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CO_2-CONCENTRATING MECHANISMS OF THE POTENTIALLY TOXIC DINOFLAGELLATE *PROTOCERATIUM RETICULATUM* (DINOPHYCEAE, GONYAULACALES)[1]

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The low CO_2 concentration in seawater poses severe restrictions on photosynthesis, especially on those species with form II RUBISCO. We found that the potentially toxic dinoflagellate *Protoceratium reticulatum* Clap. et J. Lachm. possesses a form II RUBISCO. To cast some light on the mechanisms this organism undergoes to cope with low CO_2 availability, we compared cells grown at atmospheric (370 ppm) and high (5000 ppm) CO_2 concentrations, with respect to a number of physiological parameters related to dissolved inorganic carbon (DIC) acquisition and assimilation. The photosynthetic affinity for DIC was about one order of magnitude lower in cells cultivated at high [CO_2]. End-point pH-drift experiments suggest that *P. reticulatum* was not able to efficiently use HCO_3^- under our growth conditions. Only internal carbonic anhydrase (CA) activity was detected, and its activity decreased by about 60% in cells cultured at high [CO_2]. Antibodies raised against a variety of algal CAs were used for Western blot analysis: *P. reticulatum* extracts only cross-reacted with anti-ß-CA sera, and the amount of immunoreactive protein decreased in cells grown at high [CO_2]. No pyrenoids were observed under all growth conditions. Our data indicate that *P. reticulatum* has an inducible carbon-concentrating mechanism (CCM) that operates in the absence of pyrenoids and with little intracellular CO_2 accumulation. Calculations on the impact of the CA activity to photosynthetic growth [CO_2] suggest that it is an essential component of the CCM of *P. reticulatum* and is necessary to sustain the photosynthetic rates observed at ambient CO_2.

Key index words: **carbonic anhydrase; CCM; elevated CO_2; HCO_3^-; photosynthesis; pyrenoid; RUBISCO II**

Abbreviations: **^{14}C-DMO, 5,5-dimethyl-2[^{14}C]oxalidine 2,4-dione; CA, carbonic anhydrase; CCM, carbon-concentrating mechanism; DIC, dissolved inorganic carbon; eCA, extracellular carbonic anhydrase; iCA, intracellular carbonic anhydrase; pCO_2, partial pressure of CO_2; PFD, photon flux density; PVP-40, polyvinylpyrrolidone 40**

The enzyme RUBISCO catalyzes the primary fixation of inorganic carbon (Calvin and Benson 1948, Bassham and Calvin 1957, Bowes et al. 1971). Because of the relatively high $K_M^{CO_2}$ (half-saturation constant for CO_2) values of RUBISCO (Raven 1997a) and the low CO_2 concentration in seawater (11 $\mu mol \cdot L^{-1}$ at 20°C, a salinity of 30 PSU, in equilibrium with a gas phase containing 370 ppm CO_2), photosynthesis in phytoplankton may suffer from CO_2 limitation. This limitation may be especially important in phytoplankton with form II RUBISCO, which is usually characterized by a higher $K_M^{CO_2}$ and a lower selectivity factor (τ) than form I RUBISCO (Whitney and Andrews 1998, Giordano et al. 2005). Most microalgae overcome or reduce this limitation by activating carbon-concentrating mechanisms (CCMs; Giordano et al. 2005). A typical biophysical CCM in eukaryotic microalgae includes systems for uptake of inorganic carbon (Amoroso et al. 1998) and carbonic anhydrases (CAs), localized in the periplasmic space and/or inside the cell, which catalyze the reversible dehydration of HCO_3^- to CO_2 (Badger and Price 1992, Badger and Price 1994, Giordano et al. 2005). The synergic action of all these elements typically leads to an accumulation of inorganic carbon inside the cell (Badger et al. 1998, Giordano et al. 2005).

