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EFFECTS OF NITROGEN AND PHOSPHORUS AVAILABILITY ON THE EXPRESSION OF THE COCCOLITH-VESICLE V-ATPASE (SUBUNIT C) OF *PLEUROCHRYSIS* (HAPTOPHYTA)¹

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The coccolithophores *Emiliania* and *Pleurochrysis* demonstrate increased coccolith production when growth is reduced by nitrate or phosphate limitation. The function of enhanced coccolith production under these conditions and its regulation have not been resolved. Studies at the molecular level are ideally suited to determine the exact relationship between calcification and other cellular functions. In a previous study we provided evidence for the presence of a vacuolar H⁺-ATPase on coccolith vesicle membranes of *P. carterae*. These trans-Golgi-derived vesicles are the sites of coccolith production, with each vesicle containing one coccolith. In this study, expression of the *vap* gene, encoding subunit c of the vacuolar H⁺-ATPase, was investigated. Our objective was to explore potential relationships between *vap* expression, nutrient-dependent growth, and calcification. Specifically, we monitored *vap* expression relative to two genes, *fcp* and *pcna*, whose expression was previously shown to vary with growth conditions; *fcp* encodes a fucoxanthin chl *a/c*-binding protein, and *pcna* encodes the proliferating cell nuclear antigen. Relative to the expression of *pcna* and *fcp*, *vap* expression was highest at nutrient concentrations where growth curves and chl *a* patterns indicated arrest of cell division. Our results indicate that the level of *vap* expression does not decrease when cell growth diminishes.

Key index words: calcification; calcifying vesicle; fucoxanthin chl *a/c* binding protein; *Pleurochrysis carterae*; proliferating cell nuclear antigen; proton pump; V-type ATPase; *vap* mRNA expression

Abbreviations: *fcp*, fucoxanthin chl *a/c* binding protein; *pcna*, proliferating cell nuclear antigen; *vap*, the gene encoding subunit c of the V-ATPase

The range of nitrate concentrations in the open ocean lies roughly between 0.5 and 30 μ M and changes

with season, depth, and proximity to land masses (Millero 1996, Varela and Harrison 1999). Phosphate concentrations range about 10-fold lower than nitrate levels. A number of coccolithophorid species have adapted to survive at nitrate levels below 0.5 μ M and phosphate levels below 0.02 μ M (Cortes et al. 2001, Haidar and Thierstein 2001). The relationship between nutrient availability, principally nitrogen and phosphorous, to rates of calcification among coccolithophores has only been examined in the laboratory. In nearly all cases, the coccolithophore strain or species examined exhibited greater rates of calcification when placed under nitrogen limitation (Baumann et al. 1978, Balch et al. 1992, Nimer and Merrett 1993, Paasche 1998, Fritz 1999, Varela and Harrison 1999, González 2000, Paasche 2002). Similarly, phosphorous limitation also stimulates calcification rates in *Emiliania huxleyi* (Linschooten et al. 1991, Paasche and Brubak 1994). Studies of coccolithophores in the open ocean have shown that the highest cell densities under nonbloom conditions have been at the lowest nutrient (N and P) levels. For example, Thierstein and collaborators (Haidar and Thierstein 2001, Cortes et al. 2001) examined coccolithophore dynamics in both the North Atlantic and mid-Pacific oceans and observed the highest abundance of coccospheres at nitrate and phosphorous levels below 0.5 μ M and 0.02 μ M, respectively, even though higher nutrient levels were available within the photic zone. However, their studies did not determine whether cells were still undergoing cell division. Calcification rates (CaCO₃ or coccoliths produced per cell per unit time) under field conditions are also unknown.

We (Araki and González 1998, Corstjens et al. 2001) have shown that the coccolith vesicle membrane of *Pleurochrysis* has an active proton-pumping V-type ATPase. Because calcification is an acidotic process, we hypothesized that this coccolith vesicle enzyme is involved in calcification (Araki and González 1998). Dietz et al. (2001), after a review of the literature on proton pumps homologous to the coccolith vesicle enzyme, hypothesized that such enzymes are of adaptive value for higher plants under stress conditions (e.g. high salinity). McConnaughey (1998) proposed that the calcification response to low nutrients is due to activation of plasma membrane transporter(s) that exchange protons for nutrient ions, although there is

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currently no direct evidence regarding the biochemistry of ion transport in coccolithophorids (de Vrind-de Jong and de Vrind 1997). Nevertheless, a biochemical/molecular consequence to the stress imposed by low nutrient levels is logical and predictable.

