Identification and Preliminary Characterization of Phosphoenolpyruvate Carboxykinase from the Marine Alga, *Emiliana huxleyi*

by

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Research Thesis Submitted in Partial Fulfillment for the Master of Science Degree

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April 2006
# TABLE OF CONTENTS

**TITLE PAGE**................................................................................................................................. 1

**TABLE OF CONTENTS**......................................................................................................................... 2

**ABSTRACT**............................................................................................................................................... 3

**ACKNOWLEDGEMENTS**........................................................................................................................ 4

**INTRODUCTION**.................................................................................................................................... 5

- **STRUCTURE, BIOCHEMISTRY, AND PHYSIOLOGY OF PHOSPHOENOLPYRUVATE CARBOXYKINASE** 5
- **CARBON ACQUISITION AND METABOLISM IN MARINE MICROALGAE** ........................................... 9
- **PHOTOSYNTHESIS AND CALCIFICATION IN *EMILIANIA HUXLEYI*** ................................................. 11

**MATERIALS AND METHODS**.............................................................................................................. 14

- **STRAINS, MEDIA, AND GROWTH CONDITIONS**.................................................................................. 14
- **CLONING, TRANSFORMATIONS AND GENETIC COMPLEMENTATION**................................................ 15
- **PEP CARBOXYKINASE ENZYME ASSAYS**......................................................................................... 17
- **CDNA SYNTHESIS AND REAL-TIME RT-PCR (QRT-PCR)** ............................................................... 18

**RESULTS**............................................................................................................................................ 20

- **SEQUENCE ANALYSIS OF A PEP CARBOXYKINASE cDNA FROM *E. HUXLEYI*** .............................. 20
- **CLONING AND HETEROLOGOUS EXPRESSION OF THE *E. HUXLEYI* PEPCK cDNA IN *E. COLI*** .......... 28
- **CLONING AND HETEROLOGOUS EXPRESSION OF THE *E. HUXLEYI* PEPCK cDNA IN YEAST** .......... 32
- **GENETIC COMPLEMENTATION OF *E. COLI* HG4 (PCK') WITH *E. HUXLEYI* HPCK cDNA CLONES** .... 34
- **DEVELOPMENT OF A FUNCTIONAL PEP CARBOXYKINASE ENZYME ASSAY IN *E. HUXLEYI*** .......... 35
- **EXPRESSION PROFILES OF *E. HUXLEYI* PEPCK USING QRT-PCR** ............................................ 36

**DISCUSSION**....................................................................................................................................... 43

**REFERENCES**.......................................................................................................................................... 49
ABSTRACT

Phosphoenolpyruvate carboxykinase catalyzes the nucleotide dependent reversible decarboxylation of oxaloacetate to phosphoenolpyruvate. PEPCK plays a variety of important roles in central and intermediary metabolism in organisms from all three domains of life, and is the key enzyme in gluconeogenic and anapleurotic pathways. In addition, this enzyme also participates in carbon concentrating mechanisms that function to increase the availability of CO$_2$ to RubisCO enzymes in many C$_4$ and CAM plants. However, there have been relatively few reports of PEPCK enzymes from unicellular algae, or the metabolic role of this enzyme characterized in these organisms. This is particularly relevant since marine algae, such as *Emiliania huxleyi*, are considered to be carbon-limited in the ocean environment due to the low concentration of dissolved CO$_2$ (~10 μM), and thus require external and internal carbonic anhydrases as a CCM. CAs are zinc-dependent metalloenzymes and the low levels of trace metals in the oceans suggest that alternative CCM pathways, such as those described in some plants, may also exist in unicellular algae. This hypothesis was recently supported by the description of a C$_4$ photosynthetic pathway in the marine diatom, *Thalassiosira weissflogii*, involving the spatial separation of pep carboxylase and pep carboxykinase enzyme activities. The research in this thesis is the first identification of a phosphoenolpyruvate carboxykinase from a marine coccolithophorid alga. Transcriptional profiles of this gene using qRT-PCR indicated that there was no significant difference in the expression levels of this transcript in response to variations in extracellular bicarbonate, providing important baseline data for future studies on the expression of the gene under CO$_2$ and trace metal limitation conditions. Recombinant *E. huxleyi* PEPCK was expressed in *E. coli* as an active enzyme, and transformation of this clone into an *E. coli* PEPCK mutant complimented this strain to wild type phenotype. This was the first report of genetic complementation in marine coccolithophorids, and demonstrates that this technique may provide a useful tool for identifying other genes involved in carbon metabolism in this globally important marine alga.
ACKNOWLEDGEMENTS

I would like to thank my thesis committee Dr. Thomas Wahlund, Dr. Betsy Read and Dr. Bianca Mothe.

I would especially like to thank my parents and friends for their love, support and patience through this process. And a special thank you to Clay and Wyatt who helped me achieve this goal on a daily basis.

Lastly, I would like to thank Tom for having faith in a little 'ol hick girl from the Midwest.
INTRODUCTION

Structure, Biochemistry, and Physiology of Phosphoenolpyruvate Carboxykinase

Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the nucleotide dependent reversible carboxylation/decarboxylation of phosphoenolpyruvate (PEP) and/or oxaloacetate (OAA) as described in the following equation:

\[
\text{OAA + ATP/GTP} \xrightleftharpoons{\text{Me}^{2+}} \text{PEP + ADP/GDP + P}_1 + \text{CO}_2
\]

All PEPCK enzymes are categorized into one of two distinct classifications based upon nucleotide preferences; GTP/ITP-dependent or ATP-dependent enzymes. The overall sequence identity between the GTP- and ATP-dependent enzymes is low, only approximately 18-20% (11). One exception is a small region of sequence that is highly conserved across all PEPCK enzymes, referred to as the PCK-specific domain. This domain is characterized by a consensus sequence containing two strictly conserved lysine residues (G-T X-Y-X-G-E-X KK) that are suggested to be the binding site of PEP and/or OAA. The sequence identity within each classification is much higher (40-80%) (11). For example, the sequence identity between the ATP-dependent PEPCK from *Escherichia coli* (E. coli) and the yeast, *Saccharomyces cerevisiae* (S. cerevisiae) is 42%. In addition to the PCK-specific domain, other highly conserved domains have been described in ATP-dependent enzymes. The Kinase-1a (G-X-X-G-X-G-K-T) and Kinase-2 (X-X-X-X-D) domains have been shown to be involved in forming hydrogen bonds with the phosphate groups of ATP. Conserved domains for binding of a metal ion and adenine nucleotide binding domains have also been described (11).

The development of x-ray crystallography in the 1950’s has allowed the
precise determination of the structure of many enzymes at angstrom resolution, including PEPCKs from a number of species. These data have allowed the determination of the tertiary and/or quaternary structure of these enzymes, and have provided important insight into experimental approaches for determination of the functions of these conserved domains. GTP-dependent PEPCKs described to date are all monomeric proteins, however, dimeric (trypanosome) (26), tetrameric (yeast) (4), and hexameric (C₄ plants) (21) PEPCKs have been identified. As described above, conserved domain identification from crystallographic studies has been successful in confirming the functions of most of these domains. For example, the ATP-dependent Kinase-1a sequence forms a phosphate binding motif called the P-loop (10), and the phosphate binding Kinase-2 sequence is closely associated with the putative divalent-metal binding structure for Mg²⁺/Mn²⁺ (11). In contrast, less conserved regions can still create functionally similar secondary and tertiary structures. An example of this is the covalent metal ion binding site or the adenine binding site sequence. These sequences are not strictly conserved but their tertiary structure performs the same function (11).

Regardless of overall domain/motif similarities among ATP-dependent PEPCKs based upon structural studies, their reaction mechanisms can show subtle, yet significant, differences. For example, kinetic studies of PEPCK from E. coli has shown that the divalent cations, Ca²⁺ and Mg²⁺, work allosterically, increasing the decarboxylating activity of PEPCK by approximately five to six times (23). Prior to this observation, numerous studies had determined that all PEPCKs were Mg-dependent enzymes, and thus the finding of a role for Ca²⁺ in the reaction mechanism was novel. The hypothesized reaction mechanism from kinetic studies suggested that the first Ca²⁺ ion interacts with the Kinase-2 site, and then a second Ca²⁺ interacts with a unique sequence motif involving glutamic acid residues E508 and E511. The study concluded that the PEPCK from E. coli was unique in showing enzyme activity increased by Ca²⁺ and the involvement of these uniquely positioned amino acids.
Despite the fact that these enzymes carry out the same chemical reaction and have similar reaction mechanisms, the sizes of PEPCKs across species vary widely: *E. coli* PEPCK (540 amino acids), *Plasmodium falciparum* (583 amino acids), *Homo sapien* (622 amino acids), *Crocus sativus* (670 amino acids).