Despite the large amount of information available for CCMs for some species, many microalgae have been largely overlooked in this respect. With rare exceptions (Berman-Frank et al. 1998, Leggat et al. 1999, Nimer et al. 1999a, Leggat et al. 2002), dinoflagellates are among these. This fact is unfortunate, because dinoflagellates are quantitatively a major component of phytoplankton in all oceans (Taylor and Pollingher 1987), and the toxic red-tide-forming species can have serious repercussions on coastal ecology and human communities (Yasumoto et al. 1985, Yasumoto et al. 1987, Maclean 1989,

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Murata et al. 1990, Burkholder et al. 2001, Goh et al. 2002, Ciminiello et al. 2003, Morono et al. 2003). The understanding of dinoflagellate CCMs may also provide insights into the pivotal symbiotic associations on which coral reefs are based (Rowan 1998, Goodson et al. 2001). Dinoflagellate intolerance to bubbling and the consequent difficulties in culturing them under different CO_2 regimes (Juhl and Latz 2002) may have hindered laboratory studies on the CCMs of these algae.

We here report on a study conducted on the dinoflagellate *Protoceratium reticulatum* cultured in a specially designed system that permits culturing under different CO_2 regimes without the direct and damaging bubbling of the cells. *Protoceratium reticulatum* is an important component of phytoplankton in the Adriatic Sea (Draisci et al. 1999, Ciminiello et al. 2003) and other regions of the world (Horstman 1980, Godhe and McQuoid 2003, Joyce 2004), where it is often associated with the presence of yessotoxin and its analogues and consequent diarrheic shellfish poisoning in humans (Yasumoto et al. 1985, Ciminiello et al. 2003). *Protoceratium reticulatum* contains peridinin as an accessory pigment, which, to date, has always been correlated with the presence of type II RUBISCO (Morse et al. 1995, Whitney and Yellowlees 1995, Whitney and Andrews 1998). To cast some light on the strategies that this organism adopts to cope with the limited CO_2 availability in the ocean, we compared cell responses to growth in equilibrium with a gas phase containing either atmospheric CO_2 concentration (370 ppm) or elevated CO_2 (5000 ppm).

MATERIALS AND METHODS

Cultures. The strain of *P. reticulatum* used for this study was kindly donated by L. Boni (University of Bologna, Bologna, Italy). Semicontinuous cultures were grown axenically in 1 L bottles filled to capacity with L1 seawater-based medium (Guillard and Hargraves 1993). The growth medium was buffered with 20 mmol·L^{-1} Tris-HCl, pH 8.1, and the nitrate concentration was increased from 0.83 to 2.5 mmol·L^{-1} in order to mitigate the unbalance in the external C:N ratio at the highest external [CO_2] used (see below). To avoid problems related to the well-known fragility of dinoflagellates to direct bubbling of the cultures (Juhl and Latz 2002), the medium was preequilibrated with a gas phase containing either 370 ppm (air) or 5000 ppm (enriched air) CO_2. The highest CO_2 concentration was obtained by mixing CO_2-free air and 100% CO_2 with a gas mixer (E-5700; Bronkhorst, Hi-Tec, Ruurlo, the Netherlands). Both gases were passed through a 17 cm × 6 cm column packed with silica gel to eliminate water vapor. The removal of water was necessary because the gas mixer worked on the basis of mass rather than gas volume. The growth medium was vigorously bubbled for 12 h with the gas mixture without any observable volume changes. The cells were then inoculated at a density of approximately 4000 cells·mL^{-1}, and the bottle was sealed. The CO_2 and dissolved inorganic carbon (DIC) in the medium were checked at the beginning and end of the experiments using an ADC 2250 Series infrared gas analyzer (ADC Bioscientific Ltd., Herds, UK) and were found to be initially at the expected level for the set pH and temperature (at 370 ppm: $CO_2 \approx 0.012$ mmol·L^{-1}, $HCO_3^- \approx 1.9$ mmol·L^{-1}, and DIC ≈ 2 mmol·L^{-1}; at 5000 ppm: $CO_2 \approx 0.16$ mmol·L^{-1}, $HCO_3^- \approx 25$ mmol·L^{-1}, and DIC ≈ 28 mmol·L^{-1}; see below for details on the calculations of DIC speciation) and not to vary appreciably during the experiments. Cultures were maintained at 20°C, under a continuous photon flux density (PFD) of 120 μmol photons·m^{-1}·s^{-1}, provided by cool-white fluorescent tubes. This light regime was chosen to avoid culture synchronization and after verifying that this treatment had no substantial effect on growth rate and cell morphology and ultrastructure. To verify whether pyrenoid formation was affected by the light regimes, some cultures were exposed to 12:12 light:dark (L:D) cycles. All experiments were carried out on cells in the exponential growth phase, allowed to grow at the given CO_2 concentration for at least four generations prior to any measurement. After a preliminary determination of the growth rate (see below), cultures for all subsequent experiments were diluted after a time corresponding to one doubling.

