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8 Stimulation of Calcification by Cold Stress in *Emiliania*
9
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11 phosphate-deprivation
12

13 **Cold Stress Stimulates Intracellular Calcification by the Coccolithophore, *Emiliania*
14 *huxleyi* (Haptophyceae) under Phosphate-Deficient Conditions**
15

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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 μM) 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

40 coccoliths on the cell surface under bright-field and polarization microscopy. These
41 results indicate that phosphate deprivation is the primary factor for stimulating coccolith
42 production, and cold stress is a secondary acceleration factor that stimulates calcification
43 under conditions of phosphate deprivation.

44

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

47

48 ***Introduction***

49 Coccolithophores, marine unicellular algae classified in the division Haptophyta, produce
50 huge blooms in the ocean. Moreover, they have gained a great deal of attention as
51 important players in charge of the global climate and the carbon cycle. These algae are
52 capable of fixing carbon by both photosynthesis to produce organic matter and
53 calcification to produce $CaCO_3$ crystals. Calcified shells of coccospheres consist of
54 calcareous blocks with complex shapes and are called coccoliths. *Emiliania huxleyi* is the
55 most cosmopolitan coccolithophore species and is used for numerous comparative
56 physiological studies as it grows well in laboratory culture (Winter et al., 1994; Paasche,
57 2002).

58 When grown under natural conditions in the ocean, *E. huxleyi* is the most
59 temperature-tolerant coccolithophore species (1–30°C) (Okada and McIntyre, 1979).
60 Such tolerance to wide temperature range is from data of various species that have
61 different temperature-dependence because of different environmental conditions of
62 isolation sites. Those various species were isolated from different waters. In the
63 laboratory, the maximum growth rate of *E. huxleyi* isolated in the Great Barrier Reef
64 occurs at 25°C and no growth was observed at 10°C. The sizes of protoplasts and
65 coccospheres increase when the growth temperature decreases. Quantitative analysis

66 revealed that coccolith production was enhanced after a lag period when the cells were
67 transferred from 20°C to 10°C in batch culture (Sorrosa et al., 2005).
68 Low temperatures also stimulate the degree of saturation and the amount of alkenones
69 (C_{37}–C_{39} long-chain ketones) in *E. huxleyi*. A number of unsaturated bond in the molecule
70 changes depending on the growth temperature. Thus, the degree of C_{37} alkenone
71 saturation has been used as an assessment of palaeotemperature in organic geochemistry
72 (Brassell et al., 1986; Prahl and Wakeham, 1987). Whether or how alkenone production
73 is related to coccolith production, however, is still unknown, although both processes are
74 stimulated at low temperatures.
75 Coccolith production is also regulated by other environmental factors. It was
76 significantly enhanced by acute phosphorous shortage accompanied by growth cessation,
77 but the ratio of carbon deposited in coccoliths to carbon converted to organic matters by
78 photosynthesis (C/P ratio) was increased by phosphate deficiency. Moreover, coccolith
79 production ceased immediately upon the re-addition of phosphate (Paasche, 1998).
80 Phosphate limitation also triggered an increase in alkaline phosphatase (AP) activity,
81 which is recognized as an indicator of phosphate deficiency in phytoplankton (Perry,
82 1972; Dyhrman and Palenik, 1999; Hoppe, 2003). *E. huxleyi* possesses two APs, one of
83 which is constitutive and the other inducible by phosphate deficiency (Riegman et al.,

84 2000). When the latter was localized to the cell surface and sequenced, it demonstrated no
85 significant similarity to previously sequenced APs (Dyhrman and Palenik, 2003; Xu et al.,
86 2006).

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

92

93 ***Materials and Methods***

94 ***Organism and Culture Conditions***. *E. huxleyi* (NIES 873) coccolithophore cells were
95 grown in artificial seawater (Marine Art SF; produced by Tomita Seiyaku Co., Ltd.,
96 Tokushima, formerly distributed by Senju Pharmaceutical Co., Osaka, Japan, and
97 recently by Osaka Yakken Co. Ltd., Osaka) and enriched with Erd–Schreiber's medium
98 containing 10 nM sodium selenate instead of soil extracts (Danbara and Shiraiwa, 1999).
99 Cells were maintained under constant illumination at 100 μmol m^{-2} s^{-1} and 20°C
100 (standard condition). Cells in early linear growth phase were transferred to 12° or 13°C
101 for low temperature treatments.