Among eukaryotes, the cellular location of PEPCK depends upon the species in question. For example, in humans, PEPCK is found in both the cytosol (pck-1) and mitochondria (pck-2) of liver cells (1, 32). In plants, PEPCK can be found in chloroplast and mitochondrial locations. In most all unicellular photosynthetic species described to date (the one exception being the diatom, *T. weissflogii*), this enzyme is found in the cytosol (31). Several species produce different isozymes of PEPCK, the expression of each which depends on the environmental conditions and/or the physical location of the isozyme in the organism. Two GTP-dependent isozymes have been identified and localized in the mouse, human and chicken; PCK-C and PCK-M located in the cytosol and mitochondria, respectively (1). Four isozymes for the ATP-dependent PEPCK have been found in the C₄ plant, *Urochloa panicoides* (25, 31) and two isozymes have been detected in the plant, *Arabidopsis* (20). The primary biochemical role of PEPCK in many organisms is in gluconeogenesis. This pathway is induced under conditions wherein the cell needs to convert short-chain, oxidized carbon skeletons (e.g. 2-4 carbon compounds) into more reduced carbon molecules for biosynthesis of nucleic acids and complex carbohydrates. Gluconeogenesis involves multiple steps in which intermediates of the pathway are converted into glucose as seen below.
The key reaction in the induction of gluconeogenesis is the conversion of OAA to PEP. PEP is then reduced to glucose via a series of reactions catalyzed by glycolytic enzymes working in the reverse direction. For example, *E. coli* cells grown on highly oxidized carbon sources (e.g. acetate, succinate) induce high levels of PEPCK activity, the key enzyme initiating the gluconeogenic pathway. The increased transcription of PEPCK and up regulation of its enzyme activity compensates for the lack of pentose and hexose sugars required for synthesis of essential macromolecules (e.g. purines, pyridines, polysaccharides, etc.) (7). If gluconeogenesis and glycolysis occurred at the same time, the results would be futile for the cell, and therefore the regulation of gluconeogenesis is a complex and intricately controlled process.

PEPCK also serves an anapleurotic role in certain bacteria, animals, and some plants. (11). In this capacity, PEPCK acts in the reverse direction, carboxylating and dephosphorylating PEP to yield OAA. This reaction replenishes the pool of OAA in the TCA cycle. Replenishing OAA back into the cycle allows intermediates to be siphoned off for biosynthetic needs, as in the formation of amino acids such as...
asparagine or aspartate. In the anoxygenic phototrophic bacterium, *Rhodopseudomonas palustris*, PEPCK has been shown to function in both anapleurotic and gluconeogenic roles (5, 6). This scenario is unusual since most organisms do not employ the same enzyme for both pathways, but utilize one enzyme for anapleurotic reactions (e.g. PEPC) and a different one for gluconeogenesis (e.g. PEPCK).

**Carbon Acquisition and Metabolism in Marine Microalgae**

In some photosynthetic organisms, PEPCK is involved in an enhanced version of the C₃ photosynthetic carbon cycle, called the C₄ (C₃ + C₁) cycle (16, 17). Some plants use this C₄ cycle as a means to fix additional CO₂ into an organic product, which is subsequently decarboxylated to concentrate CO₂ near the active site of Rubisco. These mechanisms are referred to as carbon concentrating mechanisms (CCMs) and are essential for the survival of many photosynthetic organisms. In general, photosynthetic organisms fix CO₂ into organic carbon via the Calvin cycle, wherein three molecules of CO₂ are combined with a molecule of ribulose 1-5 bisphosphate (RuBP) via the enzyme ribulose bisphosphate carboxylase/oxygenase (Rubisco). After several additional biosynthetic reactions, glucose and other organic compounds are produced. These reactions regenerate the acceptor molecule of the cycle, RuBP. However, Rubisco enzymes also have an oxygenase activity, resulting in the wasteful incorporation of CO₂ into glycerate, a process known as photorespiration. The competition between the carboxylase and oxygenase activity depends on Rubisco’s natural affinity for CO₂ (KmCO₂) and can be problematic in CO₂-limited environments. Therefore, many of the organisms have mechanisms that compensate for this low affinity by developing an enhanced version of the C₃ cycle, called the C₄ (C₃ + C₁) photosynthetic carbon cycle (16). Plants employing these multiple reactions within spatially separated compartments called bundle sheath and mesophyll cells are said to possess Kranz anatomy.
One variation of the C₄ cycle in multicellular plants involves an enzyme that catalyzes the decarboxylation of an organic compound in the vicinity of RubisCO. The traditional definitions of “C₄ photosynthesis” require the temporal and/or compartmentalized separation of carboxylation and decarboxylation reactions for generating additional CO₂ for RubisCO. However, data obtained from recent research has begun to challenge this “rather restricted” view of what has been described as a “C₄ photosynthesis” CCM. For example, the terrestrial plant, *Borszczowia aralocaspica*, performs a C₄ carbon-concentrating cycle by spatially separating the various enzymes and reactions within a single photosynthetic cell (27). The reaction consists of the enzyme pyruvate-orthophosphate dikinase (PPDK), located at one end of the cell, assisting in the transformation of pyruvate into phosphoenolpyruvate (PEP). Phosphoenolpyruvate carboxylase (PEPC) fixes CO₂ into OAA, which is then converted to the C₄ compound, malic acid, by NADP-malate dehydrogenase. The NAD(P) malic enzyme decarboxylates the malic acid, producing pyruvate and CO₂, in turn, releasing a CO₂ to RubisCO located in the chloroplast of the cell. Therefore, the reactions occur within close proximity to one another, spatially separated within a single photosynthetic cell.

A second type of CCM, usually found in unicellular algae and cyanobacteria, is referred to as a dissolved inorganic carbon (DIC) pump. In this model, inorganic carbon in the form of bicarbonate (HCO₃⁻) enters the cell and is transported to the chloroplast where it is dehydrated by carbonic anhydrase (CA), generating CO₂ for RubisCO (16, 17). Of significance for this proposal is a recent study in the unicellular marine diatom, *Thalassiosira weissflogii*, which showed evidence for a novel C₄-type photosynthesis in a unicellular organism under carbon- and zinc-limiting conditions (18). It has been largely assumed that marine algae must employ a CCM due to the low CO₂ levels in the oceans (~10 μM) and the poor affinity of RubisCO enzymes for CO₂ in these organisms (~30-60 μM). Consequently, these
organisms employ carbonic anhydrases as a CCM to compensate for CO$_2$ limitation. However, CA's are metalloenzymes, usually requiring zinc or other trace metals (e.g. Co, Cd) as a cofactor, and these trace elements are limited in the ocean environment (12). In the *T. weissflogii* study, cultures grown under $^{14}$CO$_2$- and zinc-limited conditions showed an increase in pep carboxylase (PEPC) activity localized in the cytoplasm, and interestingly with respect to this proposal, a decarboxylating PEPCK activity in the chloroplast, presumably providing CO$_2$ to RubisCO. Analysis of the fate of the labeled CO$_2$ found that a majority of the carbon (~70%) was identified in the malate compound and strongly suggested that a C$_4$-type photosynthesis was indeed occurring under these carbon and zinc-limiting conditions. The results of this study have provided convincing evidence for the coordinated activity of PEPC and PEPCK in delivering additional CO$_2$ to RubisCO in a unicellular alga (18).