Growth rate determination. Cell density was estimated with a Sedgwick-Rafter chamber (McAlice 1971). The specific growth rate, μ, was derived by daily counts of exponentially growing cells acclimated for at least four generations to the CO_2 growth regime. Acclimated cells were inoculated at a cell density of approximately 500 cells·mL^{-1}, in 1 L of L1 medium preequilibrated with the appropriate gas phase. Growth rate determination was effected on a minimum of six distinct cultures for each condition. The daily opening of bottles for cell counts may cause a loss of CO_2 from the medium equilibrated with the high partial pressure of CO_2 (pCO_2) gas phase; we therefore designed a system that allowed us to withdraw samples from the bottles with a syringe, while adding the appropriate gas phase to the air space of the bottle.

Chlorophyll and total protein determination. Chlorophyll was extracted in a mixture of 20% (v/v) tetrahydrofuran and 80% (v/v) methanol, and the concentrations of total chl, chl *a*, and chl *c* were determined according to Jeffrey and Humphrey (1975). Total proteins were measured with the method described by Peterson (1977).

CA-activity assay. Cells in the midexponential growth phase were harvested by filtration on 11 μm nylon filters (Millipore, Billerica, MA, USA) and subjected to two sequential extractions to determine the distribution of CA activity between the soluble and insoluble fractions (Karlsson et al. 1995). The cells were resuspended in an extraction medium containing 300 mmol·L^{-1} Tris-borate (pH 8.36), 5 mmol·L^{-1} EDTA, 2.5 mmol·L^{-1} diothiothreitol (DTT), 2% (w/v) PVP-40, 0.1% (w/v) TRITON X-100, and 0.5% (w/v) BSA (Giordano and Maberly 1989) and lysed with a Ten-Broek homogenizer, on ice. The slurry was centrifuged for 7 min at 12,000*g* and 4°C, and the supernatant was used for soluble CA-activity determination. The pellet was subjected to a further extraction, in an extraction medium similar to the one described above, supplemented with 10 μmol·L^{-1} $ZnCl_2$ and 200 mmol·L^{-1} KCl (Karlsson et al. 1995). The suspension was stirred on ice for 2 h and then centrifuged for 7 min at 12,000*g* and 4°C. This supernatant was used for the determination of ''insoluble'' CA activity. Intact cells and crude cell extracts were assayed for extracellular CA (eCA) and total CA by the potentiometric method of Wilbur and Anderson (1948) as modified by Miyachi et al. (1983). The assay mixture contained 25 mmol·L^{-1} phosphate buffer, pH 8.36, and 10 μmol·L^{-1} $ZnCl_2$. In control experiments, cell extract and cell suspension were substituted with an equal volume of either phosphate buffer (for eCA) or boiled extract [for intracellular CA (iCA)].

Estimates of CA catalytic rates: The activity of iCA measured by the Wilbur–Anderson method was converted into rate of CO_2 hydration and HCO_3^- dehydration by an extension (U. Riebesell, personal communication) of the procedure described by Hatch and Burnell (1990). The CA activities (a_{CA})

expressed as catalytic rates of CO_2 hydration and HCO_3^- dehydration were determined according to the following equation:

$$a_{CA} = \frac{C_{buffer}}{t_s} - \frac{C_{buffer}}{t_o} = \text{mol}\, CO_2 \cdot s^{-1} \quad (1)$$

where C_{buffer} is the buffering capacity of the assay mix, and t_s and t_o are the times required for the chosen pH variation in the presence and absence of the sample, respectively. The buffering capacity of the solution was determined by titration with a standard HCl solution. Considering that $dH^+/dt = -dCO_2/dt$, a_{CA} units are mol $CO_2 \cdot s^{-1}$. The a_{CA} value, divided by the cell concentration in the reaction mixture, yields the rate in units of mol CO_2 catalyzed $\cdot$ cell$^{-1} \cdot s^{-1}$.

