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Running title:
Stimulation of Calcification by Cold Stress in *Emiliania*

**Key words**: calcification, cold stress, coccolith, coccolithophore, *Emiliania huxleyi*, phosphate-deprivation
Cold Stress Stimulates Intracellular Calcification by the Coccolithophore, *Emiliania huxleyi* (Haptophyceae) under Phosphate-Deficient Conditions

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Abstract

Intracellular calcification by the coccolith-producing haptophyte *Emiliania huxleyi* (NIES 873) is regulated by various environmental factors. This study focused on the relationship between cold and phosphate-deficient stresses to elucidate how those factors control coccolith production. $^{45}$Ca incorporation into coccoliths was more than 97% of the total $^{45}$Ca incorporation by whole cells. In a batch culture, orthophosphate in the medium (final concentration, 28.7 $\mu$M) was rapidly depleted within 3 d, and then extracellular alkaline phosphatase (AP) activity, an indicator of phosphate deprivation, increased during the stationary growth phase. The increase in AP activity was slightly higher at 20°C than at 12°C. The calcification started to increase earlier than AP activity and the increase was much higher at 12°C than at 20°C. Such enhancement of calcification was suppressed by the addition of phosphate while AP activity was also suppressed after a transient increase. These results suggest that phosphate deprivation is a trigger for calcification and that a rather long induction period is needed for calcification compared to the increase in AP activity. While calcification was greatly stimulated by cold stress, other cellular activities such as growth, phosphate utilization, and the induction of AP activity, were suppressed. The stimulation of coccolith production by cold stress was minimal under phosphate-sufficient conditions. The high calcification activity estimated by $^{45}$Ca incorporation was confirmed by morphological observations of
coccoliths on the cell surface under bright-field and polarization microscopy. These results indicate that phosphate deprivation is the primary factor for stimulating coccolith production, and cold stress is a secondary acceleration factor that stimulates calcification under conditions of phosphate deprivation.

**Keywords:** calcification — coccolith production — coccolithophore — cold stress — *Emiliania huxleyi* — phosphate deprivation
Introduction

Coccolithophores, marine unicellular algae classified in the division Haptophyta, produce huge blooms in the ocean. Moreover, they have gained a great deal of attention as important players in charge of the global climate and the carbon cycle. These algae are capable of fixing carbon by both photosynthesis to produce organic matter and calcification to produce CaCO₃ crystals. Calcified shells of coccospheres consist of calcareous blocks with complex shapes and are called coccoliths. *Emiliania huxleyi* is the most cosmopolitan coccolithophore species and is used for numerous comparative physiological studies as it grows well in laboratory culture (Winter et al., 1994; Paasche, 2002).

When grown under natural conditions in the ocean, *E. huxleyi* is the most temperature-tolerant coccolithophore species (1–30°C) (Okada and McIntyre, 1979). Such tolerance to wide temperature range is from data of various species that have different temperature-dependence because of different environmental conditions of isolation sites. Those various species were isolated from different waters. In the laboratory, the maximum growth rate of *E. huxleyi* isolated in the Great Barrier Reef occurs at 25°C and no growth was observed at 10°C. The sizes of protoplasts and coccospheres increase when the growth temperature decreases. Quantitative analysis
revealed that coccolith production was enhanced after a lag period when the cells were transferred from 20°C to 10°C in batch culture (Sorrosa et al., 2005).

Low temperatures also stimulate the degree of saturation and the amount of alkenones (C\textsubscript{37}–C\textsubscript{39} long-chain ketones) in *E. huxleyi*. A number of unsaturated bond in the molecule changes depending on the growth temperature. Thus, the degree of C\textsubscript{37} alkenone saturation has been used as an assessment of palaeotemperature in organic geochemistry (Brassell et al., 1986; Prahl and Wakeham, 1987). Whether or how alkenone production is related to coccolith production, however, is still unknown, although both processes are stimulated at low temperatures.

Coccolith production is also regulated by other environmental factors. It was significantly enhanced by acute phosphorous shortage accompanied by growth cessation, but the ratio of carbon deposited in coccoliths to carbon converted to organic matters by photosynthesis (C/P ratio) was increased by phosphate deficiency. Moreover, coccolith production ceased immediately upon the re-addition of phosphate (Paasche, 1998).

