we were only able to compare the shape of the FITC signal in fixed cells with that of chlorophyll fluorescence in intact cells.
Discussion

Evidence for the presence of functional PYC in the plastid of *E. huxleyi*

OAA is the precursor of Asp and its derived amino acids, such as Asn, Met, Lys, Thr, and Ile. In addition, OAA can be converted into TCA cycle intermediates, such as 2-oxoglutarate, and then utilized as carbon skeletons for nitrogen assimilation. Therefore, characterizing enzymes that produce OAA is important for understanding the integration of carbon and nitrogen metabolism. In higher plants and algae, it is widely accepted that cytosolic PEPC produces the OAA that is required to replenish the TCA cycle intermediates used for biosynthesis (Siedow and Day, 2000; Izui et al., 2004). However, in the present study we unequivocally demonstrate the presence of a functional PYC in the plastid of the haptophyte alga *E. huxleyi*.

In a previous study (Tsuji et al. 2009), we could not detect any PYC activity in the crude cell extracts but detected very weak activity [0.95 nmol (mg protein)$^{-1}$ min$^{-1}$] in the partially purified plastid fraction. In the present study, we developed a reliable and reproducible method for EhPYC1 extraction by improving cell homogenization. The maintenance of PYC activity in the crude cell extracts of *E. huxleyi* was clearly achieved by the suppression of the proteolytic degradation of EhPYC1 (Fig 1A). This study presents the first experimental evidence demonstrating plastid-located PYC (Fig. 5). The localization of EhPYC1 is unique since PYC is reported as either a cytosolic or a mitochondrial enzyme in other eukaryotes (Jitrapakdee et al., 2008). The plastid of *E. huxleyi* has four envelopes of which the outermost one is composed of the endoplasmic reticulum (ER) membrane. Therefore, the nuclear-encoded plastid-targeted proteins possess the bipartite targeting signal consisting of the ER-targeting signal (signal peptide, SP) and subsequent transit peptide (TP) (Ishida, 2005). Previously, we found that the deduced sequence of EhPYC1 has a N-terminal extension that is predicted to be a bipartite plastid-targeting signal (Tsuji et al., 2009). In this study, we show that the first 47 amino acids are absent in mature...
EhPYC1 purified from *E. huxleyi* cells. Therefore, the region is considered to be the bipartite targeting signal that is cleaved off immediately after translocation of the protein into the plastid.

**Possible Function of EhPYC1 in the plastid of *E. huxleyi***

The abundance of plastid EhPYC1 did not show a significant difference between light-grown and 24-h dark-adapted cells (Fig. 3). However, EhPYC1 is expected to exhibit higher activity under illumination than in darkness since the concentrations of ATP, a substrate for PYC, and Mg$^{2+}$ are generally known to be increased in the stroma during photosynthesis (Portis and Heldt, 1976; Stitt et al., 1982).

Kinetic properties of His-EhPYC1 are characterized by the uniqueness of its response to L-malate. The Lineweaver-Burt plot analysis showed that the enzyme is inhibited by L-malate, one of the products derived from PYC and the following reactions, in a mixed non-competitive manner (Fig. 4D). The data show that L-malate is an allosteric inhibitor of EhPYC1. L-malate might induce some conformational changes of the enzyme protein by binding to EhPYC1, but not at the active site. According to the literature, no PYC was reported to show such inhibitory effects by L-malate, demonstrating the unique regulatory mechanism of EhPYC1. The inhibition by L-malate is regarded as feedback inhibition since malate is the reduced form of OAA. In contrast, another C$_4$ compound, Asp, showed only a weak inhibitory effect (Table 2, Fig. 4B). The weak inhibitory effect by Asp implies that Asp is more suitable for accumulation than malate in *E. huxleyi* cells. These properties of EhPYC1 are consistent with our previous results, which showed that Asp, but not malate, was one of the major $^{14}$C-radiolabeled compounds when $^{14}$CO$_2$ was supplied as a substrate during photosynthesis (Tsuji et al., 2009). These results suggest that EhPYC1 contributes to biosynthesis of Asp and related amino acids, rather than the accumulation of malate.
EhPYC1 may redirect the carbon flow to amino acid biosynthesis by supplying carbon skeletons in the plastid. The importance of such plastid-located β-carboxylation enzymes was recently reported in rice (Masumoto et al., 2010). Although PEPC is generally known as a cytosolic enzyme in higher plants, Masumoto et al. (2010) showed that rice has plastid-located PEPC (OsPPC4) in addition to cytosolic PEPC. By using OsPPC4 knockdown strains, OsPPC4 was shown to be essential for efficient assimilation of ammonium in rice. Like OsPPC4, plastid-located EhPYC1 is expected to support nitrogen assimilation by supplying carbon skeletons in the plastid, although other physiological roles are possible. To elucidate the physiological importance of EhPYC1, knockdown or knockout of EhPYC1 gene in *E. huxleyi* is essential. However, a method for genetic modification has not been established in haptophyte algae. Therefore, a detailed analysis using mutants must await the development of a genetic modification technique.