*Photosynthesis and Calcification in Emiliania huxleyi*

*Emiliania huxleyi* (*E. huxleyi*) is a prolific unicellular photosynthetic marine alga, distinguished by its ability to synthesize intricate calcium carbonate shells, or coccoliths, which cover the external portion of the cell (15). *E. huxleyi* is an obligate photoautotroph, using CO$_2$ (derived mainly from bicarbonate) for photosynthesis, and as for the diatom *T. weissflogii*, also thrives in carbon- and zinc-limited conditions found in the oceans (12). Data obtained based upon the competitive use of HCO$_3^-$ for both photosynthesis and calcification suggested that the calcification process may act as a type of CCM in *E. huxleyi* by producing intracellular CO$_2$ for RubisCO, using CA and a H$^+$ from calcification (2, 3). However, no increase in calcification rate was observed under carbon-limited conditions in these studies, or was evidence obtained showing that the proton by-product of calcification was used for photosynthesis. Another argument against the hypothesis that calcification acts as a CCM in *E. huxleyi* points out that since coccoliths are irreversibly extruded to the exterior of the cell, their function as a "productive" CCM was debatable (e.g. 50% of the inorganic carbon from the process is lost). In the scenario supporting a role for calcification as
a CCM, the CO₂ by-product of the calcification reaction (Ca^{2+} + 2 HCO₃⁻ \rightarrow CaCO₃ + CO₂ + H^+) occurs within the coccolith vesicle, and would somehow arrive at the chloroplast and be available to RubisCO (15-17), however, evidence to support these series of events has never been demonstrated. Based upon recent research from this laboratory, we are proposing another hypothesis that, if confirmed, could explain their fate of the CO₂ and H⁺ produced by the calcification reaction, and this is shown in Figure 2:

![Diagram of hypothesized relationships between photosynthesis and calcification in coccolithophorid algae.](image)

**Fig. 2.** Schematic representation of hypothesized relationships between photosynthesis and calcification in coccolithophorid algae. Earlier models proposed that the CO₂ and H⁺ by-products of calcification in the coccolith vesicle (CV) could serve to enhance photosynthesis, however the mechanisms by which this could occur have not been demonstrated. If confirmed, a CV-located CA could resolve these questions. This route would prevent dissolution of the precipitated calcite by consuming protons and re-capturing the CO₂ released. The HCO₃⁻ produced could be transported out of the CV, increasing the intracellular DIC for photosynthesis.

Physiological studies have shown that *E. huxleyi* uses CA (external and internal) as the primary carbon-fixing enzyme and both activities have been measured in vivo (3, 19). CA activity in chloroplast-enriched fraction has been described in *E.*
huxleyi (14), however the identification of and localization of a specific CA has not been described, until recent studies from this laboratory (22).

In a recent study, EST sequencing of over 3000 cDNA clones from *E. huxleyi* identified several transcripts with significant sequence similarity (BLASTP e-values > $10^{-65}$) to PEPCK enzymes identified from other organisms (29). As mentioned earlier, PEPCK has been shown to play a role in CCMs in organisms living under carbon- and zinc-limiting environments. Therefore, the focus of this project was to study the expression and regulation of PEPCK and its role in carbon acquisition and utilization in *E. huxleyi*. The importance of this study lies in the fact that very little data are available regarding the mechanisms and biochemical pathways employed by coccolithophorids for uptake and utilization of inorganic carbon. Although there are several studies describing the activities of external and internal CA in *E. huxleyi*, other enzymes involved in central and intermediate carbon metabolism have not been identified.
MATERIALS AND METHODS

Strains, media, and growth conditions.

*E. huxleyi* strains used in qRT-PCR experiments were grown to late log to early stationary phase (2 x 10^6 cells·ml⁻¹) and were inoculated (1:100 dilution) into 4 L flasks containing 2 L of media at 18-22°C under cool fluorescent lighting with a 12h light/12h dark cycle. The media employed was a modified F/2 artificial seawater media identified as "phosphate-replete F/2" media. This media was prepared by the addition of the following components (per liter ddH₂O): NaCl, 400 mM; KCl, 10 mM; MgSO₄·7 H₂O, 20 mM; MgCl₂·6 H₂O, 20 mM; CaCl₂·2 H₂O, 7.5 mM; HBO₃, 0.4 mM; NaNO₃, 0.88 mM; Na₂HPO₄·H₂O, 0.036 mM; trace elements (ZnSO₄·7 H₂O, 76.5 nM, CuSO₄·5 H₂O, 39 nM; CoCl₂·6 H₂O, 42 nM; MnCl₂·4 H₂O, 910 nM; Na₂MoO₄·2 H₂O, 26 nM; FeCl₂·6 H₂O, 5.8 μM; Na₂EDTA·2 H₂O, 117 μM); vitamin solution (Biotin, 50 μg; B12, 50 μg; thiamine-HCL, 100 ng). Next, 10 mM Tris buffer was added and the media pH adjusted to 7.8 with concentrated HCL prior to autoclaving. Media pH was maintained with this amount of Tris regardless of the HCO₃⁻ levels employed in this study. Filter-sterilized bicarbonate was added to the cooled media following autoclaving with varying final concentrations of .002 mM, .02 mM, 0 mM and 2mM.

Yeast strains were routinely maintained YPD media (1% yeast extract, 1% peptone, 2% glucose) in a shaking water bath at 30 °C. Growth in minimal media (0.17% yeast nitrogen base, 40 mM ammonium sulfate), was obtained with glucose, ethanol, or acetate (2% final each) supplemented as carbon sources. *S. cerevisiae* PUK 3B (MATα pck1 ura3 adel) was grown in minimal media supplemented with uracil, or in uracil dropout media for selection of the yeast shuttle vector, pYES-DEST52, and/or the recombinant plasmid constructs, pYESehpck1 and pYESehpck2.
*E. coli* strains were routinely grown aerobically in Luria-Bertania (LB) media in a shaking water bath (280 rpm) at 37 °C, and supplemented with the appropriate antibiotic as needed.

**Cloning, transformations and genetic complementation**

*E. coli* strains (ccdB-sensitive) were employed as recipient for subcloning *E. huxleyi* PEPCK cDNAs from pMAB-pck library clones into the destination vector pYES-DEST52 via the GateWay™ LR Clonase system (Invitrogen Corp.). Following transformation, cells were plated on Luria-Bertani media supplemented with the ampicillin (100 μg/ml), and colonies appearing on the plates were assumed to have undergone recombination of the *E. huxleyi pepck* cDNA inserts (pMAB-pck1 and pMAB-pck2) with the destination vector, replacing the lethal *ccdB* gene in the vector. True recombinant plasmids were confirmed by checking for chloramphenicol sensitivity and designated as pYESehpck1 and pYESehpck2. The same procedures were employed for subcloning these cDNAs into the *E. coli* expression vector, pBAD-DEST49. Recombinant plasmids were also confirmed by restriction enzyme digestion patterns of the *E. huxleyi* cDNA not present in the destination vector. Plasmid purification and transformations into *E. coli* pck mutant strains were performed by standard methods.

Experiments to optimize transformation of *S. cerevisiae* PUK 3B with pYES-DEST52, pYESehpck1 and pYESehpck2 for complementation analysis were performed using variations of the lithium acetate procedure. Following centrifugation (5 min @ 10k), the cell pellet was washed in the same media minus glucose, then resuspended in 1 ml of the uracil dropout media containing acetate or ethanol as carbon source for induction of gluconeogenesis. Cells from control and recombinant strains were quadrant streaked onto minimal-acetate and minimal-glucose plates and incubated for 48–72h at 30 °C. Complementation of *E. coli* strain HG4 was
performed by growing single colonies of each strain overnight in LB (Ap, 100 μg/ml) containing glucose or succinate as carbon source (0.4% final), diluted 1:100 in LB supplemented with arabinose (0.2% final) for induction of the promoter on pBAD-DEST49, and grown for 5 h. Cells were washed twice in M9 minus a carbon source, resuspended in the same media and quadrant streaked on the appropriate plates. For over expression of EhPEPCK for SDS-PAGE and Western blots, pBADehpck1 and 2 were transformed into Top10 (ara-; Invitrogen Inc.), and inductions performed for 5 h (0.2% arabinose) prior to preparation of soluble extracts. Thioredoxin fusion proteins were analyzed using anti-Thioredoxin antibody as described by the manufacturer (Invitrogen, Inc.).