The *vap* gene encodes subunit *c* (also known as the "16-kDa proteolipid"), 1 of 12 or 13 subunits of the V-ATPase. Subunit *c* is the proton-binding component of the homomultimeric V_o domain that spans the vesicle membrane (Schumaker and Sze 1990). Because of its strategic location in the coccolith vesicle and its proton-pumping function, the V-ATPase is presumed to have a role in calcification (Araki and González 1998, Corstjens et al. 2001). Because calcification of cells in the field is typically examined at or near the end of a bloom, presumably when nutrient levels have been depleted, we wondered whether enhanced calcification, under these conditions, is a response to nutrient limitation. In this study we examined the relationship between the expression of *vap* and nutrient availability. The approach we used examined expression dynamics during different nutrient states. The expression of *vap* was compared with the only two other (partially) characterized growth-associated nuclear encoded genes of *P. carterae*, namely *fcp* and *pcna*. The former encodes a fucoxanthin chl *a/c* binding protein of the light-harvesting complex; the latter encodes the proliferating cell nuclear antigen. Because enhanced calcification is observed during conditions of nutrient limitation, we assume a similar increase in production of the coccolith vesicle membrane, including subunit *c* of the V-ATPase proton pump. Whereas calcification increases as nutrients become limiting, *pcna* expression decreases (Lin and Corstjens 2002). We hypothesize a similar expression pattern of *fcp* and *pcna* and distinctly different expression pattern for *vap* under the conditions of nutrient availability tested here.

MATERIALS AND METHODS

Algal strain and cultivation. *Pleurochrysis carterae* (Braarud & Fagerland) Christensen, PLY 156 from the Plymouth Culture Collection (*Hymenomonas carterae* strain 136 in van der Wal et al. 1983) was grown in artificial seawater supplemented with nutrients (including 26 nM FeCl_3 and EDTA) as described for f/2 medium (Guillard 1975) at 18°C. This medium with or without N or P was used in all experiments and for simplicity is herein referred to as "f/2" with the concentration of N or P indicated as appropriate. Illumination was provided with cool white fluorescent lights with a photon flux of $100 \mu\text{mol photons} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$ and a photoperiod of 12:12-h light:dark.

Determination of chl *a* content. Culture samples of 0.5 mL were centrifuged (1 min $\times$ 10,000g) and the pellet extracted in 0.25 mL dimethyl formamide. The absorption spectrum between 350 and 700 nm was determined, and the absorption maximum at 664 nm was taken as a relative measure to quantify chl *a*. Cell counts were performed in a hemocytometer (Sigma-Aldrich, St. Louis, MO, USA) on samples to which 0.1% (v/v) Lugol solution (Sigma-Aldrich) was added. Cell counts were performed in triplicate. The chl *a* content was determined in triplicate and divided by the average cell number to obtain the chl *a* per cell.

RNA isolation. RNA was extracted from 20- to 200-mL culture samples using Trizol reagent (GIBCO BRL, Gaithersburg, MD, USA) according to the manufacturer's recommendations. An additional centrifugation step after lysis and homogenization, to remove cell debris, was essential. In general, a pellet obtained from 10^7 cells was treated with 1 mL of Trizol reagent. Because of low yields obtained from the slow growing cultures, the RNA samples (destined for RT-PCR) isolated from triplicate cultures were pooled. All RNA samples underwent a second Trizol treatment to remove polysaccharide and DNA contaminants. In the case where RNA samples were destined for RT-PCR analysis, a DNase treatment was performed (1 U DNase I per 100 μg RNA, according to the manufacturer's protocol; Promega, Madison, WI, USA) before the second Trizol treatment. Quantification of RNA was performed spectrophotometrically and calculated from the A_{260} value. Integrity of the RNA was evaluated by analysis of the rRNA bands on ethidium bromide-stained agarose gels (Fig. 1). The RNA samples were stored at -80°C .