In addition to plastid EhPYC1, putative mitochondrial PYC (EhPYC2) and PEPC (EhPEPC) are found in the genome of *E. huxleyi* (Table S1). The predicted localization of EhPEPC is unique since no mitochondrial PEPC has been reported. Experimental confirmation of putative mitochondrial β-carboxylases, especially for EhPEPC, will be necessary to reveal the complexity of the OAA production pathway in *E. huxleyi*.

**Distribution of PYC among eukaryotic algae**

Among photosynthetic organisms, the presence of PYC was demonstrated in dinoflagellates (Appleby et al., 1980), but PYC activity has not been detected in algae as diverse as brown algae (Akagawa et al., 1972), diatoms (Appleby et al., 1980; Descolas-Gros and Oriol, 1992), and cryptophyte algae (Descolas-Gros and Oriol, 1992). Despite the lack of PYC activity, putative
PYC genes were identified in diatoms and a brown alga (Table 4). Based on our database search indicate, we identified putative PYC genes in diverse eukaryotic algae such as Chlorophyta (green algae), and Heterokontophyta including diatoms and brown algae (Table 4).

At the amino acid level, PYC in Chlorophyta shows 44–52% identity to EhPYC1, while those of Heterokontophyta show 54–66% identity (Table 4). Both Haptophyta and Heterokontophyta have evolved through the secondary endosymbiosis of red algae and therefore might share their evolitional origin. In the diatom Phaeodactylum tricornutum, comprehensive prediction of the subcellular localization of enzymes indicates that PYC might be located in the plastid and mitochondrion (Kroth et al. 2008). If such a prediction is correct, the existence of plastid-located PYC is not a feature specific to E. huxleyi, but could be conserved among various algae. In addition to the plastid PYC in E. huxleyi, unique characteristics of primary carbon metabolism are suggested in a diatom (Kroth et al. 2008).

In conclusion, we demonstrate the existence of functional PYC in the plastid of the haptophyte alga E. huxleyi. Based on the data presented and genomic analyses, E. huxleyi has complex multiple OAA-producing pathways that are driven by EhPYC1 in the plastid and by EhPYC2 and/or EhPEPC possibly located in the mitochondrion. The multiplicity of OAA-producing pathways in E. huxleyi differs from the general metabolic model established in higher plants in which cytosolic PEPC produces OAA. Haptophyte algae have evolved through secondary endosymbiosis and are therefore expected to have distinct characteristics compared with higher plants. To elucidate the actual diversity and evolution of photosynthetic carbon metabolism, further studies on secondary symbiotic algae are essential.
Materials and Methods

Organism and Culture Conditions

The organism used in this study was the haptophyte alga *Emiliania huxleyi* (NIES 837). Cells were grown in artificial seawater, Marine Art SF (produced by Tomita Seiyaku Co., Ltd., Tokushima, Japan, and distributed by Osaka Yakken Co. Ltd., Osaka) enriched with Erd–Schreiber medium (MA-ESM), in which soil extract was replaced with 10 nM sodium selenite (Danbara and Shiraiwa, 1999). Cells used for experiments were grown under constant illumination (100 µmol photons m⁻² s⁻¹) at 20°C unless otherwise stated.