102

103 ***Assays*.** The inorganic phosphate concentration in the medium was assessed by the

104 molybdenum blue method (Murphy and Riley, 1962). Cells were removed from an

105 aliquot of the culture medium by filtration before assessment.

106 AP activity was measured according to Reichardt et al. (1967) using *p*-nitrophenyl

107 phosphate (*p*-NPP) as a substrate. Then 100 µL of 36 mM *p*-NPP and 700 µL of 200 mM

108 3-(cyclohexylamino)-1-propane sulfonic acid (CAPS) buffer at pH 10.0 were added to

109 200 µL of the cell suspension and incubated at 40°C for 15 min. The reaction was

110 terminated by the addition of 100 µL 4 M NaOH. The absorbance at 397 nm was

111 measured using a UV-VIS spectrophotometer (UV mini 1240; Shimadzu, Kyoto, Japan).

112

113 ***^{45}Ca Tracer Experiments for Calcification*.** First, 4.2 kBq/mL of $^{45}CaCl_2$ (692.69

114 MBq/mg; Perkin-Elmer, Waltham, MA) was injected to initiate the ^{45}Ca-labeling reaction.

115 At intervals 400 µL of culture was harvested and the cells collected by centrifugation

116 (10,000 × *g*, 5 min). The pellet was washed with fresh medium and transferred to a

117 scintillation vial containing 0.5 mL of scintillation cocktail, Hionic-Fluor (Perkin-Elmer).

118 The radioactivity incorporated into the coccosphere and the cytosolic space together was

119 determined, the cells harvested by filtration using an Ultrafree-MC filter (pore size, 0.65

120 µm; Millipore, Billerica, MA), and the coccosphere was removed by washing in medium
121 adjusted to pH 3.0 with HCl. Naked cells obtained on the filter were washed twice with
122 the acidic medium and the radioactivity determined using a liquid scintillation counter
123 (LS 5000TD; Beckman, Fullerton, CA).
124
125 ***Microscopic Observation.*** Cell number was determined by counting under a
126 microscope (BX50; Olympus, Tokyo, Japan). Coccoliths surrounding the cells were
127 observed with polarized light, and micrographs were taken using a microscope (DMR;
128 Leica, Wetzlar Germany) equipped with a fluorescence microscope digital camera
129 (Keyence, Osaka, Japan).
130
131 ***Results***
132 ***Distribution of ^{45}Ca Incorporated into Coccoliths and the Cytosolic Space of E. huxleyi***
133 ***Cells.*** Figure 1 shows that ^{45}Ca incorporated into *E. huxleyi* cells was primarily
134 distributed to the coccospheres. During the initial 10 h of growth, less than 90% was
135 incorporated, but the amount gradually increased to over 97% over 2 d (Figure 1). The
136 results demonstrated that the amount of ^{45}Ca incorporated by whole cells can be
137 considered as that used for calcification including coccolith production.

138

139 **Figure 1**

140

141 ***Effects of Phosphate Concentration and Cold Stress on Calcification by E. huxleyi.***

142 Growth of *E. huxleyi* was suppressed when the temperature was suddenly lowered from

143 20° to 12°C during logarithmic phase growth. The cells reached stationary phase

144 following an initial 2 d lag in growth (Figure 2).

145

146 Orthophosphate added to the medium (final concentration, 28.7 μM) was depleted in

147 the batch culture during 2.5 d at 20°C, although the rate of Pi depletion and time needed

148 for P*i*-depletion changed depending on pre-culture conditions and initial cell density. The

149 decrease in temperature to 12°C suppressed growth and caused inorganic phosphate (P_i)

150 depletion and an increase in AP activity. Extracellular AP, known to be synthesized *de*

151 *novo* under P_i deprivation (Xu et al., 2006), began to increase 4 d after the initiation of

152 culture, irrespective of temperature change. It took 1–2 d to initiate AP induction after P_i

153 was removed from the medium. The increase in AP activity was less at 12°C than that at

154 20°C, although the difference was small, suggesting that the timing of the enzyme

155 induction is mainly regulated by P_i availability in the medium and the extent is largely