Phosphate limitation also triggered an increase in alkaline phosphatase (AP) activity, which is recognized as an indicator of phosphate deficiency in phytoplankton (Perry, 1972; Dyhrman and Palenik, 1999; Hoppe, 2003). *E. huxleyi* possesses two APs, one of which is constitutive and the other inducible by phosphate deficiency (Riegman et al.,
When the latter was localized to the cell surface and sequenced, it demonstrated no significant similarity to previously sequenced APs (Dyhrman and Palenik, 2003; Xu et al., 2006).

In the present study, we investigated how cell growth, extracellular AP activity, and coccolith production, as estimated by $^{45}$Ca incorporation, are associated with changes in temperature and phosphate availability in *E. huxleyi*. This information is important for our understanding of the relationship between phosphate availability and temperature to oceanic carbon flux.

**Materials and Methods**

**Organism and Culture Conditions.** *E. huxleyi* (NIES 873) coccolithophore cells were grown in artificial seawater (Marine Art SF; produced by Tomita Seiyaku Co., Ltd., Tokushima, formerly distributed by Senju Pharmaceutical Co., Osaka, Japan, and recently by Osaka Yakken Co. Ltd., Osaka) and enriched with Erd–Schreiber’s medium containing 10 nM sodium selenate instead of soil extracts (Danbara and Shiraiwa, 1999). Cells were maintained under constant illumination at 100 $\mu$mol m$^{-2}$ s$^{-1}$ and 20°C (standard condition). Cells in early linear growth phase were transferred to 12° or 13°C for low temperature treatments.
Assays. The inorganic phosphate concentration in the medium was assessed by the molybdenum blue method (Murphy and Riley, 1962). Cells were removed from an aliquot of the culture medium by filtration before assessment.

AP activity was measured according to Reichardt et al. (1967) using \( p \)-nitrophenyl phosphate (\( p \)-NPP) as a substrate. Then 100 µL of 36 mM \( p \)-NPP and 700 µL of 200 mM 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS) buffer at pH 10.0 were added to 200 µL of the cell suspension and incubated at 40°C for 15 min. The reaction was terminated by the addition of 100 µL 4 M NaOH. The absorbance at 397 nm was measured using a UV-VIS spectrophotometer (UV mini 1240; Shimadzu, Kyoto, Japan).

\( ^{45} \text{Ca Tracer Experiments for Calcification.} \) First, 4.2 kBq/mL of \( ^{45} \text{CaCl}_2 \) (692.69 MBq/mg; Perkin-Elmer, Waltham, MA) was injected to initiate the \( ^{45} \text{Ca} \)-labeling reaction. At intervals 400 µL of culture was harvested and the cells collected by centrifugation (10,000 \( \times \) g, 5 min). The pellet was washed with fresh medium and transferred to a scintillation vial containing 0.5 mL of scintillation cocktail, Hionic-Fluor (Perkin-Elmer). The radioactivity incorporated into the coccospHERE and the cytosolic space together was determined, the cells harvested by filtration using an Ultrafree-MC filter (pore size, 0.65
µm; Millipore, Billerica, MA), and the coccosphere was removed by washing in medium adjusted to pH 3.0 with HCl. Naked cells obtained on the filter were washed twice with the acidic medium and the radioactivity determined using a liquid scintillation counter (LS 5000TD; Beckman, Fullerton, CA).

Microscopic Observation. Cell number was determined by counting under a microscope (BX50; Olympus, Tokyo, Japan). Coccoliths surrounding the cells were observed with polarized light, and micrographs were taken using a microscope (DMR; Leica, Wetzlar Germany) equipped with a fluorescence microscope digital camera (Keyence, Osaka, Japan).

Results

Distribution of $^{45}$Ca Incorporated into Coccoliths and the Cytosolic Space of $E$. huxleyi

Cells. Figure 1 shows that $^{45}$Ca incorporated into $E$. huxleyi cells was primarily distributed to the coccospheres. During the initial 10 h of growth, less than 90% was incorporated, but the amount gradually increased to over 97% over 2 d (Figure 1). The results demonstrated that the amount of $^{45}$Ca incorporated by whole cells can be considered as that used for calcification including coccolith production.
Effects of Phosphate Concentration and Cold Stress on Calcification by E. huxleyi.

Growth of E. huxleyi was suppressed when the temperature was suddenly lowered from 20°C to 12°C during logarithmic phase growth. The cells reached stationary phase following an initial 2 d lag in growth (Figure 2).