Preparation of Crude Extracts for Enzyme Assay

Cells were grown in 3-l culture vessels and harvested at the logarithmic phase (OD₇₅₀, ~0.2) by centrifugation at 2000 × g for 10 min. Cells were washed with 20 ml extraction buffer containing 50 mM HEPES–NaOH (pH 7.5), 5 mM MgCl₂, 2 mM Na₂EDTA, 100 mM KCl, 5 mM ascorbate, and 2 mM dithiothreitol (DTT). Cells were then resuspended into 4 ml of the extraction buffer and disrupted with French pressure cell treatment or a bath-type sonicator (Bioruptor UC100-D; Olympus, Tokyo, Japan). The supernatant obtained after centrifugation (35,000 × g, 20 min) was applied to a PD10 desalting column (GE Healthcare Bio-Sciences KK, Tokyo, Japan) pre-equilibrated with the extraction buffer. The desalted crude extracts were used for the enzyme assay. All steps were carried out at 4°C or on ice. Protein concentration was determined with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as a standard.

Enzyme Assays of Pyruvate Carboxylase (PYC)

PYC activity in the crude extracts of *E. huxleyi* was determined by measuring...
substrate-dependent $^{14}$CO$_3^{-}$ fixation activity. A reaction mixture (0.5 ml) containing 100 mM Tris–HCl (pH 8.0), 5 mM MgCl$_2$, 10 mM NaH$^{14}$CO$_3$ (6.8 MBq mmol$^{-1}$), 2.5 mM ATP, 2 mM DTT, 0.125 mM NADH, 2.5 units malate dehydrogenase, 100 mM KCl, and 25 µl of crude extract was preincubated at 30°C for 2 min. The reaction was initiated by addition of 5 mM pyruvate. After 15 min of incubation at 30°C, the reaction was terminated by the addition of 50 µl acetate. A blank assay was performed without pyruvate.

Then radioactivity in acid-stable compounds was determined as described in Tsuji et al. (2009).

For measuring recombinant EhPYC1 activity, the formation of OAA was determined in a reaction coupled with malate dehydrogenase. The oxidation of NADH was monitored at $A_{340}$ with a spectrophotometer. The reaction mixture in the spectrophotometric assay was the same as that used in the $^{14}$C fixation assay except that $^{14}$C-bicarbonate was replaced by nonradioactive bicarbonate.

**Generation of the Anti-EhPYC1 Antibody**

A cDNA fragment encoding K1035 to P1206 of EhPYC1 (GenBank Accession Number BAH22705) was amplified using the primer pair EhPYC_Nde_F/EhPYC_Eco_R (Table S2). The amplified fragment was introduced into the NdeI/EcoRI site of the pET28a vector (Novagen, Madison, WI). The partial EhPYC1 polypeptide was expressed as a His-tagged recombinant protein in *E. coli* Rosetta2 (DE3) (Novagen). The His-tagged partial EhPYC1 was purified with a His GraviTrap (GE Healthcare Bio-Sciences KK) according to the manufacturer’s protocol and then used as antigen for generating the rabbit polyclonal antibody.

**Western Blot Analysis**

For Western blot analysis, proteins separated by SDS-PAGE were electrophoretically
transferred onto a PVDF membrane. The membrane was then blocked with 0.5% (w/v) skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBS-T) for 1 h. A rabbit anti-EhPYC1 serum (diluted 1:1000) was used as the primary antibody, and alkaline phosphatase-conjugated anti-rabbit IgG (Bio-Rad Laboratories; diluted 1:20,000) was used as the secondary antibody. Immunologically positive signals from protein bands were visualized using the CDP-star detection reagent (GE Healthcare Bio-Sciences KK) and LAS 4000 mini (Fuji Photo Film, Tokyo, Japan). No signal was obtained when preimmune serum was used (data not shown).