Probe sequences used for hybridizations. The isolation and characterization of the *P. carterae* *pcna* (Lin and Corstjens 2002) and *vap* (Corstjens et al. 1996) genes has been described previously, and their nucleic acid sequences are available at GenBank as AF368193 and U81519, respectively. The *fcp* probe was obtained from nucleic acid sequence analysis of 20 randomly selected clones from a *P. carterae* cDNA library. Briefly, the DNA probe was 391 base pair (GenBank accession number AY289119) with high homology to a *P. tricornutum* *fcp* (62 identical and 14 similar within a 98 AA region) and with amino acid sequence homology with an *fcp* sequence (GenBank accession number AY289120) derived from an *E. huxleyi* cDNA library (Corstjens et al. 1998). Southern blot analysis (not shown) revealed that this gene is a member of a gene family containing at least six copies. Sequence variation between the gene members was not investigated; therefore, *fcp* was only used for Northern analysis and not in RT-PCR experiments. The specificity of the two probes used for dot-blot analyses was tested on Northern blots using formaldehyde gels; RNA samples (obtained from logarithmic growing cells) were denatured before electrophoresis. Hybridizations and detection was performed as described previously (Corstjens et al. 2001).

RNA slot-blot analysis. For initial analysis of *fcp* and *vap* expression profiles, Northern slot-blot analysis was used. Four hundred milliliters of a culture (early stationary phase) was added to a 5-L flask containing 1.6 L medium without nitrate. The culture was allowed to adapt for 48 h before 200-mL samples for RNA isolation were taken. Sampling was performed for 6 consecutive days during the middle of the light period. This experiment was repeated twice (with minor modifications, i.e. 2-day sampling time intervals and bigger sampling volume) to collect larger quantities of RNA for certain control experiments. RNA was isolated, and samples containing 5 μg of total RNA were applied to 0.2 μm mesh nylon membranes (Nytran, Schleicher & Schuell, Keene, NH, USA) in $6 \times \text{SSC}$ ($1 \times = 0.9 \text{ M NaCl}$, $0.09 \text{ M Na}_3\text{C}_6\text{H}_5\text{O}_7$) with 20% formaldehyde. The RNA was fixed to the membrane for 2 h at 80°C in a vacuum oven. Blots were hybridized at 50°C in DIG Easy Hyb (Roche Molecular Biochemicals, Indianapolis, IN, USA) with 10 ng/mL of the appropriate digoxigenin-11-dUTP-labeled cDNA inserts.

Comparison of *pcna* and *vap* expression in nitrate- or phosphate-depleted cultures via semiquantitative RT-PCR. Nitrate-depleted cultures were initiated by growing a culture of *P. carterae* in f/2 for 22 days (until chl *a* per cell indicated depletion of nitrate). Cells were collected by low speed centrifugation (2000g) and suspended in their original volume of f/2 without added N. To investigate the effect of nitrate depletion on gene

FIG. 1. Demonstration of probe specificity and transcript size of *vap* (lane A) and *fcp* (lane B) by Northern blot analyses. Bars indicate the RNA size markers, from top to bottom: 7400, 2800, 1600, 1000, 600, 400, 300. The arrowhead indicates the position of *vap* and *fcp* signals. Gels for Northern blot analysis were performed under denaturing conditions (RNA samples were denatured before electrophoresis). A typical nondenatured RNA sample to show the integrity of the rRNA bands (gel stained with EtBr) is shown on the left. For comparison, the dotted lines indicate the approximate position of two RNA size markers.

expression and cell growth, the nitrate-depleted culture was divided and diluted 1:9 (in f/2 without N). At this point, nitrate was added to achieve concentrations of 824 (100% as compared with f/2), 206, 103, or 0 μM in separate treatment flasks. Cultivation was performed in triplicate using 150-mL cultures in 500-mL flasks. Cell growth and chl *a* levels were followed for 14 days. The RNA was isolated from 20 mL of the 824- and 206- μM nitrate cultures on days 0, 7, and 14. The samples were collected during the middle of the light period.