Table of strains and plasmids used in this study

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<th>Strains</th>
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</tr>
</tbody>
</table>

Plasmids

| pMAB58        | A yeast shuttle vector used for cDNA for library construction |
| pYES-DEST52   | A destination cloning vector designed for cloning and inducible expression of genes in S. cerevisiae (Gateway Technology, Invitrogen, Inc.) |
| pBAD-DEST49   | A destination cloning vector designed for cloning and expression of HP-thioredoxin |
| pMAB-peck1    | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone B02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
| pMAB-peck2    | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone B02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
| pYES-dest52   | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone C02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
| pBAD-dest52   | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone C02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
| pBAD-peck1    | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone C02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
| pBAD-peck2    | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone C02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
| pBAD-dest52   | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone C02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
| pBAD-peck1    | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone C02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
| pBAD-peck2    | A 1.5 kb fragment containing the pep carboxykinase cDNA (clone C02) from E. huxleyi cloned into the NotI-EcoRV restriction sites of pMAB58 |
**PEP carboxykinase enzyme assays**

*E. coli* strains were grown overnight to obtain an OD<sub>600</sub> reading of 1.0-3.0. Cells were harvested (25-50 ml) by centrifugation (10,000 xg, 10 min) at 4°C. Pellets were washed two times (1mM EDTA, 50 mM Tris-HCl, pH 7.5) and then resuspended in 10 ml of lysis buffer (50 mM Tris-HCl, pH7.5, 100 mM NaCl, 5 mM DTT, 1 mM PMSF). The cells were lysed by one of the three following methods: (1) French pressure cell (2 passes at 10,000 lbs./in²), (2) sonication (30 sec. pulses/15 sec intervals, 4 min), or (3) enzymatic lysis (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5% glycerol, 5mM DTT, 1 mM PMSF, 300 µg/ml lysosyme) incubated at 4°C for 30 minutes. Crude cell extract was prepared by centrifugation at 10,000 xg for 20 minutes. High spin cell extracts were centrifuged for an additional hour at 60,000 xg. The supernatant was aliquoted into chilled sterile eppendorf tubes and used immediately in enzyme assays. Protein concentrations were determined by the Bradford assay (BioRad kit). The methods employed for lysis of yeast strains, *S. cerevisiae* PUK3B and MaV203 strains for enzyme assays were essentially the same as that of the *E. coli* strains. The lysis buffer contained the following components: 50 mM Tris-HCl, pH7.5, 50 mM NaCl, 5 mM DTT, 1 mM PMSF, and the protease inhibitors leupeptin and pepstatin (1 µg/ml).

The enzyme assay used was a coupled assay involving the formation of OAA coupled to its conversion to malate by the NADH-dependent enzyme, malate dehydrogenase (MDH).

\[
\text{(PEPCK)} \\
(1) \text{PEP} + \text{ADP} + \text{CO}_2 \rightarrow \text{OAA} + \text{ATP} \\
(2) \text{OAA} + \text{NADH} \rightarrow \text{Malate} + \text{NAD}^+ 
\]

The oxidation of NADH is measured spectrophotometrically in a continuous assay.
and therefore one can quantitatively measure the amount of NADH oxidized by following the decrease in absorbance at 340nm per unit of time. The oxidation of NADH by MDH will be converted to specific enzyme activity (nmol NADH oxidized/min/mg protein). Specific activity was confirmed by showing dependency of the reaction on substrate (PEP), extract, and other reaction components (ADP, Mg^{2+}, HCO_3^-). The assays were performed in a total volume of 700 µl and were initiated with the addition of either cell extract or PEP. Each assay contained the following components: extract, 10 mM NaHCO_3, 5 mM PEP, 2 mM ADP, 0.2 mM NADH, 2.4 units of MDH, and 100 mM of various buffers (pH 6.6-8.3).

cDNA synthesis and real-time RT-PCR (qRT-PCR)

RNA was extracted from cultures grown as described above at 4, 7 & 10 day time periods during the growth stages. All cultures were treated with 11 ml of a 0.1 M HCl solution to dissolve the calcium carbonate coccoliths prior to RNA extraction, and 14.3 ml of a 0.1 NaOH to neutralize the acid treatment. The cells were centrifuged at 10,000 xg for 20 minutes at 18°C. Following resuspension of cell pellets in extract buffer, the cells were lysed by grinding in liquid nitrogen. A standard guanidine isothiocyanate extraction buffer was added and a phenol extraction procedure was used to isolate the RNA. Additional purification was done using a lithium chloride precipitation. RNA purity and concentration was measured spectrophotometrically at 260 nm and 280 nm using the Nanodrop 1000 (Nanodrop Technologies, Inc.). The samples were run on an RNA agarose gel at 80 volts for 2 hours to check for possible degradation. Samples were then aliquoted and frozen at -70°C until further use.

The Omniscript Reverse Transcription (RT) kit (Qiagen, Valencia, CA) was used to make cDNA from isolated *E. huxleyi* total RNA. Based on the total RNA concentration readings, each RT reaction contained 2 µg of total RNA, 2 µl of 1X
buffer reverse transcriptase (RT), 0.5 mM deoxynucleoside triphosphate, 1 μM of Oligo (dT) primers (Invitrogen, Carlsbad, CA), 10 U of RNase inhibitor, 4 U of Omniscript reverse transcriptase and DEPC treated water to bring the final reaction volume to 20 μl. The reaction mixture was incubated for 60 minutes at 37 °C. The cDNA was aliquoted and frozen at -70°C until further use. The cDNA was diluted 1:100 and 5 μl of the dilution was used in the total reaction solution for qRT-PCR. The forward and reverse PCR primers for PEPCK were 5'-GGGTCGCCCAAGTCATGTTC-3' and 5'-GGAGAGCCAGGCGACTTC-3', respectively. The primers were designed using the Primer Express software version 1.0 (PE Applied Biosystems) and synthesized by Invitrogen (Carlsbad, CA). PCR amplification and detection was performed in the BioRad iCycler-IQ 1000 with the use of SYBR green as the fluorescent. The reactions were performed in a 96-well plate containing 25 μl of total reaction solution. The reaction solution consisted of 5 μl of cDNA and 20 μl of a fluorescent mixture 8.1 2 X SYBER Green Master Mix, .3 μM (each) of forward and reverse primers and 12.9 of DEPC treated water). The plates were covered with optical tape and centrifuged at 500 xg for 1 minute. The cycling conditions for amplification included a 10-min. polymerase activation at 95°C followed by 40 cycles of 95°C for 10 seconds, 60°C for 30 seconds, and 82°C for 30 seconds. Fluorescence was measured at 82°C at each cycle. Melt curves were generated by denaturing the sample for 1 minutes at 95°C, cooling it to 55°C for 1 minute and then ramping the temperature 0.5 °C every 10 seconds beginning at 55°C until a final temperature of 95.0°C was reached. Each sample was run in triplicate. In addition, each plate contained a “No Template” (NT) control and a standard, both in triplicates. The standard consisted of the pMABehpck2, ranging in concentration from 1 x 10^-11 to 1 x 10^-7. The forward and reverse primers mentioned previously were used for both the genomic cDNA and the standard wells.
RESULTS

*Sequence analysis of a pep carboxykinase cDNA from* *E. huxleyi*

EST sequence analysis of over 3000 cDNA clones from a library constructed from cells grown under calcifying conditions revealed five transcripts that showed significant homology to pep carboxykinase enzymes (29). Full length sequencing of these clones revealed transcripts of various lengths, and suggested that they were all transcribed from the same gene. Two of these clones, *ehPck1* and *ehPck2*, were employed in the initial analysis since they represented pepck transcript sequences with the longest open reading frames (ORF). The most complete transcript, clone *ehPck2*, was used for further characterization, and the full-length sequence is shown in Figure 3. After trimming of 5' and 3' vector sequence, the *ehPck2* transcript revealed an ORF 1551 bp in length. This clone lacked an obvious ATG start codon and started with a CTC (leucine) codon, continuing in frame to a putative TAA stop codon. Although attempts to employ 5'RACE to obtain upstream sequence of *ehPck2* were unsuccessful, BLASTN analysis of this cDNA sequence against the *E. huxleyi* genome traces file identified an in-frame ATG (Met) 15 bp upstream of the initiating CTC in the clone. This 15 bp sequence would theoretically add five amino acids (MPDSY: Fig. 3, light gray shading) to the functional protein (521 total), and may represent the translational start of the *E. huxleyi* PEPCK. However, confirmation that these 5 amino acids are part of the *E. huxleyi* protein will have to be determined experimentally.
Fig. 3. Nucleotide sequence and deduced amino acid sequence of 1551-bp ehPck2 eDNA from *E. huxleyi*. The putative translational start of the 1556 proposed ORF is underlined (bp 1-3) and the translation termination codon (TAA) is indicated with an asterisk. The in-frame ATG (Met 1) defining the putative start of the Pck region has not been experimentally determined and was deduced from the genome sequence trace files in the NCBI database. The 5' and 3' primers used to amplify the Pck eDNA for subcloning are shown with arrows.

Translation of this eDNA sequence (starting with Leu 6) predicted a protein of 516 amino acids, with a theoretical molecular weight of 57.3 kDa and a pi of 5.44. A
detailed analysis of the amino acid sequence of this transcript identified each of the conserved domains characteristic of ATP-dependent pep carboxykinases (Fig. 4). These include the Kinase 1a, Kinase 2, Pck-specific domain, Covalent metal binding site and Adenine binding site, and thus provide convincing evidence for encoding a PEPCK enzyme.