Expression and Purification of Recombinant His-EhPYC1 in *Streptomyces lividans*

To obtain recombinant EhPYC1, we used the inducible expression system established in *Streptomyces* (Herai et al., 2004). A cDNA fragment encoding the His-tag (fragment-1) was amplified from the pET28 vector (Merck-Novagen, Frankfurt, Germany) with the primer pair His_F/His_R (Table S2). Another cDNA fragment encoding mature EhPYC1 (fragment-2, corresponding to V47 to E1274 of EhPYC1) was amplified from EhPYC1 cDNA with the primer pair HisPYC_F/HisPYC_R (Table S2). The 3’ end of fragment-1 and 5’ end of fragment-2 contain overlapping sequences introduced from the primers. Both fragments were purified and then fused by second-round PCR with the primer pair His_F/HisPYC_R (Table S2). After the second-round PCR, a cDNA encoding His-tagged EhPYC1 (His-EhPYC1) was amplified. The resulting PCR product was cloned into the pGEM T-easy Vector (Promega) and the sequence verified. The His-EhPYC1 cDNA fragment was excised from the pGEM T-easy Vector through HindIII/XbaI digestion and then ligated with the HindIII/XbaI-digested pSH19 expression vector, which was a kind gift from Prof. M. Kobayashi, University of Tsukuba. The ligation product was introduced into *S. lividans* TK24 using the PEG-mediated transformation method (Bibb et al., 1978).

After selection with thiostrepton, plasmids were extracted from the transformants and the
introduction of His-EhPYC1 cDNA into pSH19 was confirmed by digestion. The transformant was grown in baffled flasks containing yeast extract–malt extract (YEME) liquid medium with thiostrepton (20 µg ml^{-1}) for 48 h at 28°C on a rotary shaker. At 48 h, an inducer (ε-caprolactam, 0.1%, w/v; at final conc.) and biotin (2 µg ml^{-1} at final conc.) were added, and the transformants were further cultured for 24 h at 28°C. The induced His-EhPYC1 showed no PYC activity (data not shown) without the addition of biotin, suggesting that additional biotin may be required for efficient biotinylation of newly synthesized His-EhPYC1. Cells from 200 ml of culture were harvested and washed with 20 ml of purification buffer containing 50 mM HEPES–NaOH (pH 7.5), 300 mM NaCl, 20 mM imidazol, and 0.5% (v/v) protease inhibitor cocktail (product no. P8849; Sigma, St. Louis, MO). The washed cells were resuspended in 10 ml of purification buffer and then disrupted by French press. The homogenate was then centrifuged at 35,000 × g for 20 min. The recombinant His-EhPYC1 in the resulting supernatant was purified with a His GraviTrap column (GE Healthcare Bio-Sciences KK) according to the manufacturer's instructions.

**Indirect Immunofluorescence Labeling of EhPYC1**

The following procedures were carried out at room temperature unless otherwise stated. *E. huxleyi* cells at the logarithmic growth phase were harvested by centrifugation and washed with 200 mM EDTA (pH 8.0) to remove cell-covering calcareous plates (coccoliths). The cells were washed with 3% NaCl and then fixed in 3% paraformaldehyde dissolved in PHEM buffer (60 mM PIPES, 25 mM HEPES, 10 mM EGTA, 2 mM MgCl_2, pH 6.9) supplemented with 3% NaCl for 30 min. The cells were then affixed to a poly-L-lysine-treated cover glass and washed in phosphate buffered saline (PBS) containing 2% NaCl. To remove lipid bilayer membranes, cells were treated with 1% Triton-X100 for 60 min with gentle shaking and washed three times in PBS. The
permeabilized cells were blocked with blocking reagent (Blocking One; Nacalai Tesque, Kyoto, Japan) at 30°C for 1 h. Then cells were treated with diluted (1:50) anti-EhPYC1 antibody at 37°C for 1 h. After washing three times in PBS containing 0.05% Tween 20 (PBS-T), cells were treated with diluted (1:100) FITC-conjugated secondary antibody (anti rabbit IgG) at 30°C for 1 h. After washing three times in PBS-T, cells on the cover glass were mounted on a slide glass with SlowFade (Invitrogen, Carlsbad, CA) and observed under a fluorescence microscope (BX50; Olympus). For observation of FITC fluorescence and chlorophyll fluorescence, the Olympus NIBA filter cube (excitation, 470–490 nm; emission, 515–550 nm) and Olympus WU filter cube (excitation, 330–385 nm; emission, >420 nm), respectively, were used. Since chlorophylls are lost from cells after permeabilization treatment, chlorophyll fluorescence was not observed in fixed cells; therefore, we could not compare FITC signal and chlorophyll fluorescence from the same preparation, but only separately from fixed and intact cells, respectively.