156 affected by temperature. Coccolith production, however, was greatly stimulated at low
157 temperatures, and 6 d after exposure to cold stress, the amount of ^{45}Ca incorporation was
158 about five times higher at 12°C than at 20°C. Notably, calcification was initiated about 1
159 d earlier than the induction of AP (Figure 2C). When *Emiliania* cells were suspended in
160 the medium with sufficient-Pi and without addition of Pi, cell growth ceased about 2 and
161 3 d after the initiation of culture, respectively. The increase in AP activity that is due to *de*
162 *novo* synthesis by Pi-limitation was obvious at 3 d in Pi-limited culture but very little in
163 Pi-sufficient culture. Similarly, calcification, expressed as ^{45}Ca-incorpotation, was
164 stimulated in Pi-limited culture but no or less in Pi-sufficient culture (Figure 3A). The
165 stimulation of AP and calcification by phosphate deprivation was negated by the addition
166 of phosphate to the medium following a transient increase in AP and a lag of several hours
167 in calcification, respectively (Figure 3B).
168
169 **Figure 2**
170
171 **Figure 3**
172
173 AP activity and ^{45}Ca incorporation of calcification clearly increased when cells were

174 transferred to P_i-deficient conditions at low temperature (Figure 4). When cells were
175 transferred from 20° to 12°C, cell growth was not significantly affected by P_i availability.
176 AP activity and calcification, however, were greatly stimulated by P_i-deficiency. ^{45}Ca
177 incorporation increased slightly but then ceased altogether until day 3 at 12°C, even under
178 phosphate-sufficient conditions, although AP was not induced (Figure 4C). This suggests
179 that cold stress itself may also function as a trigger for calcification since the calcification
180 began prior to AP induction when the cells were exposed to both stressors. Calcification
181 was primarily regulated by P_i availability while cold stress functioned as a stimulating
182 factor (Figure 4).
183
184 **Figure 4**
185
186 The physiological data on coccolith production was confirmed by morphological
187 observations under bright-field and polarizing microscopes (Figure 5). The cells in
188 logarithmic growth phase had almost no coccoliths and were approximately 3–4 μm in
189 diameter (Figure 5B-a). When the culture was allowed to grow until it became
190 P_i-deprived at constant temperature, the cells began to increase in size to 5 μm, but the
191 production of coccoliths on the cell surface was very low (Figure 5B-b). In contrast, *E.*

192 *huxleyi* transferred to low temperature (13°C) under P_i-deficient conditions increased its
193 cell size and produced many coccoliths (Figure 5B-c). However, the cells grown at the
194 lower temperature under phosphate-sufficient conditions produced no coccoliths on the
195 cell surface, although cell size increased similarly to cells grown in phosphate-deficient
196 medium (Figure 5B-d). As *Emiliania* cells transferred to 13°C increased slightly in cell
197 size (Figure 5B-d), the calcification process may be proceeding even though it is not yet
198 observable under the microscope. These data demonstrate the significant stimulatory
199 effect of cold stress on coccolith production in the coccolithophore *E. huxleyi* under
200 P_i-deficient conditions.
201
202 **Figure 5**
203
204 ***Discussion***
205 *E. huxleyi* is phylogenetically closely related to *Isochrysis galbana*, a
206 non-coccolith-producing haptophyte (Edvardsen et al., 2000; Fujiwara et al., 2001). Our
207 previous results demonstrated that incorporation of ^{45}Ca by *I. galbana* was several
208 hundred times less than that by *E. huxleyi* even though the cell sizes are nearly equivalent
209 (Sorrosa et al., 2005). The data presented here in Figure 1 confirm that ^{45}Ca absorbed by

210 *E. huxleyi* cells is primarily used for coccolith production and that only a tiny percentage

211 of the ^{45}Ca remains inside the cells themselves. This suggests that haptophyte algae

212 require a high concentration of calcium that is primarily used for coccolith production and

213 not for other cellular activities. Therefore, the total ^{45}Ca incorporated by *E. huxleyi* can be

214 thought of as a quantitative measure of coccolith production. Coccolith production by a

215 coccolithophore such as *E. huxleyi* was confirmed to be a mechanism for the storage of

216 huge amounts of calcium and inorganic carbon.