Orthophosphate added to the medium (final concentration, 28.7 μM) was depleted in the batch culture during 2.5 d at 20°C, although the rate of Pi depletion and time needed for Pi-depletion changed depending on pre-culture conditions and initial cell density. The decrease in temperature to 12°C suppressed growth and caused inorganic phosphate (P_i) depletion and an increase in AP activity. Extracellular AP, known to be synthesized de novo under Pi deprivation (Xu et al., 2006), began to increase 4 d after the initiation of culture, irrespective of temperature change. It took 1–2 d to initiate AP induction after Pi was removed from the medium. The increase in AP activity was less at 12°C than that at 20°C, although the difference was small, suggesting that the timing of the enzyme induction is mainly regulated by Pi availability in the medium and the extent is largely
affected by temperature. Coccolith production, however, was greatly stimulated at low
temperatures, and 6 d after exposure to cold stress, the amount of $^{45}$Ca incorporation was
about five times higher at 12°C than at 20°C. Notably, calcification was initiated about 1
d earlier than the induction of AP (Figure 2C). When *Emiliania* cells were suspended in
the medium with sufficient-Pi and without addition of Pi, cell growth ceased about 2 and
3 d after the initiation of culture, respectively. The increase in AP activity that is due to *de
novo* synthesis by Pi-limitation was obvious at 3 d in Pi-limited culture but very little in
Pi-sufficient culture. Similarly, calcification, expressed as $^{45}$Ca-incorporation, was
stimulated in Pi-limited culture but no or less in Pi-sufficient culture (Figure 3A). The
stimulation of AP and calcification by phosphate deprivation was negated by the addition
of phosphate to the medium following a transient increase in AP and a lag of several hours
in calcification, respectively (Figure 3B).

**Figure 2**

**Figure 3**

AP activity and $^{45}$Ca incorporation of calcification clearly increased when cells were
transferred to $P_i$-deficient conditions at low temperature (Figure 4). When cells were transferred from 20° to 12°C, cell growth was not significantly affected by $P_i$ availability. AP activity and calcification, however, were greatly stimulated by $P_i$-deficiency. $^{45}$Ca incorporation increased slightly but then ceased altogether until day 3 at 12°C, even under phosphate-sufficient conditions, although AP was not induced (Figure 4C). This suggests that cold stress itself may also function as a trigger for calcification since the calcification began prior to AP induction when the cells were exposed to both stressors. Calcification was primarily regulated by $P_i$ availability while cold stress functioned as a stimulating factor (Figure 4).

**Figure 4**

The physiological data on coccolith production was confirmed by morphological observations under bright-field and polarizing microscopes (Figure 5). The cells in logarithmic growth phase had almost no coccoliths and were approximately 3–4 μm in diameter (Figure 5B-a). When the culture was allowed to grow until it became $P_i$-deprived at constant temperature, the cells began to increase in size to 5 μm, but the production of coccoliths on the cell surface was very low (Figure 5B-b). In contrast, *E.*
huxleyi transferred to low temperature (13°C) under P$_i$-deficient conditions increased its cell size and produced many coccoliths (Figure 5B-c). However, the cells grown at the lower temperature under phosphate-sufficient conditions produced no coccoliths on the cell surface, although cell size increased similarly to cells grown in phosphate-deficient medium (Figure 5B-d). As Emiliania cells transferred to 13°C increased slightly in cell size (Figure 5B-d), the calcification process may be proceeding even though it is not yet observable under the microscope. These data demonstrate the significant stimulatory effect of cold stress on coccolith production in the coccolithophore E. huxleyi under P$_i$-deficient conditions.

Figure 5

Discussion

E. huxleyi is phylogenetically closely related to Isochrysis galbana, a non-coccolith-producing haptophyte (Edvardsen et al., 2000; Fujiwara et al., 2001). Our previous results demonstrated that incorporation of $^{45}$Ca by I. galbana was several hundred times less than that by E. huxleyi even though the cell sizes are nearly equivalent (Sorrosa et al., 2005). The data presented here in Figure 1 confirm that $^{45}$Ca absorbed by
**E. huxleyi** cells is primarily used for coccolith production and that only a tiny percentage of the $^{45}$Ca remains inside the cells themselves. This suggests that haptophyte algae require a high concentration of calcium that is primarily used for coccolith production and not for other cellular activities. Therefore, the total $^{45}$Ca incorporated by *E. huxleyi* can be thought of as a quantitative measure of coccolith production. Coccolith production by a coccolithophore such as *E. huxleyi* was confirmed to be a mechanism for the storage of huge amounts of calcium and inorganic carbon.