**Purification of Biotin Enzymes and N-Terminal Sequencing of EhPYC1**

Since biotin carboxylases contain a covalently attached biotin molecule, the enzymes can be captured using avidin, which has the ability to bind biotin. For the Western blotting and N-terminal sequencing of EhPYC1, biotin enzymes were purified from crude extracts using Streptavidin Paramagnetic Particles (SA-PMPs; Promega). The purification process was carried out at 4°C unless otherwise stated. Cells (500 ml suspension) at the logarithmic growth phase was harvested and resuspended in 2 ml 100 mM HEPES–NaOH containing 4 mM EDTA (pH 7.5). The cells were then disrupted in a bath-type sonicator (Bioruptor UC100-D; Olympus) at intensity 4 for 2 min at 5-s intervals. After centrifugation, the supernatant was desalted through a PD10 column (GE Healthcare Bio-Sciences KK) and then immediately mixed with the protease inhibitor cocktail (cat. no. P9599; final concentration, 0.5% (v/v); Sigma). SA-PMPs collected
from 0.3 ml of suspension were washed in 100 mM HEPES–NaOH (pH 7.5) containing 0.1% (w/v) BSA to prevent nonspecific binding of proteins to the SA-PMPs. The prewashed SA-PMPs were added to the crude extracts and then incubated for 1 h with gentle rotation. After incubation, SA-PMPs were collected using a magnetic stand and then washed three times with 1 ml HEPES–NaOH (pH 7.5). The biotin enzymes captured by SA-PMPs were released by the addition of 50 µl SDS sample buffer and subsequent boiling. The purified biotin enzyme fraction was used for Western blotting or sample preparation for the N-terminal sequence of EhPYC1. After SDS-PAGE separation, the purified biotin enzymes were transferred onto a PVDF membrane. After CBB-staining of the membrane, a band corresponding to EhPYC1 was excised and subjected to a protein sequencer (ABI Procise 494HT; Applied Biosystems/Life Technologies Corp, Carlsbad, CA).

**Database Searches**

To identify PYC genes in various organisms in a database, the amino acid sequence of EhPYC1 (GenBank Accession No. BAH2705) was used as the query sequence for BLASTP searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

**Funding**

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Acknowledgments

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References


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Tables

**Table 1** PYC activity determined in the crude extracts of *E. huxleyi* cells. Crude extracts were prepared by cell disruption in a bath-type sonicator and subsequent centrifugation. PYC activity was determined as the activity of substrate-dependent $\text{H}^{14}\text{CO}_3^-$ fixation. Mean values ± SD (n = 3) are shown.

<table>
<thead>
<tr>
<th>Components</th>
<th>Rate of $^{14}$C fixation [nmol (mg protein)$^{-1}$ min$^{-1}$]</th>
<th>% relative to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete (Control)</td>
<td>7.35 ± 0.33</td>
<td>100</td>
</tr>
<tr>
<td>-MgCl$_2$</td>
<td>0.44 ± 0.04</td>
<td>5.7</td>
</tr>
<tr>
<td>-ATP</td>
<td>0.34 ± 0.12</td>
<td>4.6</td>
</tr>
<tr>
<td>-Pyruvate</td>
<td>0.44 ± 0.04</td>
<td>6.0</td>
</tr>
<tr>
<td>Complete + Avidin$^a$</td>
<td>0.35 ± 0.04</td>
<td>4.7</td>
</tr>
</tbody>
</table>

$^a$Avidin, an inhibitor of biotin carboxylases, was added to a final concentration of 0.5 mg ml$^{-1}$.
<table>
<thead>
<tr>
<th>Organism</th>
<th>Accession No.</th>
<th>Localization</th>
<th>$K_m$ values (mM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>for ATP</td>
<td>for pyruvate</td>
</tr>
<tr>
<td>Emiliania huxleyi</td>
<td>AB461363</td>
<td>Plastid</td>
<td>0.048</td>
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<tr>
<td>Amphidinium carterae</td>
<td>-a</td>
<td>-b</td>
<td>0.030</td>
<td>0.13</td>
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<tr>
<td>Yeast</td>
<td>CAA96765</td>
<td>Cytosol</td>
<td>0.065</td>
<td>0.50</td>
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<tr>
<td>Human</td>
<td>AAA82937</td>
<td>Mitochondrion</td>
<td>0.22</td>
<td>0.22</td>
</tr>
</tbody>
</table>

*a* Not identified.