These catalytic rates, obtained at saturating CO_2, were converted to the catalytic rates in culture conditions by a three-step procedure. First, the expected activity for one unit of enzyme (a_{CAm}) was calculated from the Michaelis–Menten kinetics according to equation 2 (Pocker and Deits 1984):

$$a_{CAm} = \frac{k_{cat}^{CO_2}[CO_2]_{assay}}{K_M^{CO_2} + [CO_2]_{assay}} \quad (2)$$

where $k_{cat}^{CO_2}$ is the catalytic rate constant, $K_M^{CO_2}$ is the half-saturation constants, and $[CO_2]_{assay}$ is the CO_2 concentration used in the assay. The measured activity (a_{CA}) was then divided by the Michaelis–Menten predicted activity (a_{CAm}) to obtain the CA content per cell (C_{CA}). Finally, forward (CA-catalyzed hydration rate, $a_{CA}^{CO_2}$) and reverse (CA-catalyzed dehydration rate, $a_{CA}^{HCO_3^-}$) reaction rates in vivo were estimated from the C_{CA} activity using the following equations:

$$a_{CA}^{CO_2} = C_{CA}\frac{k_{cat}^{CO_2}[CO_2]}{K_M^{CO_2} + [CO_2]} \quad (3)$$

$$a_{CA}^{HCO_3^-} = C_{CA}\frac{k_{cat}^{HCO_3^-}[HCO_3^-]}{K_M^{HCO_3^-} + [HCO_3^-]} \quad (4)$$

where $k_{cat}^{CO_2}$ and $k_{cat}^{HCO_3^-}$ are the catalytic rate constants, $K_M^{CO_2}$ and $K_M^{HCO_3^-}$ are the half-saturation constants, and $[CO_2]$ and $[HCO_3^-]$ are the intracellular CO_2 and HCO_3^- concentrations. The catalytic rate constants used were those for higher plant intracellular β-CAs: $k_{cat}^{CO_2} = 4.6 \times 10^5 \cdot s^{-1}$ is that of *Pisum sativum* CA (Johansson and Forsman 1993); $k_{cat}^{HCO_3^-} = 3.4 \times 10^5 \cdot s^{-1}$ is that of *Spinacia oleracea* CA (Pocker and Miksch 1978). The K_M values used were those reported by Graham et al. (1984) for higher plant intracellular β-CAs, with typical values of about 30 mmol $\cdot$ L^{-1}. The effect of temperature on the rates was estimated assuming that the Q_{10} was equal to that for maize CA (Burnell and Hatch 1988).

PAGE and Western blotting. Cell-free extracts, obtained as described in the paragraph on CA-activity determination, were loaded on 12% (v/v) SDS-PAGE gels (Laemmli 1970) in a Mini-Protean III slab-gel apparatus (Bio-Rad, Hercules, CA, USA). After SDS-PAGE, gels were equilibrated for 10 min in a transfer buffer [200 mM $NaHCO_3$, 60 mM Na_2CO_3, 20% (v/v) methanol]; the polypeptides were then transferred electrophoretically to a nitrocellulose membrane (Extra Blotting Sartorius, Goettingen, Germany) at 250 mA for 4 h at 4°C, using a Mini Trans-Blot Cell (Bio-Rad). Immune reactions on the blots were visualized after incubation with a horseradish peroxidase-linked secondary antibody using enhanced chemoluminescence (ECL kit; Amersham Pharmacia Biotech, Piscataway, NJ, USA). The primary antibodies used for Western blots were raised against RUBISCO type I of *Chlamydomonas reinhardtii* P. A. Dang., RUBISCO type II of *Gonyaulax* sp., cytosolic β-CA of *Coccomyxa* sp. (CoCA) and mitochondrial β-CA of *C. reinhardtii* (mtCA; kindly donated by G. Samuelsson, Umeå University, Sweden), chloroplast β-CA of *Phaeodactylum tricornutum* Bohlin (PtCA; kindly donated by Y. Matsuda, Kwansei-Gakuin University, Sanda, Hyogo, Japan), and α-CA of *Thalassiosira weissflogii* (Grunow) G. Fryxell et Hasle (TwCA; kindly donated by F. M. M Morel, Princeton University, Princeton, NJ, USA). The hybridized membranes were scanned in reflection with a Sharp JX-300 scanner (Sharp Electronics Corporation, Mahwah, NJ, USA) and analyzed densitometrically with the Image Master 1D software (Amersham Biosciences).