<table>
<thead>
<tr>
<th>Domain</th>
<th>E. huxleyi</th>
<th>Y. lipolytica</th>
<th>A. nidulans</th>
<th>S. cerevisiae</th>
<th>U. panicoides</th>
<th>C. sativus</th>
<th>T. brucei</th>
<th>T. pseudonana</th>
<th>S. aureus</th>
<th>R. mellioti</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td>PKC-specific</td>
<td>172 FGGP----DFT IYNAGFPAN RFTGEMSNT TIDINFKMERIL GTEYAGEMKK</td>
<td>173 FHP----DIV IYNAGFPAN RFTGEMSNT TIDINFKEMERIL GTEYAGEMKK</td>
<td>173 FHP----DIV IYNAGFPAN RFTGEMSNT TIDINFKEMERIL GTEYAGEMKK</td>
<td>172 FGGP----DFT IYNAGFPAN RFTGEMSNT TIDINFKMERIL GTEYAGEMKK</td>
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<td>173 FHP----DIV IYNAGFPAN RFTGEMSNT TIDINFKEMERIL GTEYAGEMKK</td>
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<td>173 FHP----DIV IYNAGFPAN RFTGEMSNT TIDINFKEMERIL GTEYAGEMKK</td>
</tr>
</tbody>
</table>

**Fig. 4.** Selected portion of an amino acid sequence alignment of *E. huxleyi* and various ATP-dependent PEPCKs showing conserved domains (boxed regions). Highlighted amino acids reflect residues that are strictly conserved in ATP-dependent pep carboxykinase enzymes.
Table 5 shows the results from BLASTP analysis of the translated *E. huxleyi* PEPCK sequence against representative sequences from ATP-dependent PEPCKs from eukaryotes and prokaryotes, and indicated significant identity across these diverse species. *E. huxleyi* PEPCK was most similar to the enzyme from yeasts (*Y. lipolytica*, 76%; *A. nidulans*, 74%, and; *S. cerevisiae*, 73%), and plants (*C. sativus*, 70%, and *U. panicoides*, 67%). However, significant similarity was also seen with the enzyme from the marine diatom, *T. pseudonana* (61%) and the bacterium, *E. coli* (57%), indicating a high degree of conservation of this enzyme over evolutionary time.

Figure 6 shows the phylogenetic relationships of the PEPCK sequences from the organisms listed in Table 5 and shows the early divergence of the prokaryotic versus eukaryotic enzyme. The overall sequence similarities suggest selection via lateral transfer events of PEPCK genes over time as opposed to vertical evolutionary selection.

<table>
<thead>
<tr>
<th>Species</th>
<th>Classification</th>
<th>Identity</th>
<th>Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Y. lipolytica</em></td>
<td>Yeast</td>
<td>62%</td>
<td>76%</td>
</tr>
<tr>
<td><em>A. nidulans</em></td>
<td>Fungus</td>
<td>62%</td>
<td>74%</td>
</tr>
<tr>
<td><em>S. cerevisiae</em></td>
<td>Yeast</td>
<td>59%</td>
<td>73%</td>
</tr>
<tr>
<td><em>C. sativus</em></td>
<td>Plant</td>
<td>56%</td>
<td>70%</td>
</tr>
<tr>
<td><em>U. panicoides</em></td>
<td>Plant</td>
<td>53%</td>
<td>67%</td>
</tr>
<tr>
<td><em>T. brucei</em></td>
<td>Trypanosome</td>
<td>51%</td>
<td>66%</td>
</tr>
<tr>
<td><em>T. pseudonana</em></td>
<td>Diatom</td>
<td>44%</td>
<td>61%</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>Bacteria</td>
<td>44%</td>
<td>63%</td>
</tr>
<tr>
<td><em>R. meliloti</em></td>
<td>Bacteria</td>
<td>44%</td>
<td>60%</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>Bacteria</td>
<td>40%</td>
<td>57%</td>
</tr>
</tbody>
</table>
Figure 6. Phylogram showing the evolutionary relationship of the *E. huxleyi* PEPCK enzyme with those from other eukaryotes and prokaryotes.

A comparison alignment of full length PEPCKs shows that the primary sequences vary widely in size (Figure 7), with the prokaryotic sequences being generally shorter than eukaryotic sequences. In addition, although most all GTP-dependent PEPCKs are monomers, the ATP-specific enzymes are generally multimeric, with two, four or six identical subunits per holoenzyme (11). The one exception is the bacterial enzyme, all of which characterized to date have been monomeric (11). The quaternary structure of the *E. huxleyi* PEPCK has not yet been determined.
E. huxleyi
Y. lipolytica
A. nidulans
S. cerevisiae
U. panicoides
C. sativus
T. brucel
T. pseudonana
S. aureus
R. meliloti
E. coli

---
Fig. 7. Amino acid sequence alignment of E. huxleyi PEPCK with enzymes from a variety of prokaryotic and eukaryotic species. Amino acids that are identical to E. huxleyi are shaded in black, and conservative replacements are shaded in grey.
Cloning and heterologous expression of the *E. huxleyi* PEPCK cDNA in *E. coli*.

A variety of strategies were employed in this thesis research to subclone the two PEPCK cDNA clones (ehPck1 and ehPck2) from the library vector, pMAB58 (Fig. 8) for heterologous expression and analysis of the recombinant protein.

![Figure 8. Map of vector pMAB58](image)

Although the pMAB58 library vector was not designed as an expression vector, we decided to “test” the abilities of *E. coli* to transcribe DNA from spurious promoter elements by transforming the two PEPCK constructs into the *E. coli* DH5α strain and analyzing cell extracts for PEPCK enzyme activities versus control strains minus plasmid under gluconeogenic conditions. In *E. coli*, gluconeogenesis is induced when cells are grown on highly oxidized carbon sources, such as acetate or succinate, and repressed on reduced carbon sources such as glucose. Cultures of the parent and recombinant strains were grown overnight in M9 minimal media containing glucose or succinate, and cell extracts assayed for enzyme activity. Specific activities of
PEPCK in control and recombinant DH5α strains were determined, and fold-change differences calculated from baseline PEPCK activities of wild type DH5α grown on succinate (normalized to “1”). Since specific activities are normalized to total cell protein, then fold changes greater than 1 in the recombinant strains could be attributed to recombinant *E. huxleyi* enzyme provided by the multicopy plasmids in these cells. The results of these experiments are shown in table 6.

**Table 6.** Specific activities of PEPCK in *E. coli* control and recombinant strains

<table>
<thead>
<tr>
<th><em>E. coli</em> strain</th>
<th>Glucose</th>
<th>Succinate</th>
<th>FCb</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α (no plasmid)</td>
<td>1.76</td>
<td>16.5</td>
<td>9.4</td>
</tr>
<tr>
<td>DH5α (pMAB-ehPckc)</td>
<td>3.82</td>
<td>15.7</td>
<td>4.1</td>
</tr>
</tbody>
</table>

*a Specific activity is in nmol of NADH oxidized min⁻¹ mg⁻¹ by malate dehydrogenase in coupled assay via reduction of OAA to malate.
b FC, fold change calculated as the ratio of the PEPCK specific activity under gluconeogenic conditions with succinate versus glucose as carbon source.
c pMAB-ehPck contains the transcript identified as ehpck2

As expected, a significantly higher PEPCK activity was observed in all strains grown on succinate as a carbon source versus glucose (Table 6). However, there was no difference in PEPCK activities between the DH5α strains containing the pMAB-ehPck plasmids and the DH5α(-) plasmid when grown on succinate versus glucose (Table 6). In fact, average specific activities were significantly higher in cells lacking the recombinant plasmid (9.4-fold versus 4.1-fold, respectively), suggesting an adverse effect of the recombinant plasmid on *E. coli* PEPCK enzyme activity.

The results of these studies necessitated employment of a vector suitable for inducible expression of cloned cDNA. The cDNA library vector, pMAB58, is a Gateway-compatible cloning “entry” vector (invitrogen, Inc.), and thus in order to
evaluate expression of our pepck cDNAs, we subcloned ehPck1 and ehPck2 into the Gateway destination vector pBAD-DEST49 (Fig. 9).