217 P_i limitation is known to increase the ratio of carbon deposition into coccoliths

218 produced by calcification to organic materials produced by photosynthesis, expressed as

219 the C/P ratio (Paasche and Brubak, 1994; van Bleijswijk et al., 1994). Moreover, an

220 increase in temperature from 10° to 15°C was reported to lead to a slight increase in the

221 C/P ratio since calcification was stimulated and the production of organic matter was

222 slightly reduced (van Bleijswijk et al., 1994). By estimating calcification activity using

223 the incorporation of ^{45}Ca into coccoliths, the present study clearly showed that P_i

224 deprivation induces both AP activity and coccolith production (Figures 2, 3), as

225 previously observed by Paasche (1998) and Riegman et al. (2000). AP was shown to

226 localize on the cell surface in *E. huxleyi* by biochemical and gene analyses (Landry et al.,

227 2006; Xu et al., 2006). In addition, our results clearly show that coccolith production can

228 be triggered by a low temperature signal prior to the AP induction when cells are exposed
229 to the double stresses of cold and P_i limitation (Figure 4). Calcification was greatly
230 stimulated when both a cold stress and a P_i-deficient signal were given simultaneously
231 (Figures 4, 5).

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

242 Cold stress on a plant leads to the modification of metabolism in two ways. The first is
243 through a mechanism that improves stress tolerance, and the second adjusts metabolic
244 processes to the consequences of cold stress (Guy et al., 2008). Cold stress is known to
245 induce the expression of a desaturase and to enhance the production of unsaturated fatty

246 acids in order to increase membrane flexibility at low temperatures (Wada et al., 1994;
247 Los et al., 1997). In this case, the physiological function of the increase in the desaturase
248 activity is very clear. The physiological importance of the stimulation of coccolith
249 production under cold and P_i-deficient conditions, however, remains to be elucidated.

250 P_i limitation generally induces the suppression of photosynthetic carbon metabolism
251 by reducing the amount of phosphate compounds and the activities of some enzymes in
252 the plastid C_3 cycle, including via ATP synthesis (Rao and Terry, 1989). Coccolith
253 production occurs in a coccolith vesicle, a separate compartment considered to be derived
254 from the Golgi apparatus and probably associated with the nuclear envelope during the
255 initial stage of calcification in *E. huxleyi* (Westbroek et al., 1989; Pienaar, 1994; de
256 Vrind-de Jong and de Vrind, 1997). Electron microscopy studies indicate that the
257 coccolith vesicle disappears during cell division (Linschooten et al., 1991). The cessation
258 or decrease in cell division may be needed to promote coccolith production, as suggested
259 by previous reports (Paasche and Brubak, 1994; Paasche, 1998).

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

264 The intracellular status of phosphate should be investigated to elucidate the P_i-deficient
265 induction of calcification.
266 The enhancement of coccolith production by low temperatures under P_i-limiting
267 conditions may be one of the key factors as to why coccolithophore blooms have been
268 observed in the ocean at high latitudes where the temperature is low. Further studies are
269 necessary to elucidate the detailed molecular mechanism that allows cold stress and
270 phosphate deficiency to regulate coccolith production. Studying the regulatory
271 mechanisms will also provide insight into the physiological role of coccolithophorean
272 coccoliths.
273

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280

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393

394 **Figure legends:**

395 **Figure 1.** Time courses of ^{45}Ca-incorporation into the coccoliths on the cell surface and in

396 the cytosolic space of *Emiliania huxleyi*.

397 A, ^{45}Ca-incorporation into coccoliths (closed circles) and protoplasts (open circles). B,

398 Ratio of ^{45}Ca incorporation into coccoliths *vs*. whole cells. Temperature for cell growth

399 and experiments was 20ºC.

400

401 **Figure 2.** Changes in inorganic phosphate concentration, alkaline phosphatase activity,

402 and calcification during growth of *Emiliania huxleyi*.

403 The cells were grown at 20ºC and then half of the culture was transferred to 12ºC (open

404 symbols) while the remainder was maintained at 20ºC as a control (closed symbols) at 0 h

405 (vertical line). A, Cell numbers (circles) and inorganic phosphate concentration in the

406 medium (squares). B, Alkaline phosphatase (AP) activity. C, ^{45}Ca incorporation into

407 whole cells.

408

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

410 *Emiliania huxleyi*.