Phosphate-starved cultures were initiated by dilution of a log-phase culture (1:9 in f/2 without phosphate) and incubated for 11 days until the growth curve indicated diminished cell division. Cells were collected by low speed centrifugation and suspended in their original volume of P-free medium. The phosphate-starved culture was divided and diluted 1:9 in f/2 with added P to achieve concentrations of 29 (12.5% as compared with f/2), 7.2, 1.8, or 0 μM phosphate. The RNA was isolated from the 29- and 7.2- μM phosphate cultures on days 0, 7, 14, and 21. Cultivation and sampling conditions were similar to the conditions used in the nitrate depletion experiment.

The RT-PCR was performed on 300 ng total RNA. The RT reactions were performed using the cDNA Cycle Kit (Invitro-

gen, Carlsbad, CA, USA) according to the manufacturer's protocol. The RT-primers for *vap* and *pcna* (Table 1) were combined in a single-tube reaction to avoid differences caused by pipetting errors and potential differences in RNA degradation; the two RT-PCR primers did not interfere with each other. Of the resulting 20- μL sample, 1 μL was used for radioactive PCR amplification. The reverse PCR primers were chosen (nested) upstream from the RT primers (Table 1). Radioisotope labeling and analysis of PCR product accumulation for both genes showed exponential amplification for up to 30 cycles (Lin and Corstjens 2002). In general, 27 amplification rounds were used. A PCR cycle consisted of 94° C for 30 s, 60° C for 30 s, and 72° C for 45 s. The specific PCR fragments were then analyzed and separated from free label on agarose gels, blotted on nylon membranes (downward turbo-blot system with 0.2- μm mesh Nytran membranes, Schleicher & Schuell), and analyzed by autoradiography. The radioactive bands on the blots were excised and quantified in a Beckman scintillation counter (Beckman Coulter, Inc., Fullerton, CA). A small dilution series of the sample with the highest expected activity was used to validate quantification. Experiments were performed in triplicate. The sample value with the highest count (per experiment and for *vap* and *pcna* individually) was used for normalization. The *vap*:*pcna* ratios were calculated from normalized values.

RESULTS

Northern blot data (Fig. 1) confirm both the specificity of our DNA probes for *fcp* and *pcna* and the overall quality of RNA from a standard RNA isolation. Both probes hybridized specifically with transcripts, as expected, just below 1000 bases. *Pleurochrysis*, like other coccolithophores, is adapted for growth under oligotrophic conditions. Not surprisingly, we observed differences in hybridization of specific gene probes to total mRNA from cells before and after transfer to nutrient-depleted medium. After 72 h in nitrate-free medium, the expression of *fcp* became noticeably reduced, whereas the expression of *vap* was relatively constant (Fig. 2).

The pattern of gene expression during a nutritional recovery period was examined with cells that were submitted to a regimen that minimized internal nitrogen stores. This enabled us to compare cells that approached N-replete status, as they recovered in medium containing high levels of nitrate. Comparisons were made with parallel cultures that remained without added nitrate. To achieve low chl per cell values (demonstrating N depletion), we grew cells to high density in batch culture for 22 days. Starved cells were then collected and divided into four aliquots in fresh f/2 with added N to achieve nitrate concentrations from 0 to 824 μM . Increases in cell numbers were observed within 4 days after transfer to the nutrient-supplemented medium, whereas cell numbers remained constant in the cultures without nitrate. The most rapid growth rate was observed for cells in 824 μM nitrate (Fig. 3A). Cells in 824 μM nitrate (full-strength f/2 at T_0) were "replete" for up to 7 days after transfer as indicated by chl per cell values (Fig. 3B). However, cells in 206 μM nitrate (and lower) showed signs of N starvation by 7 days posttransfer. This experiment enabled us to select appropriate treatment concentrations and posttransfer periods for the RT-PCR analyses.

TABLE 1. RT and PCR primers used in this study.