![Gateway destination vector map of pBAD-DEST49.](image)

This vector is designed for regulated expression of N-terminal HP-thioredoxin fusion proteins in *E. coli*. The advantages of this vector include a tightly controllable, dose-dependent arabinose promoter (P_{BAD}), and an N-terminal thioredoxin fusion protein for increased solubility of heterologous proteins in *E. coli*. The recombinant plasmids were transformed into an *E. coli* pep carboxykinase mutant, strain HG4. Given the fact that gluconeogenesis in *E. coli* is repressed by glucose, and also by other reduced carbon sources such as arabinose, the *E. coli* strain chosen for evaluation of pBAD-ehPck expression did not require a *pck* mutant since expression of *E. huxleyi* PEPCK was under control of the arabinose-inducible promoter, pBAD-DEST49. However, the HG4 mutant strain was used in our genetic complementation experiments, and therefore was employed with the pBAD-ehPck construct in the expression experiments described below for consistency. Overnight cultures of HG4 (*pck^−*) and HG4 (pBAD-ehPck2) strains were inoculated into fresh media and grown to an OD_{600} 0.5-0.8 and induced for an additional 5 hours with the addition of arabinose (0.02%
v/v) and assayed for PEPCK activity. A low, but significant PEPCK activity (ca. 2-fold) was detected in recombinant strains induced with arabinose, and was undetectable in the same strains when grown on glucose, or in HG4 alone when grown on glucose or arabinose (Table 7). Given the fact that HG4 does not possess a functional PEPCK, the specific activities shown in Table 7 must be attributed to a functional *E. huxleyi* enzyme activity in these cells.

<table>
<thead>
<tr>
<th>Specific Activity&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Glucose</th>
<th>Arabinose</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>E. coli</em> Extract</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HG4(-)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>HG4(pBAD-ehPck1)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1.88</td>
</tr>
<tr>
<td>HG4(pBAD-ehPck2)</td>
<td>nd&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.08</td>
</tr>
</tbody>
</table>

<sup>a</sup> specific activities expressed as nmol of NADH oxidized min<sup>-1</sup> mg<sup>-1</sup> by malate dehydrogenase in an assay coupled to reduction of OAA to malate

<sup>b</sup> nd = Not detectable

Attempts to identify the expressed recombinant EhPEPCK protein in these *E. coli* extracts using Western Blots were inconclusive. Figure 10 shows the results of induction experiments described above, and these same extracts were also used for SDS-PAGE and Western analysis. The expected size of the PEPCK product encoded in the *E. huxleyi* cDNA is ~55 kDa, and the total size of the recombinant fusion protein was ~68 kDa (13 kDa thioredoxin protein on the N-terminus). Although no protein signal of the expected size was detected, the anti-thioredoxin antibody identified a band at around 30 kDa that was originally assumed to be a PEPCK degradation product. However, upon further analysis the faint signal appearing in the *E. coli* control lanes (lanes 1 and 4) suggested that the thioredoxin antisera obtained from invitrogen was of poor quality, and most likely contaminated with antibodies against *E. coli* proteins. Further efforts at employing this expression system were stopped to focus on a eukaryotic heterologous expression system for over expression and purification of recombinant EhPEPCK2.
Cloning and heterologous expression of the *E. huxleyi* PEPCK cDNA in yeast.

In order to obtain more convincing data supporting the identification of a functional PEPCK from *E. huxleyi*, we decided to try to complement a PEPCK mutant yeast strain, *S. cerevisiae* PUK3B with the *E. huxleyi* cDNA. The rationale for this was based upon the close sequence similarity between *E. huxleyi* and yeast strains, *S. cerevisiae* and *Y. lipolytica* PEPCK sequences (see Fig. 5). The ehPck1 and ehPck2 cDNA inserts were subcloned into the Gateway expression vector, pYES-DEST52 (Fig. 11) for transformation into *S. cerevisiae* PUK3B prior to induction and complementation experiments.
This Gateway destination shuttle vector is designed for galactose-inducible expression of cloned inserts in *S. cerevisiae*. Despite repeated efforts, transformations with pYES-ehPck plasmids were unsuccessful using standard *S. cerevisiae* protocols and competent cell preps, although control transformations with the pYES-DEST52 vector alone were positive (Table 8). Since we were employing a PEPCK-mutant strain of *S. cerevisiae* to make competent cells and not the standard strain transformation host strain, MAV204, experiments were performed to evaluate important variables that could affect transformation efficiency, including cell growth stage, cell density, plasmid amounts, and time of DNA incubation. In order to determine if the negative transformations were specifically related to the *E. huxleyi* *pepck* cDNA insert, we picked a random cDNA from our library for which we had obtained the full sequence (calreticulin) and subcloned it into pYES-DEST52. Transformations with this recombinant plasmid were also negative. It was subsequently assumed that the high GC content of the *E. huxleyi* (65%) may be incompatible with the replication machinery of the yeast (50% GC), preventing establishment of yeast recombinant strains for subsequent induction and complementation experiments.
Table 8. Apparent incompatibility of *E. huxleyi*’s cDNA inserts in *S. cerevisiae* strain PUK 3B

<table>
<thead>
<tr>
<th>Plasmid/Clone</th>
<th>Transformation Efficiency</th>
<th>Transformants/µg DNA</th>
<th>Transformants/Viable Cells [DNA]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0.05</td>
<td>0.1</td>
</tr>
<tr>
<td>pYES-DEST52</td>
<td></td>
<td>2.9 x 10^2</td>
<td>4.0 x 10^2</td>
</tr>
<tr>
<td>pYES-ehpck1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd&lt;sup&gt;a&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>pYES-ehpck2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
<tr>
<td>pYES-ehcalret&lt;sup&gt;c&lt;/sup&gt;</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strain PUK 3B is a pep carboxykinase-deficient mutant successfully employed as a host in heterologous complementation experiments with pep carboxykinase genes from a variety of eukaryotic organisms.

<sup>b</sup> Two cDNA encoding putative pep carboxykinases from *E. huxleyi* cloned into the yeast expression vector, pYES-DEST52.

<sup>c</sup> The calreticulin gene from *E. huxleyi* used as a recombinant control plasmid in transformation reactions.

<sup>d</sup> nd, no transformants detected.

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**Genetic complementation of *E. coli* HG4 (pck<sup>−</sup>) with *E. huxleyi* ehpck cDNA clones.**

Final confirmation that *E. huxleyi* ehpck encoded a functional enzyme was obtained by complementation of *E. coli* HG4 (pck<sup>−</sup>) with the *E. huxleyi* pYES-ehpck recombinant plasmids (Zheng et al., manuscript in preparation) (Figure 11). *E. coli* wild type (DH5α), pck-minus mutant (HG4), and HG4 strains containing recombinant clones pYES-ehpck<sub>1</sub> and pYES-ehpck<sub>2</sub> were grown over night in M9 minimal media with glucose. Cells (1 ml) were spun down and inoculated into 10 ml minimal media (1:20) containing succinate or acetate to induce gluconeogenesis. Galactose (0.2% v/v) was added to induce transcription from the pGAL promoter and the cells were grown for another 24 hours. Each culture was streaked onto minimal...
media plates and incubated for 24 h, and the results shown in Figure 11. As expected, all strains grew well with glucose as carbon source. However, strain HG4 minus plasmid showed no evidence for growth under gluconeogenic conditions, whereas HG4 strains containing each of the *E. huxleyi* PEPCK cDNAs grew on both acetate and succinate. Growth was less dense on acetate, which was not surprising since it is well known that succinate is a better inducer of gluconeogenesis in *E. coli* than acetate. The results of this complementation provide convincing data showing that the *E. huxleyi* PEPCK cDNAs encode a functional PEPCK, and support the heterologous enzyme assay data described earlier (see Table 7). In addition, the results from this complementation indicate that *E. coli* may be an efficient host for identifying genes involved in metabolic pathways in marine coccolithophorids, such as *E. huxleyi*.

**Figure 11.** Phenotypic complementation of *E. coli pepck* mutant by *E. huxleyi* pep carboxykinase cDNA. *E. coli* strains were as follows: WT, DH5α control; pck-, HG4 pepck mutant; pehpck1 and pehpck2, strain HG4 containing *E. huxleyi* pepck cDNAs (Zheng, et. al., manuscript in preparation).

*Development of a functional pep carboxykinase enzyme assay in E. huxleyi.*

Heterologous complementation does not provide any information regarding the regulatory mechanisms, cellular role(s), or subcellular location of PEPCK in *E. huxleyi*. Therefore, in order to begin investigation of the expression of this gene and protein, experiments were undertaken to establish conditions to assay this enzyme.
activity in *E. huxleyi* cells. Enzyme assays were performed on *E. huxleyi* strain 2090 grown in F/2 media. A summary of the variables evaluated is shown in Table 9. Despite significant efforts to identify and evaluate enzyme assay variables in *E. huxleyi* cell extracts, PEPCK activity was not detected under the conditions employed. PEPCK enzymes are often expressed at low levels in plants and bacteria, and in C₄ plants their expression is regulated by light. Given the fact that conditions for maximal expression of PEPCK in *E. huxleyi* are presently unknown, efforts were directed towards evaluating gene expression using qRT-PCR in order to obtain preliminary data on the expression profile of this transcript in *E. huxleyi*.