$\text{P}_i$ limitation is known to increase the ratio of carbon deposition into coccoliths produced by calcification to organic materials produced by photosynthesis, expressed as the C/P ratio (Paasche and Brubak, 1994; van Bleijswijk et al., 1994). Moreover, an increase in temperature from 10° to 15°C was reported to lead to a slight increase in the C/P ratio since calcification was stimulated and the production of organic matter was slightly reduced (van Bleijswijk et al., 1994). By estimating calcification activity using the incorporation of $^{45}$Ca into coccoliths, the present study clearly showed that $\text{P}_i$ deprivation induces both AP activity and coccolith production (Figures 2, 3), as previously observed by Paasche (1998) and Riegman et al. (2000). AP was shown to localize on the cell surface in *E. huxleyi* by biochemical and gene analyses (Landry et al., 2006; Xu et al., 2006). In addition, our results clearly show that coccolith production can
be triggered by a low temperature signal prior to the AP induction when cells are exposed
to the double stresses of cold and P\textsubscript{i} limitation (Figure 4). Calcification was greatly
stimulated when both a cold stress and a P\textsubscript{i}-deficient signal were given simultaneously
(Figures 4, 5).

Figure 5 clearly indicates that the cell diameter increased when cells were placed
under cold stress and P\textsubscript{i}-deficient conditions. Sorrosa et al. (2005) also observed cell
enlargement under cold stress but did not investigate P\textsubscript{i} limitation. A close relationship
between cell enlargement and the stimulation of coccolith production was described
previously (Shiraiwa, 2003; Sorrosa et al., 2005). Here we show that \textit{E. huxleyi} coccolith
production and cell size are strongly regulated by changes in environmental factors and
growth status. During rapid cell division in the logarithmic growth phase, cells remained
small in size without coccolith production on the cell surface (Figure 5). These results
suggest that coccolith production may be important in less active cells to avoid stresses
that may result in cell damage.

Cold stress on a plant leads to the modification of metabolism in two ways. The first is
through a mechanism that improves stress tolerance, and the second adjusts metabolic
processes to the consequences of cold stress (Guy et al., 2008). Cold stress is known to
induce the expression of a desaturase and to enhance the production of unsaturated fatty
acids in order to increase membrane flexibility at low temperatures (Wada et al., 1994; Los et al., 1997). In this case, the physiological function of the increase in the desaturase activity is very clear. The physiological importance of the stimulation of coccolith production under cold and \( P_i \)-deficient conditions, however, remains to be elucidated.

\( P_i \) limitation generally induces the suppression of photosynthetic carbon metabolism by reducing the amount of phosphate compounds and the activities of some enzymes in the plastid C\(_3\) cycle, including via ATP synthesis (Rao and Terry, 1989). Coccolith production occurs in a coccolith vesicle, a separate compartment considered to be derived from the Golgi apparatus and probably associated with the nuclear envelope during the initial stage of calcification in \textit{E. huxleyi} (Westbroek et al., 1989; Pienaar, 1994; de Vrind-de Jong and de Vrind, 1997). Electron microscopy studies indicate that the coccolith vesicle disappears during cell division (Linschooten et al., 1991). The cessation or decrease in cell division may be needed to promote coccolith production, as suggested by previous reports (Paasche and Brubak, 1994; Paasche, 1998).

One study has suggested relationship between cold stress and \( P_i \) deficiency in photosynthetic organisms; it showed that low temperatures lead to rapid accumulation of phosphorylated metabolites and the depletion of \( P_i \) (Hurry et al., 1994). The intracellular status of phosphate metabolism is expected to integrate calcification via such processes.
The intracellular status of phosphate should be investigated to elucidate the P_i-deficient induction of calcification.

The enhancement of coccolith production by low temperatures under P_i-limiting conditions may be one of the key factors as to why coccolithophore blooms have been observed in the ocean at high latitudes where the temperature is low. Further studies are necessary to elucidate the detailed molecular mechanism that allows cold stress and phosphate deficiency to regulate coccolith production. Studying the regulatory mechanisms will also provide insight into the physiological role of coccolithophorean coccoliths.
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References


Figure legends:

**Figure 1.** Time courses of $^{45}$Ca-incorporation into the coccoliths on the cell surface and in the cytosolic space of *Emiliania huxleyi*.

A, $^{45}$Ca-incorporation into coccoliths (closed circles) and protoplasts (open circles). B, Ratio of $^{45}$Ca incorporation into coccoliths vs. whole cells. Temperature for cell growth and experiments was 20ºC.

**Figure 2.** Changes in inorganic phosphate concentration, alkaline phosphatase activity, and calcification during growth of *Emiliania huxleyi*.