*b* Not investigated.
Table 3 Effect of various metabolites and compounds on the activity of His-EhPYC1. Mean values ± SD (n = 3) are shown. The activity in control assay was 1.72±0.014 µmol (mg protein)\(^{-1}\) min\(^{-1}\).

<table>
<thead>
<tr>
<th>Additive</th>
<th>Concentration</th>
<th>Activity of His-EhPYC1 (% relative to control(^a))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetyl-CoA</td>
<td>0.1 mM</td>
<td>100 (±7.5)</td>
</tr>
<tr>
<td>Avidin(^a)</td>
<td>0.1 mg ml(^{-1})</td>
<td>3 (±3.9)</td>
</tr>
<tr>
<td>Asp</td>
<td>4 mM</td>
<td>94 (±1.3)</td>
</tr>
<tr>
<td>Asn</td>
<td>4 mM</td>
<td>101 (±1.3)</td>
</tr>
<tr>
<td>Glu</td>
<td>4 mM</td>
<td>81 (±0.8)</td>
</tr>
<tr>
<td>Gln</td>
<td>4 mM</td>
<td>93 (±2.2)</td>
</tr>
<tr>
<td>3-Phosphoglyceric acid</td>
<td>4 mM</td>
<td>89 (±2.7)</td>
</tr>
<tr>
<td>Glucose 6-phosphate</td>
<td>4 mM</td>
<td>96 (±1.1)</td>
</tr>
<tr>
<td>Fructose 6-phosphate</td>
<td>4 mM</td>
<td>99 (±1.1)</td>
</tr>
<tr>
<td>L-Malate</td>
<td>4 mM</td>
<td>73 (±2.2)</td>
</tr>
<tr>
<td>2-Oxoglutarate</td>
<td>4 mM</td>
<td>86 (±2.8)</td>
</tr>
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</table>

\(^a\)An inhibitor of biotin enzymes.
<table>
<thead>
<tr>
<th>Division</th>
<th>Organism</th>
<th>Accession No.</th>
<th>Identity (similarity) to EhPYC1</th>
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</thead>
<tbody>
<tr>
<td>Chlorophyta</td>
<td><em>Chlamydomonas reinhardtii</em></td>
<td>XP_001696348</td>
<td>44% (59%)</td>
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<tr>
<td></td>
<td><em>Volvox carteri</em></td>
<td>XP_003080664</td>
<td>51% (67%)</td>
</tr>
<tr>
<td></td>
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</tr>
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<tr>
<td></td>
<td><em>Micromonas pusilla</em></td>
<td>XP_003058393</td>
<td>51% (65%)</td>
</tr>
<tr>
<td></td>
<td><em>Ostreococcus tauri</em></td>
<td>XP_003080664</td>
<td>51% (66%)</td>
</tr>
<tr>
<td></td>
<td><em>Ostreococcus lucimarinus</em></td>
<td>XP_001419085</td>
<td>52% (68%)</td>
</tr>
<tr>
<td>Heterokontophyta</td>
<td><em>Phaeodactylum tricornutum</em></td>
<td>XP_002183906</td>
<td>66% (82%)</td>
</tr>
<tr>
<td></td>
<td><em>Phaeodactylum tricornutum</em></td>
<td>XP_002184364</td>
<td>56% (72%)</td>
</tr>
<tr>
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<td><em>Thalassiosira pseudonana</em></td>
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<td>66% (80%)</td>
</tr>
<tr>
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<td><em>Thalassiosira pseudonana</em></td>
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<td><em>Aureococcus anophagefferens</em></td>
<td>EGB11977</td>
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<td><em>Aureococcus anophagefferens</em></td>
<td>EGB06496</td>
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<td></td>
<td><em>Ectocarpus siliculosus</em></td>
<td>CBN79581</td>
<td>54% (68%)</td>
</tr>
</tbody>
</table>
**Figure legends**

**Fig. 1** Improvement to preparing active EhPYC1 in *E. huxleyi* cells. Crude cell extracts were prepared by treatment with a French pressure cell (A) and a bath-type sonicator (B). To examine the degradation of EhPYC1 by endogenous proteases, crude extracts in each sample were incubated for 1 h at 25°C in the absence (−) or presence (+) of 1% (v/v) of the protease inhibitor cocktail (Sigma-Aldrich, cat. no. P9599). Each sample was subjected to Western blot analysis using anti-EhPYC1 serum.