**Table 9.** Enzyme assay data for PEPCK in *Emiliania huxleyi*

<table>
<thead>
<tr>
<th>Buffer</th>
<th>NaHCO₃ [mM]</th>
<th>MgCl₂ [mM]</th>
<th>MnCl₂ [mM]</th>
<th>Tris-Cl [mM]</th>
<th>ADP [mM]</th>
<th>PEP [mM]</th>
<th>NADH [μM]</th>
<th>MDH [1.5 U]</th>
<th>Extract [μg]</th>
<th>Spin</th>
<th>SA</th>
</tr>
</thead>
<tbody>
<tr>
<td>T</td>
<td>10-50</td>
<td>2</td>
<td>100</td>
<td>1.2-2</td>
<td>2.5-5</td>
<td>.25</td>
<td>1.5-4 U</td>
<td>.344-9.06</td>
<td>C,U</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10-50</td>
<td>2</td>
<td>100</td>
<td>1.2-2</td>
<td>2.5-5</td>
<td>.25</td>
<td>1.5-4 U</td>
<td>.344-9.06</td>
<td>C,U</td>
<td>nd</td>
<td></td>
</tr>
<tr>
<td>P</td>
<td>10-50</td>
<td>2</td>
<td>100</td>
<td>1.2-2</td>
<td>2.5-5</td>
<td>.25</td>
<td>1.5-4 U</td>
<td>.344-9.06</td>
<td>C,U</td>
<td>nd</td>
<td></td>
</tr>
</tbody>
</table>

* Buffers employed: T, Tris-HCl; B, bicine; P, Pipes.
* All values refer to final concentration or concentration ranges evaluated in the experiments.
* Extract supernatant centrifugation; C, crude extract (7000 xg, 10 min); U, ultracentrifugation (100,000 xg, 60 min).
* SA, specific activities

**Expression Profiles of *E. huxleyi* PEPCK using qRT-PCR**

Next, experiments were designed to evaluate the expression of the *E. huxleyi* *pepck* gene in response to bicarbonate over the growth cycle stages. Cells were grown in F/2 media until mid-stationary phase and RNA extracted on days 4, 7 and 10-day periods for approximately thirty cell generations (i.e. ca. three sequential transfers) under the same carbon conditions, designated T1, T2, and T3. Two strains
were employed in these experiments: the noncalcifying strain 2090, and the calcifying strain B39 and bicarbonate concentrations were varied from 2 mM to 0 mM (ambient atmospheric CO$_2$; Table 10).

**Table 10.** Experimental design for qRT-PCR analysis of *E. huxleyi* pepck expression.

<table>
<thead>
<tr>
<th>Media</th>
<th>F/2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Transfer</td>
<td>1/2/3</td>
</tr>
<tr>
<td>Days</td>
<td>4/7/10</td>
</tr>
<tr>
<td>2090[HCO$_3^-$]</td>
<td>2 mM .02 mM .002 mM 0 mM</td>
</tr>
<tr>
<td>B39 [HCO$_3^-$]</td>
<td>2 mM .02 mM .002 mM 0 mM</td>
</tr>
</tbody>
</table>

Figure 12 shows an example of a typical standard curve and melt curve profile used for calculations of *pepck* transcript copy number in the bicarbonate limitation experiments described below.
In order to obtain baseline data for developing experimental approaches for determining if *E. huxleyi* possesses a C₄-type CCM mechanism (and one that involves PEPCK), real time RT-PCR was performed using RNA extracted from the non-calcifying strain 2090 grown under marine HCO₃⁻ concentrations [2 mM] (Fig. 13A), versus HCO₃⁻-minus (ambient atmospheric CO₂) levels (Fig. 13B). Strain 2090 was employed to eliminate any effects of calcification on *pepck* expression. The overall expression levels of *pepck* at 2, 0.2, and 0.002 mM HCO₃⁻ showed little difference in transcript copy number (data not shown). However, expression in strain 2090 at ambient CO₂ as sole carbon source (0 mM HCO₃⁻) versus 2 mM HCO₃⁻ did show some small, but significant difference in transcript copy number during log phase (Days 7 and 10) in T3 cells (Fig. 13). This was not the case with the calcifying strain B39 (Fig. 14, T3, Days 7 and 10).
In general, there were no obvious trends in the temporal expression levels of pepck transcript over the 4, 7, and 10-day samples in either strain over the thirty generations. However, a general trend did appear regarding copy number of the pepck transcript from T1 cells at both 2 mM and 0 mM HCO$_3^-$, showing a significant increase over that observed in T2 and T3 cell cultures in both strains 2090 (noncalcifying) and B39 (calcifying). In contrast, pepck expression appeared relatively the same at 0 mM or 2 mM HCO$_3^-$ in T2 and T3 cell cultures (ca. generations 10-30) in both the noncalcifying strain 2090 and calcifying strain B39 cells (Figs. 13 and 14).

To obtain a general picture of the possible significance of these observations, the average copy number in T1, T2 and T3 cultures was calculated at both inorganic carbon levels (2 and 0 mM) in strain 2090 (and B39) and the results shown in Figure 15. The average copy numbers in T2 cells in strain 2090 showed no significant difference with ambient atmospheric CO$_2$ as carbon source than at 2mM HCO$_3^-$ (Fig. 15). However, the pepck expression levels in T3 cells were about 2.1-fold higher at ambient atmospheric CO$_2$ than at 2 mM HCO$_3^-$ in T3 cells. In contrast, there was no difference in average expression levels of pepck in strain B39 T2 and T3 cells in response to carbon source (Fig. 15). The largest expression differences were seen in the T1 as compared to T2 and T3 cultures of both strains. As seen in Figure 15, copy numbers in B39 T1 culture were 42- and 19-fold higher at 0 mM HCO$_3^-$, than observed in T2 and T3 cells. The expression levels in strain 2090 T1 cells at 2 mM HCO$_3^-$ were 17- and 5-fold higher, respectively than observed in T2 and T3 cells. It is logical to hypothesize that the difference in the pepck copy number in the T1 cultures may be directly related to physiological differences in carbon acquisition and metabolism in calcifying (strain B39) versus noncalcifying (strain 2090) cells grown in F/2 media.
Figure 13. Temporal expression of *E. huxleyi* strain 2090 *pepck* over thirty cell generations in (A) HCO$_3^-$-replete versus (B) ambient atmospheric CO$_2$ as sole carbon sources, and under noncalcifying conditions.
Figure 14. Temporal expression of *E. huxleyi* strain B39 *pepck* over thirty cell generations in (A) HCO$_3^-$-replete versus (B) ambient atmospheric CO$_2$ as sole carbon sources and under calcifying conditions.
Average PEPCK Copy Number: Strains 2090 and B39

Figure 15. Average copy number of *E. huxleyi pepck* in strains 2090 and B39 over thirty generations.
DISCUSSION

Phosphoenolpyruvate carboxykinase is a key enzyme in gluconeogenic pathways in many organisms, being induced when cellular pools of reduced carbon skeletons required for biosynthesis are low. In human type II diabetes mellitus, the ability of insulin to suppress gluconeogenesis is impaired, and results in the overexpression of the key rate-controlling enzyme in this process, PEPCK (24, 28). Deficiencies in mitochondrial PEPCK (a rare autosomal recessive condition) result in blood acidemia, hypoglycemia, loss of muscle tone, liver enlargement, and growth abnormalities (9). PEPCK is ubiquitous in C₄ flowering plants, with some plants possessing a single PEPCK gene and others with multigene families encoding several isozymes (8). The presence of this enzyme in unicellular algae is less well described, and has recently been described in the marine diatom, *T. weissflogii* (18), where it was shown to be involved in a C₄-type CCM, and is also present in the marine coccolithophorid *E. huxleyi*, wherein it was initially identified and described solely from EST sequence analysis (13, 29, 30). Perhaps the most important role played by these enzymes is the anapleurotic carboxylation of the C₃ phosphoenolpyruvate or pyruvate to yield oxaloacetate (OAA). OAA is the carbon skeleton for the synthesis of several amino acids (asparate, asparagines, threonine) and pyrimidines, and is also the acceptor molecule for extracting electrons from the remaining four glucose carbons (2-acetyl-~CoA molecules) in the TCA cycle. In addition to these many functions, PEPCK has also been shown to play a role in nitrogen metabolism and pH regulation (8). In many organisms (invertebrates, bacteria, some protozoans) it is thought that PEPCK acts as a carboxylase in vivo because of it’s low affinity for OAA and/or due to elevated environmental CO₂. In most plants, however, it has usually been shown to act as a decarboxylase, primarily being involved in gluconeogenesis and in C₄ and CAM photosynthesis (33). The role(s) that this enzyme plays in the biology of coccolithophorid algae has not been determined. In addition, it is not yet known if *E. huxleyi* possesses more than one isozyme, or where
in the cell the protein encoded by the cDNA clone described in this thesis is located. The completion of the genome sequencing of *E. huxleyi* could shed light on the former question; however, the latter question will require experiments that are beyond the scope of this thesis. The primary goals of this thesis research were as follows: (1) to develop a biochemical assay for PEPCK in *E. huxleyi* and determine its expression profile under calcifying versus noncalcifying conditions, and in response to variations in inorganic carbon species and concentrations; (2) to develop a system for heterologous expression of the *E. huxleyi* cDNA in either a yeast or bacterial host, for purification of the recombinant PEPCK, and generation of antibodies for localization experiments, and; (3) to evaluate the transcriptional profiles of *E. huxleyi pepck* under calcifying and noncalcifying conditions under variations in inorganic carbon.