411 A, Cells pre-grown in P*i*-sufficient culture at 20ºC were transferred to P*i*-sufficient

412 (closed circles) and -deficient (open circles) conditions at time 0. B, Dibasic potassium

413 phosphate solution was added (final concentration, 27.8 μM) to cells grown in

414 P*i*-deficient medium for 2.8 d. In both A and B: a, Cell number. b, Alkaline phosphatase

415 (AP) activity. c, ^{45}Ca incorporation into whole cells.

416

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

418 *Emiliania huxleyi*.

419 The cells were grown at 20ºC and then transferred to 12ºC under phosphate-sufficient

420 (closed symbols) or -deficient (open symbols) conditions at 0 h (vertical line). For

421 phosphate-sufficient culture, the phosphate concentration was maintained above 20 μM

422 by the addition of dibasic potassium phosphate solution. For phosphate-deficient culture,

423 phosphate concentration in the medium was rapidly decreased. A, Changes in cell

424 number (circles) and inorganic phosphate concentration in the medium (squares). B,

425 Alkaline phosphatase (AP) activity. C, ^{45}Ca incorporation into cells.

426

427 **Figure 5.** Microscopic observations of *Emiliania huxleyi* cells grown at 20ºC and 13ºC

428 under phosphate-sufficient and -deficient conditions.

429 Cells in logarithmic growth at 20ºC were transferred to 13ºC under phosphate-sufficient

430 and -deficient conditions at 0 h (vertical line). Inorganic phosphate concentration in the

431 medium was quantified every day and dibasic potassium phosphate solution was added

432 periodically to maintain phosphate-sufficient conditions. A portion of the culture was

433 maintained at 20ºC as a control. (A), Changes in cell number. Closed triangles, 20ºC

434 without a supply of phosphate (control); Open triangles, 13ºC without a supply of

435 phosphate; Open circles, 13ºC with supply of phosphate. (B), Photographs taken under

436 bright-field microscopy (left) and polarization microscopy (right). a, Cells in logarithmic

437 growth (time 0); b and c, Cells grown at 20ºC and 13ºC for 4 d without a supply of

438 phosphate (phosphate-deprived conditions), respectively; d, Cells grown at 13ºC for 4 d

439 with a supply of phosphate (phosphate-sufficient conditions). Magnification of

440 photographs is the same in a–d. Scale bar, 5 µm.