**Fig. 2** Cross-reactivity of biotin carboxylases purified from *E. huxleyi* cells against anti-EhPYC1 antiserum. Biotin enzymes (B) were purified from crude cell extracts (C) using avidin paramagnetic beads and then subjected to Western blot analysis. SDS-PAGE was performed on a 6% acrylamide gel. Proteins were visualized after silver staining (Ag-stain) and Western blotting (WB) using anti-EhPYC1 antiserum. EhPYC1 and a band expected to be EhPYC2 are indicated by white and black arrowheads, respectively.

**Fig. 3** Effect of light on the abundance of EhPYC1 in *E. huxleyi* cells. Crude extracts prepared from light-grown cells (L) and 24-h dark-adapted cells (D) were subjected to Western blot analysis with anti-EhPYC1 antiserum. Each lane contained 10 µg of protein. SDS-PAGE was performed with a 10% acrylamide gel.

**Fig. 4** Kinetic analysis for testing the effects of C₄-metabolites on the activity of His-EhPYC1. (A) Expression and purification of a recombinant His-EhPYC1 in *Streptomyces lividans*. After 24-h expression, soluble proteins were extracted from transformants harboring either the empty pSH19 vector (lane 1) or pSH19 containing His-EhPYC1 cDNA (lane 2). Purified His-EhPYC1
(lane 3) was also subjected to SDS-PAGE. Proteins were visualized with CBB and Western blot analysis using the anti-His-tag antibody (Anti-His). (B) Comparison of inhibitory effects of L-malate (circles) and Asp (diamonds) on His-EhPYC1. His-EhPYC1 activity was measured at pH 8.0 as described in Materials and Methods except for the addition of Asp or L-malate. Measurements were performed in triplicate and mean values are shown. (C) Effect of L-malate on the kinetics of His-EhPYC1. The enzyme activities measured at different concentrations of pyruvate in the absence (open circles) or presence (filled circles) of L-malate. Data are means of four replicates. (D) The Lineweaver-Burk plot of the kinetic curve shown in Fig. 4C.

Fig. 5 Determining the subcellular localization of EhPYC1 using an indirect immunofluorescence labeling assay. For immunofluorescence labeling of EhPYC1, anti-EhPYC1 serum and FITC-conjugated anti-rabbit IgG were used as primary and secondary antibodies, respectively. (A) FITC fluorescence probed with both the primary and secondary antibodies. (B) FITC signal in a negative control experiment in which anti-EhPYC1 serum was absent. (C) Chlorophyll fluorescence from the chloroplasts of intact *E. huxleyi* cells. Magnified pictures of each cell are shown below panels A and C. Scale bars indicate 10 µm. As the FITC fluorescence method does not allow maintenance of chlorophyll fluorescence in the fixed cells, algal samples used in each pictures were different (see Materials and Methods).
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Fig. 4 Kinetic analysis for testing the effects of C4-metabolites on the activity of His-EhPYC1. (A) Expression and purification of a recombinant His-EhPYC1 in Streptomyces lividans. After 24-h expression, soluble proteins were extracted from transformants harboring either the empty pSH19 vector (lane 1) or pSH19 containing His-EhPYC1 cDNA (lane 2). Purified His-EhPYC1 (lane 3) was also subjected to SDS-PAGE. Proteins were visualized with CBB and Western blot analysis using the anti-His-tag antibody (Anti-His). (B) Comparison of inhibitory effects of L-malate (circles) and Asp (diamonds) on His-EhPYC1. His-EhPYC1 activity was measured at pH 8.0 as described in Materials and Methods except for the addition of Asp or L-malate. Measurements were performed in triplicate and mean values are shown. (C) Effect of L-malate on the kinetics of His-EhPYC1. The enzyme activities measured at different concentrations of pyruvate in the absence (open circles) or presence (filled circles) of L-malate. Data are means of four replicates. (D) The Lineweaver-Burk plot of the kinetic curve shown in Fig. 4C.
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