Electron microscopy. Protoceratium reticulatum cells were taken from different culture conditions, washed with 0.4 M PBS, and fixed with 3% glutaraldehyde in 0.4 M PBS for 30 min without osmium to preserve antigenicity on the sections. The fixed cells were washed three times in PBS and water, dehydrated with a standard ethanol series, and embedded in LR White resin (EMS, Hatfield, PA, USA) polymerized for 24 h at 60°C in gelatin capsules. Thin sections were cut for TEM, poststained with uranyl acetate, and observed using a JEOL JEM 100S microscope (JEOL, Tokyo, Japan) operating at 80 kV. Antibodies raised against the cytosolic β-CA of *Coccomyxa* sp (anti-CoCA) or the chloroplastic β-CA of *Ph. tricornutum* (anti-PtCA) were tested for immunolabeling at concentrations (1/100 and 1/200 for anti-CoCA; 1/50 and 1/100 for anti-PtCA) roughly 10 times those used to obtain signals on Western blots. As a secondary antibody, 20 nm gold-labeled IgG (BBInternational, Cardiff, UK) was tested at 1/50, 1/100, and 1/200 dilutions; no specific labeling of the cell sections was observed under any condition with either antibody.

Gas-exchange measurements. Photosynthetic O_2 evolution was measured with a Clark-type O_2 electrode (Chloroview 2; Hansatech, King's Lynn, UK). Cells in the exponential growth phase were harvested by gravity filtration on 11 μm nylon filters (Millipore), resuspended at a density of 1.5–2 $\times$ 10^5 cell $\cdot$ mL^{-1} in DIC-free culture medium, placed in the O_2 electrode chamber, and stirred at the slowest rate allowed by the system to avoid mechanical damage to the cells. The chamber was closed, and the light turned on at a saturating (for growth and photosynthesis) PFD of 470 μmol photons $\cdot$ m$^{-2} \cdot$ s^{-1} provided by a red (660 nm) light-emitting diode source (Hansatech). The measurements were carried out at a temperature of 20°C. The cells were allowed to reach the CO_2 compensation point. The rate of O_2 evolution was then measured, following the sequential addition of incremental $NaHCO_3$ aliquots. Respiratory rates were determined in the same system by measuring O_2 consumption in the dark, 5 min after the onset of darkness.

pH-drift experiments. The pH-drift experiments were carried out to investigate whether *P. reticulatum* was able to use HCO_3^- as a source of DIC for photosynthesis. Cells in the exponential growth phase were harvested by gravity filtration on 11 μm nylon filters (Millipore) and resuspended, at a cell density of 4000 cells $\cdot$ mL^{-1}, in unbuffered growth medium. The initial pH of the medium was adjusted to 8.1 with HCl. Glass vials were filled to capacity (8 mL) with this cell suspension and closed tightly with screw caps. Cells were incubated in the same conditions used for growth until pH was stable (usually after 24 h of incubation).

DIC speciation. Inorganic carbon speciation was calculated using the dissociation constants in Goyet and Poisson (1989), the measured pH, temperature, and salinity, and the concentration of dissolved CO_2 calculated according to Henry's law for the appropriate conditions (Weiss 1974). For the pH drift experiments, the alkalinity was determined by Gran tritration, and the concentration of DIC species was calculated according to Maberly (1996), using the dissociation constants of Goyet and Poisson (1989) for the salinity of the growth medium.

Silicone oil centrifugation. The silicone oil centrifugation technique was used to estimate the intracellular concentration of inorganic carbon, the internal pH, and the cell volume. Cells in the exponential growth phase were treated as described in