Name	Gene	Application	Position ^a	GenBank reference
PP01	<i>vap</i>	PCR primer	166–183/forward	U81519
PP02	<i>vap</i>	RT primer	1017–1000/reverse	U81519
PP03	<i>vap</i>	PCR primer	824–805/reverse	U81519
PP40	<i>pcna</i>	PCR primer	113–132/forward	AF368193
PP42	<i>pcna</i>	PCR primer	1078–1059/reverse	AF368193
PP43	<i>pcna</i>	RT primer	1691–1672/reverse	AF368193

^aLocation of the primers is given with respect to their position on the genomic DNA. The size of the resulting RT-PCR fragment is calculated taking into account the length of intervening intron sequences. RT-PCR amplification results in a 261-base pair *vap* fragment and a 312-base pair *pcna* fragment.

The expression of *pcna* is an indicator of cell division and is therefore an indicator of growing cultures. Expression of *pcna* and *vap* were monitored by RT-PCR during the nitrate repletion study shown in Figure 3. Overall, expression levels for *pcna* began a rapid increase as the cultures were transferred to the higher nutrient medium (824 μ M N). Higher rates of *pcna* expression were expected after renewal of nitrate supply. In contrast, the general trend for *vap* expression was moderately high at time T_0 , decreased temporarily at the beginning of the posttransfer period and then increased. This increase was more pronounced in the 206-mM N culture as the culture more readily used up the available N.

The RT-PCR reactions for both sets of primers were carried out in the same tube, allowing presentation of results as a ratio of *vap:pcna* expression (Fig. 4). We are aware that, in general, expression profiles are normalized to constitutively expressed genes. However, because of scanty molecular data (in particular, specific nucleic acid sequences and respective RNA expression profiles) on coccolithophorids, such probes were not available for *P. carterae*. The *vap:pcna* ratio provides an alternative internal expression reference that is required because RNA extraction efficiencies between different samples can be expected to be variable. The *vap:pcna* ratios for two batches of cells recovering from

nutrient depletion are shown (824 and 206 μ M, Fig. 4). Starved cells, at the time of transfer (i.e. T_0), showed a *vap:pcna* ratio of 4. Seven days after transfer the *vap:pcna* ratios were down to <1 and <2, for the 824 and 206 μ M nitrate, respectively. By day 14, however, nitrate was depleted (Fig. 2A), and the *vap:pcna* ratios increased (Fig. 4A). The *fcp* slot blots (Fig. 4B) support the idea that cultures analyzed in Figure 3 were undergoing nutrient stress at the time of transfer. Moreover, the chl *a* per cell profile (Fig. 3B) and the *fcp* slot blot indicate that the cells in 824 μ M nitrate had recovered their ability to express *fcp* mRNA around day 7. In contrast, the equivalent factors remained low for culture supplemented with 206 μ M nitrate.

A similar experiment was designed to investigate the effect of phosphate depletion (in the presence of normal nitrate levels) on the expression of *vap*. A high cell density batch culture was starved for 11 days by growing the cells in phosphate-free medium after a 1:9 dilution in fresh f/2. Starved cells were divided into equal batches and allowed to recover in medium containing 0, 1.8, 7.2, and 29 μ M phosphate. During the recovery period, the highest growth rates were seen between days 7 and 14 in 29 μ M phosphate (Fig. 5A). Cultures exposed to phosphate concentrations higher than 29 μ M did not show greater increase in growth rate (not shown). Chl per cell values decreased during the 21 days of recovery (Fig. 5B), with the largest decrease observed for the faster growing cultures. However, the decrease was much less than that observed in the nitrate experiment (Fig. 3B). These results highlight the contrast between cells that are replete with respect to nitrate and those that are not. One should emphasize that at T_0 the cultures differed only in the amount of phosphate in the medium.

As noted above, the slope inflection point on the growth curve after 14 days of recovery (Fig. 5A) suggests onset of nutrient limitation. This is reflected in the *vap:pcna* ratio (Fig. 6A). This ratio increased more rapidly for cells grown in 7.2 μ M phosphate as compared with 29 μ M phosphate. The *vap:pcna* ratio increased by a factor of 3 after 14 days in the 7.2- μ M phosphate culture, whereas the 29- μ M phosphate culture remained approximately the same. Additional support for replete conditions was again provided by

FIG. 2. Slot-blot analysis of *fcp* (bottom) and *vap* (top) mRNA in nitrate-depleted cells. Slots contained 5 μ g of total RNA isolated at 24-h intervals from day 2 to day 7 after inoculation.