Fig. 1

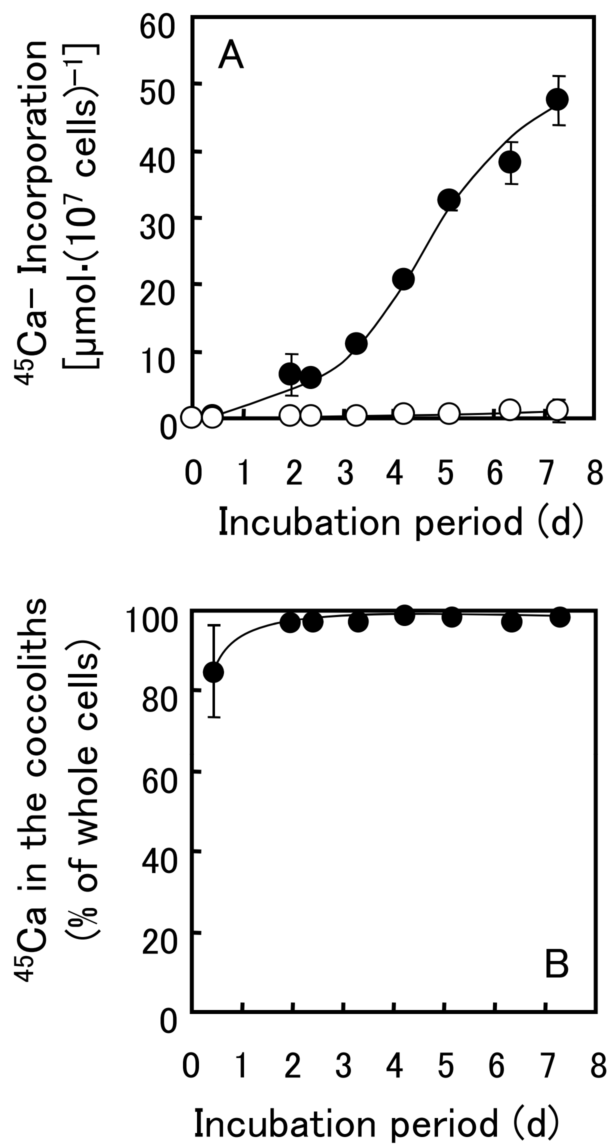

Fig. 2

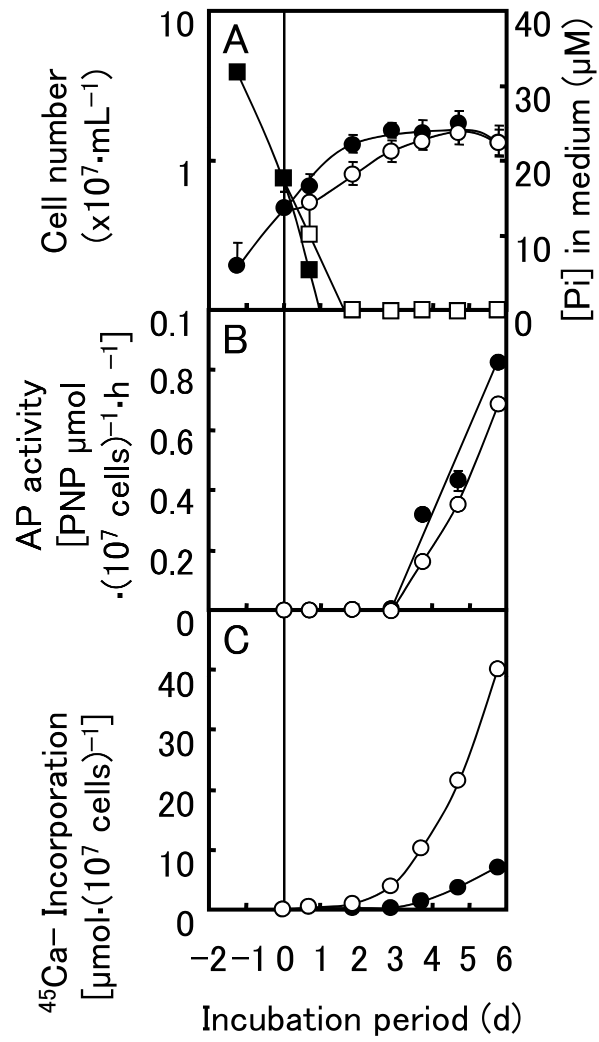