the previous paragraph and allowed to reach the CO_2 compensation point in the O_2 electrode chamber. The intracellular concentration of DIC was determined by a slight modification of the method described by Leggat et al. (1999). Two-hundred microliters of a cell suspension with a density of 2.5×10^5 cell $\cdot$ mL^{-1} was transferred into a 400 μL tube (Starlab, Ahrensburg, Germany) containing 100 μL of silicone oil mixture (AR200:AR20 = 2:1; Sigma-Aldrich, St. Louis, MO, USA) layered over 20 μL of killing solution (1 M NaOH and 10% v/v MeOH). Cells were then preilluminated for 2 min with a saturating PFD of 470 μmol photons $\cdot$ m^{-2} $\cdot$ s^{-1}. Subsequently, a $NaH^{14}CO_3$ (Amersham, Little Chalfont, UK) solution with a working specific activity of 74 GBq $\cdot$ mol^{-1} was added to the cell suspension. Cells were incubated in the presence of this solution for 30 s, at the same PFD used for the preillumination, prior to being spun at 12000*g* for 20 s through the silicone oil mixture and into the killing solution. Total extracellular and intracellular volumes were calculated after a 1 h incubation in the presence of ^3H_2O (specific activity 162 KBq $\cdot$ mL^{-1}; Amersham) followed by a 10 s incubation with a ^{14}C-mannitol solution (specific activity 2.18 GBq $\cdot$ mmol^{-1}; Amersham; Badger et al. 1980). The intracellular pH was measured after 15 min of incubation with 14.8 KBq $\cdot$ mL^{-1} 5,5-dimethyl-2[^{14}C]oxalidine 2,4-dione (^{14}C-DMO; specific activity 1850 MBq $\cdot$ mmol^{-1}; Amersham; Badger et al. 1980). These incubation times were chosen after careful preliminary trials, to ensure that ^3H_2O and ^{14}C-DMO were in equilibrium between the inside and the outside of the cells and to minimize the risk of ^{14}C-mannitol entry into the cells.

Statistics. The data are expressed as the mean ± standard deviation of measurements obtained from at least three distinct cultures. Statistical significance of differences was determined with one-tailed *t*-test, with the significance level set at 0.05.

RESULTS

Growth and cell size, and chl and protein content. Specific growth rates were significantly higher in cells grown in high CO_2, with μ values 1.4-fold higher than those of cells grown in low CO_2 (Table 1). Neither cell volume (Table 1) nor protein or chl content (Table 2) was appreciably affected by the pCO_2 in the gas phase in equilibrium with the growth medium ($P > 0.05$).

Cell ultrastructure. Protoceratium reticulatum contained numerous chloroplasts, both adjacent to the nucleus and more peripherally (Fig. S1, a, see supplementary material), similar to plastids in *Amphidinium* (Jenks and Gibbs 2000) and *Gonyaulax* (Nassoury et al. 2001), respectively. However, unlike these other dinoflagellates, pyrenoids were not observed in the plastids of *P. reticulatum* grown under constant light at either atmospheric or elevated CO_2 concentration (Fig. S1, b and c, see supplementary material). Cells were also grown under L:D cycles, as these conditions have been observed to influence pyrenoid formation in other microalgae (Nassoury et al. 2001, Cook et al. 1976). However, no pyrenoid was observed in *P. reticulatum* cells cultured under these conditions (Fig. S1, d–g, see supplementary material). Cells grown under L:D cycles appeared to contain less vacuolar space and proportionally more cytoplasm (Fig. S1, see supplementary material), but time of day following entrainment to L:D cycles did not affect either the distribution of plastids within the cells (Fig. S1, d and f, see supplementary material) or the spacing between thylakoid stacks (Fig. S1, e and g, see supplementary material).

CA. To determine if photosynthetic performance could be correlated with CA activity, the activity of these enzymes was measured for both intact cells and crude extracts (Table 3). No CA activity was detected outside the intact cells or in the insoluble fraction of crude extracts, regardless of the CO_2 concentration used for growth. The only CA activity detected was associated with the soluble fraction of cell extracts (iCA): this activity, expressed in Wilbur–Anderson units, was 3-fold higher in cells acclimated to atmospheric CO_2 concentration than in those cultured in a medium equilibrated with a gas phase with a pCO_2 of 5000 ppm. Wilbur–Anderson units, however, are not satisfactory if the quantitative contribution of CA activity to photosynthesis and CCM is to be assessed. We therefore converted these units into catalytic rates and determined that the

TABLE 1. Specific growth rate (μ) and cell volume of *Protoceratium reticulatum* cells cultivated in media in equilibrium with gas phases containing either 370 or 5000 ppm of CO_2.

pCO_2 (ppm)	μ (d^{-1})	Cell volume (μm^3)
370	0.14 ± 0.06	2733 ± 919
5000	0.20 ± 0.02	2786 ± 215

The results are shown as the means ± SD of measurements carried out on six (growth rate) or three (cell size) distinct cultures. pCO_2, partial pressure of CO_2.