The cells were grown at 20ºC and then half of the culture was transferred to 12ºC (open symbols) while the remainder was maintained at 20ºC as a control (closed symbols) at 0 h (vertical line). A, Cell numbers (circles) and inorganic phosphate concentration in the medium (squares). B, Alkaline phosphatase (AP) activity. C, $^{45}$Ca incorporation into whole cells.

**Figure 3.** Effect of phosphate on alkaline phosphatase activity and calcification in *Emiliania huxleyi*.

A, Cells pre-grown in Pi-sufficient culture at 20ºC were transferred to Pi-sufficient
(closed circles) and -deficient (open circles) conditions at time 0. B, Dibasic potassium phosphate solution was added (final concentration, 27.8 µM) to cells grown in Pi-deficient medium for 2.8 d. In both A and B: a, Cell number. b, Alkaline phosphatase (AP) activity. c, \(^{45}\)Ca incorporation into whole cells.

**Figure 4.** Combined effect of phosphate deprivation and cold stress on calcification by *Emiliania huxleyi.*

The cells were grown at 20ºC and then transferred to 12ºC under phosphate-sufficient (closed symbols) or -deficient (open symbols) conditions at 0 h (vertical line). For phosphate-sufficient culture, the phosphate concentration was maintained above 20 µM by the addition of dibasic potassium phosphate solution. For phosphate-deficient culture, phosphate concentration in the medium was rapidly decreased. A, Changes in cell number (circles) and inorganic phosphate concentration in the medium (squares). B, Alkaline phosphatase (AP) activity. C, \(^{45}\)Ca incorporation into cells.

**Figure 5.** Microscopic observations of *Emiliania huxleyi* cells grown at 20ºC and 13ºC under phosphate-sufficient and -deficient conditions.

Cells in logarithmic growth at 20ºC were transferred to 13ºC under phosphate-sufficient
and -deficient conditions at 0 h (vertical line). Inorganic phosphate concentration in the medium was quantified every day and dibasic potassium phosphate solution was added periodically to maintain phosphate-sufficient conditions. A portion of the culture was maintained at 20°C as a control. (A), Changes in cell number. Closed triangles, 20°C without a supply of phosphate (control); Open triangles, 13°C without a supply of phosphate; Open circles, 13°C with supply of phosphate. (B), Photographs taken under bright-field microscopy (left) and polarization microscopy (right). a, Cells in logarithmic growth (time 0); b and c, Cells grown at 20°C and 13°C for 4 d without a supply of phosphate (phosphate-deprived conditions), respectively; d, Cells grown at 13°C for 4 d with a supply of phosphate (phosphate-sufficient conditions). Magnification of photographs is the same in a–d. Scale bar, 5 µm.
Fig. 1

A

$^{45}\text{Ca}$ Incorporation 
\[ \text{[umol}(10^7 \text{ cells})^{-1}] \]

Incubation period (d)

B

$^{45}\text{Ca}$ in the coccoliths 
\[ \% \text{ of whole cells} \]

Incubation period (d)
Fig. 2

![Graph showing cell number, AP activity, and Ca^2+ incorporation over time.](image-url)

- **Cell number**: Cell number (x10^7 mL^-1)
- **AP activity**: AP activity [PNP umol (10^7 cells)^(-1) h^-1]
- **Ca^2+ Incorporation**: Ca^2+ Incorporation [μmol (10^7 cells)^(-1)]

**Incubation period (d)**: -2 -1 0 1 2 3 4 5 6

**[P] in medium (μM)**: 0 10 20 30 40

Legend:
- Solid line
- Dotted line
Fig. 3

A

Cell number (x10^7 mL^-1)

AP activity [PNP µmol (10^7 cells) h^-1]

[^45]Ca Incorporation [µmol (10^7 cells) h^-1]

Incubation period (d)

B

Cell number (x10^7 mL^-1)

AP activity [PNP µmol (10^7 cells) h^-1]

[^45]Ca Incorporation [µmol (10^7 cells) h^-1]

Incubation period (d)
Fig. 4

![Graph showing cellular growth, enzyme activity, and calcium incorporation over time.](image)
Fig. 5

A

![Graph showing cell number (x 10^7 mL^{-1}) vs. incubation period (d)]

B

![Images of cells labeled a, b, c, and d]

- **a**: Cell shape in light microscopy.
- **b**: Cell fluorescence under light microscopy.
- **c**: Cell morphology in light microscopy.
- **d**: Cell fluorescence under light microscopy.