FIG. 3. Effects of nitrate addition to N-starved cells. (A) Cell density (cells per mL). (B) Chl *a* per cell ($A_{664} \times 10^6$). Cells were grown for 22 days in f/2 to the point that chl *a* was minimal and then brought up in f/2 with either 824, 206, 103, or 0 μM nitrate and sampled for 14 days. Error bars represent SD for triplicate cultures.

fcp expression. Despite the qualitative nature of the slot-blot data, a trend for much higher expression for the first 7 days of recovery for both cultures (Fig. 6B) was observed. The higher expression levels were not maintained as phosphate was depleted (7.2-μM culture). The culture with the highest level (29 μM) of added phosphate exhibited no clear evidence of phos-

FIG. 5. Effects of phosphate addition to P-starved cells. (A) Cell density (cells per mL). (B) Chl *a* per cell ($A_{664} \times 10^6$). Cells were starved for 11 days and subsequently brought up to 29, 7.2, 1.8, or 0 μM phosphate and sampled for 21 days. Error bars represent SD for triplicate cultures.

FIG. 4. Effects of nitrate addition on the expression of RNA in N-starved cells. (A) Expression of *vap* and *pcna* (RT-PCR). (B) Expression of *fcp* (slot-blot analysis). Results with cells grown in 824 μM (stripped bars on the left) or 206 μM (dotted bars on the right) nitrate. Error bars represent SD for triplicate cultures.

phate depletion during the course of the experiment. For this culture, observed differences in expression may reflect nitrate depletion because comparable cultures with higher phosphate levels (up to 234 μM) did deviate in growth curves and chl per cell quantity.

DISCUSSION

A variety of observations indicate that the V-ATPases in general are adaptive for plants under stress (Dietz et al. 2001). It is not known whether stress-induced changes in enzyme activity reflect simultaneous adjustments in expression of the genes encoding the several subunits of the enzyme or whether regulation is imposed upon a single key component (such as subunit *c*) or the result of posttranscriptional mechanisms controlling activity. Nevertheless, in *Pleurochrysis* (González 2000) (as in *Emiliania*; Paasche and Brubak 1994)

FIG. 6. Effects of phosphate addition on the expression of RNA in P-starved cells. (A) Expression of *vap* and *pcna* (RT-PCR). (B) Expression of *fcp* (slot-blot analysis). Striped bars on the left represent results obtained with cells recovering in 29 μM phosphate; dotted bars on the right, 7.2 μM phosphate. Error bars are SD from triplicate cultures.

enhanced calcification is correlated to nutrient limitation (nutrient stress). Higher rates of calcification suggest an increase in the number of calcifying vesicles produced by the cell and a concomitant increase in the proteins needed for calcification.

We previously showed that the V (vacuolar)-ATPase is the proton pump of the coccolith vesicle. We suspect that this enzyme plays a role during removal of protons produced during dehydration of bicarbonate in the coccolith vesicle. Subunit *c* (encoded by *vap*) of the ATPase is the membrane-spanning multimeric core that anchors the enzyme in the coccolith vesicle membrane. It is reasonable to hypothesize that the expression of this subunit is necessary for the assembly of the ATPase holoenzyme in the membrane.

At the very least, our results indicate constitutive expression of the *vap* gene. We further show that the *vap:pcna* expression ratio increased with the occurrence of nutrient depletion. This could mean that the potential for the synthesis of subunit *c* persisted after cells had stopped dividing. However, the expression ratio is most sensitive to dramatic decreases of *pcna* expression when nutrient stress imposes cessation of cell division. A more narrow range of time-scale experiments that take into account the total RNA per cell and RNA isolation efficiencies are needed to further clarify this issue. Constitutively expressed mRNAs for *Pleurochrysis* are also needed for normalization purposes. Moreover, translation activity, turnover rates of subunit *c*, and the proportion of the protein that is targeted to the calcifying vesicle (as compared with the Golgi) need to be addressed.

Our results reveal that the expression of an important component of the calcification-associated V-ATPase of the coccolith vesicle is either slightly induced or is constitutive even as the stress imposed by nutrient depletion contributes to the arrest of cell division.

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