Fig. 3

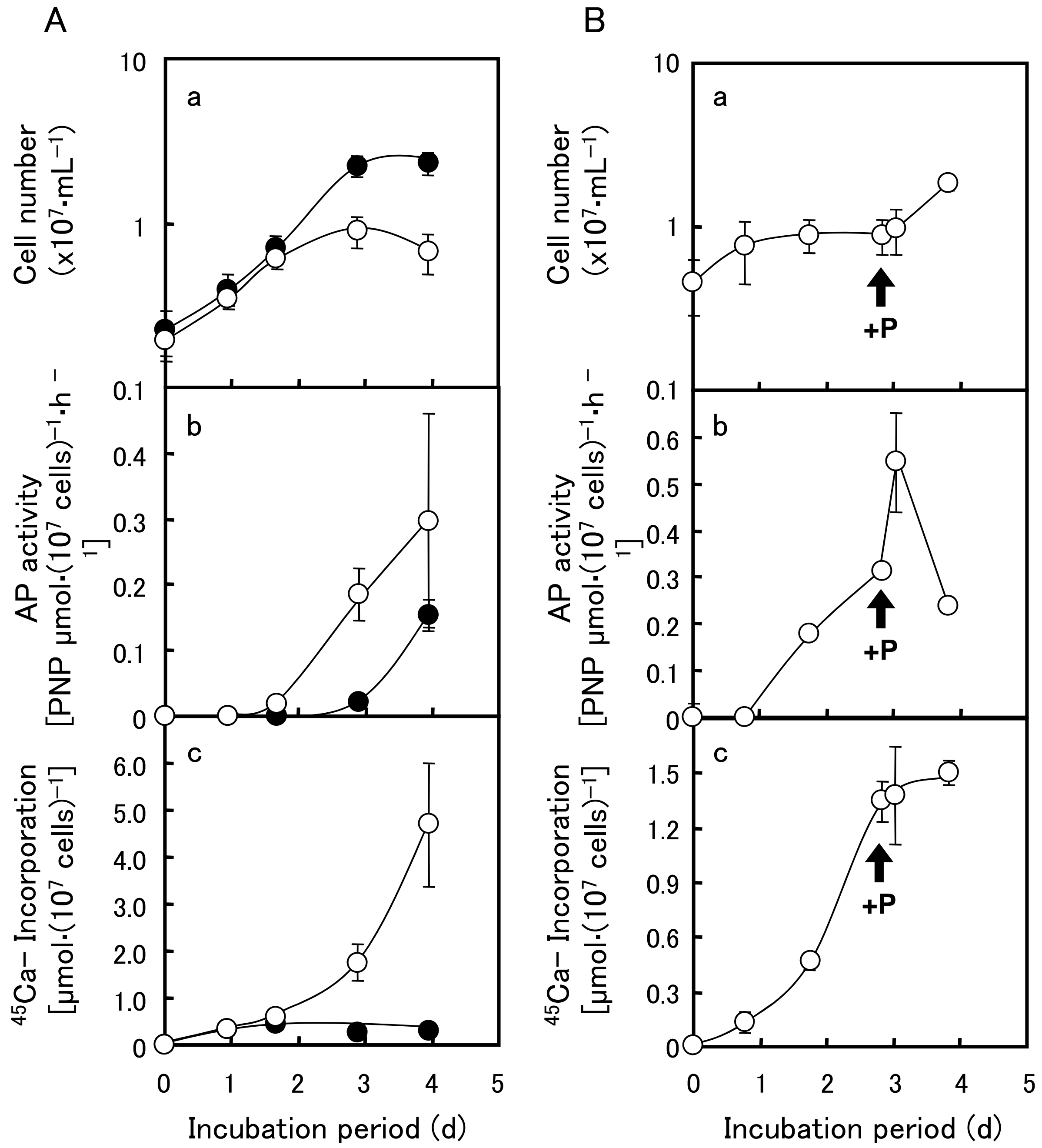

Fig. 4

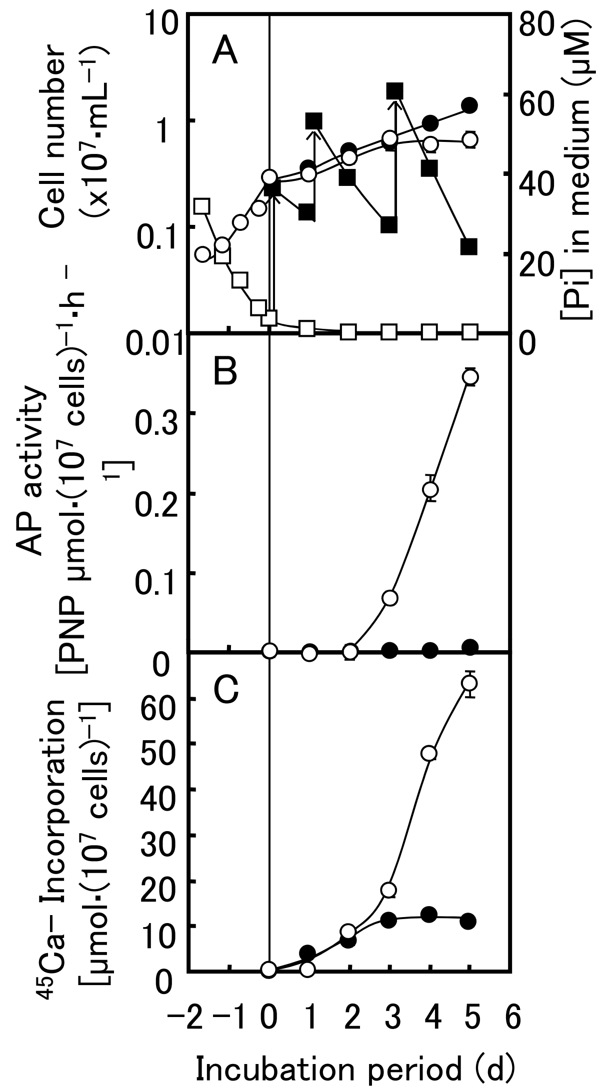

Fig. 5