Sequence analysis of the cDNA clone suggested that the ORF of this 1551 bp transcript (516 aa) was truncated, as no ATG start codon was found in the clone. BLAST analysis of this sequence against the *E. huxleyi* trace archive revealed an upstream in frame ATG that may represent the start of the ORF, and therefore the putative protein may consist of 521 amino acids. The deduced primary amino acid sequence of the cDNA indicated that all the highly conserved domains and motifs present in other ATP-dependent PEPCKs were also present in the *E. huxleyi* sequence. This includes the strictly conserved lysine residues (K168 and K169 in the *E. huxleyi* sequence). Most all ATP-dependent PEPCKs are also multimeric, being composed of identical subunits. Although the size of the *E. huxleyi* holoenzyme has not yet been determined, preliminary results from heterologous expression, SDS-PAGE and Western blot experiments lend support for the deduced size of the this protein as ca. 57.3 kDa (Zheng, pers. comm.). Phylogenetic analysis indicated that the *E. huxleyi* enzyme sequence formed a tight cluster with those from yeasts, plants, and trypanosomes (protozoan), and was only distantly related to the bacterial and diatom enzymes. This is intriguing given the recent discovery of a novel carbonic anhydrase from *E. huxleyi* that formed a unique class of enzymes with the diatom
carbonic anhydrase (22), and given the common evolutionary histories of coccolithophorids and diatoms. These phylogenetic data suggest that horizontal transfer of genes encoding metabolically important enzymes has occurred in this species from different ancestral lineages.

Data obtained from heterologous expression of ehpck in E. coli confirmed that this clone encoded a functional PEPCK enzyme. Two lines of evidence supported this conclusion: (1) PEPCK specific activities in an E. coli pck mutant (strain HG4) containing the ehpck gene under control of an arabinose-inducible promoter were significantly higher (ca. 2-fold) in cells grown on succinate versus glucose (no enzyme activity with glucose as carbon source), and (2) the ehpck cDNA was able to genetically compliment the E. coli PEPCK mutant to wild type phenotype. This was the first demonstration of heterologous complementation of a coccolithophorid gene, and suggests that this approach may provide a useful tool for identifying other genes involved in central and intermediary carbon metabolism in E. huxleyi. Although the yeast, S. cerevisiae, was used as the host in preparation for our initial complementation experiments, the failure to obtain transformants with two E. huxleyi pepck cDNA clones, and a randomly selected calreticulin clone, suggested that the yeast replication machinery was incompatible with the high GC content E. huxleyi DNA. Eukaryotic expression systems were not exhaustively investigated in this study due to the successful use of E. coli for PEPCK expression. Experiments are planned to evaluate other eukaryotic expression systems for expression of E. huxleyi proteins (e.g. Pichia, Xenopus), as one system may not “fit” all proteins.

The confirmation of a functional PEPCK enzyme activity in E. coli directed research efforts towards measuring endogenous PEPCK activity in E. huxleyi cells under various growth conditions. The expression, regulation, and activity of a particular enzyme are dependent upon extra- and intracellular communication pathways, and therefore require that enzyme activity be measured in the host cell
environment. Although attempts to develop a biochemical assay for detection of PEPCK enzyme activity in *E. huxleyi* cells were unsuccessful, the number of experimental variables tested in this study will provide important data for the developing this assay in *E. huxleyi* in future studies. The presence of this particular PEPCK enzyme in *E. huxleyi* raises questions regarding its primary function (activity profiles) in *E. huxleyi* during calcification and photosynthesis. PEPCK enzymes perform a variety of functions in cellular metabolism, and this enzyme has not been previously described from any coccolithophorid alga. Interestingly, the *ehpck* transcript was identified from sequence analysis of 3000 ESTs from cells grown under calcifying conditions (29). In that study, multiple copies of this *pepck* transcript were present from a limited and random, sequencing effort. This observation was somewhat surprising given that PEPCK expression in most organisms is generally expressed at low levels and is strictly regulated. The immediate interpretation from the EST study was that PEPCK expression was up-regulated under calcifying conditions. Calcification and photosynthesis are competing processes in that each requires inorganic carbon as a substrate, and thus compete for intracellular DIC. The obvious question from these observations was the following: “Is PEPCK upregulated in calcifying cells as part of a CCM to enhance CO₂ availability for photosynthesis?” In the diatom, *T. weissflogii*, a PEPCK decarboxylation enzyme activity was detected in the chloroplast, providing CO₂ to RubisCO under conditions wherein carbonic anhydrase was inhibited (18). This was the first identification of a C₄-type CCM in a unicellular alga, and demonstrated that this mechanism is not restricted to multicellular Kranz anatomy-type plants. Given the common evolutionary histories of *E. huxleyi* and *T. weissflogii* under low atmospheric CO₂ levels (15), it was reasonable to focus on the *E. huxleyi* PEPCK as a possible participant in an alternative (non-carbonic anhydrase-mediated) CCM. Consequently, experiments were designed to measure the expression levels of *pepck* using qRT-PCR in response to inorganic carbon variations in calcifying and noncalcifying cells. The working hypothesis was that we would not expect to see any
significant difference in \textit{pepck} transcript levels in response to bicarbonate limitation and at ambient atmospheric \(\text{CO}_2\) levels in calcifying and noncalcifying cells. In \textit{T. weissflogii}, the \(\text{C}_4\) photosynthesis story required culture conditions that inhibited carbon anhydrase (zinc and cobalt limitation) and reduced \(\text{CO}_2\) levels. Given the multiple roles of PEPCK enzymes in cellular metabolism, data on \textit{pepck} expression in response to extracellular inorganic carbon would provide a baseline for future studies under trace metal and \(\text{CO}_2\) limitation conditions.

The result of the qRT-PCR experiments largely supported the hypothesis, as \textit{pepck} expression did not vary significantly with inorganic carbon source (2mM \(\text{HCO}_3^-\) versus atm, \(\text{CO}_2\)) in either calcifying or noncalcifying cells. As this cDNA is the only PEPCK identified thus far in \textit{E. huxleyi}, it will be important to determine it's cellular location, as other isoforms may exist that would not be involved in a \(\text{C}_4\) pathway. In the diatom, \(^{14}\text{C}\)-labelling studies identified a pep carboxylase activity in the cytoplasm yielding a labeled OAA, and a decarboxylating pep carboxykinase activity in the chloroplast. The results herein with the \textit{E. huxleyi} transcript will be important for following expression in zinc and cobalt-limited cells under variations in \(\text{CO}_2\), and these studies are now under way in this laboratory. In addition, transcriptional profiles in response to changes in light over time will determine if \textit{E. huxleyi} PEPCK follows a diel or circadian expression pattern.

One of the most important, and oft cited, contributions attributed to \textit{E. huxleyi} is its role in the global cycling of carbon via its ability to incorporate this inorganic carbon into biomineralized and photosynthetic product. In spite of the numerous elegant ecophysiological studies of marine coccolithophorids, an understanding of the genetic and molecular events underlying these processes is lacking. The recent description of two novel carbonic anhydrases from this laboratory, coupled with microarray analysis identifying genes related to calcification, are the first such studies described in these algae. The identification of the first pep carboxykinase in \textit{E.}}
huxleyi and its expression profiles in response to extracellular bicarbonate as described in this thesis has shaped the direction for future studies in our laboratories. This approach will focus on the metabolic route(s) of assimilated carbon that occur downstream of carbonic anhydrase-mediated uptake of bicarbonate and CO₂ in E. huxleyi, including the question on the operation of a C₄ photosynthetic pathway in these algae.
REFERENCES