TABLE 2. Total chl, chl *a* and *c*, and total protein of *Protoceratium reticulatum* cells cultured in a media equilibrated with a gas phase containing either 370 or 5000 ppm of CO_2.

pCO_2 (ppm)	Total protein (ng $\cdot$ cell^{-1})	Chl *a*	Chl *c*	Total chl
		(pg $\cdot$ cell^{-1})		
370	0.80 ± 0.21	6.01 ± 0.68	2.82 ± 1.03	8.82 ± 1.45
5000	0.81 ± 0.27	5.87 ± 0.94	3.75 ± 2.93	9.62 ± 2.76

Values shown are the means ± SD of determination effected on five distinct cultures. pCO_2, partial pressure of CO_2.

TABLE 3. Extracellular and intracellular carbonic anhydrase (CA) activity in cells of *Protoceratium reticulatum* cultivated in equilibrium with gas phases containing either 370 or 5000 ppm CO_2.

pCO_2 (ppm)	eCA activity	Soluble iCA activity (WAU × 10^9 cell)	Insoluble iCA activity
370	n.d.	6.8 ± 1.1	n.d.
5000	n.d.	2.5 ± 2.3	n.d.

The values are the means ± SD of four independent replicates. eCA, extracellular carbonic anhydrase; iCA, intracellular carbonic anhydrase; pCO_2, partial pressure of CO_2; WAU, Wilbur–Anderson units; n.d., not detected.

TABLE 4. Catalytic rates of intracellular carbonic anhydrase (CA) in cells of *Protoceratium reticulatum* cultivated at either 370 or 5000 ppm of CO_2 in the gas phase.

pCO_2 (ppm)	$a_{CA}^{HCO_3^-}$* (pmol · cell^{-1} · s^{-1})	$a_{CA}^{CO_2}$* (pmol · cell^{-1} · s^{-1})	CA content (fmol · cell^{-1})
370	329 ± 188	0.62 ± 0.35	7.65 ± 4.40
5000	69.9 ± 70.1	0.20 ± 0.22	9.61 ± 0.10

$a_{CA}^{HCO_3^-}$, CA-catalyzed rates of HCO_3^- dehydration; $a_{CA}^{CO_2}$, CA-catalyzed rates of CO_2 hydration; pCO_2, partial pressure of CO_2. The values are the means ± SD (n = 4).

*Catalytic rates were calculated using CO_2 and HCO_3^- intracellular concentration values determined by silicon oil centrifugation experiments.

CA-catalyzed rate of HCO_3^- dehydration was greater than the CA-catalyzed rate of CO_2 hydration in both cells grown in low CO_2 and high CO_2 (Table 4; $P < 0.05$). More interestingly, the catalytic rate of HCO_3^- dehydration was 10-fold higher in cells grown in low CO_2 than in their counterparts grown in high CO_2 (Table 4; $P < 0.05$).

To confirm these findings, both the soluble fraction and the insoluble fraction of the extracts were subjected to immunoblot analysis using antibodies raised against CAs from different microalgae. No cross-reaction was observed with the insoluble fraction of *P. reticulatum* extracts. Instead, a 45 kDa polypeptide in the soluble fraction cross-reacted with both the antibodies raised against CoCA and those raised against PtCA (Fig. 1). No hybridization of *P. reticulatum* polypeptides was detected with antibodies directed against mtCA of *C. reinhardtii* and TwCA.

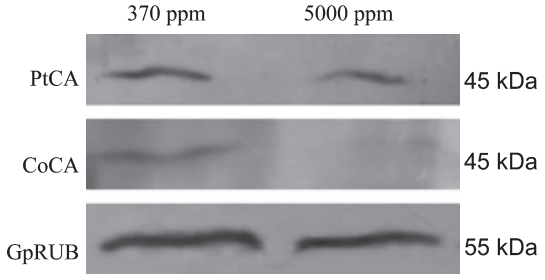

FIG. 1. Abundance of carbonic anhydrase (CA) protein in cells of *Protoceratium reticulatum* cultivated in medium equilibrated with gas phases containing either 370 or 5000 ppm of CO_2. The immunodetection was carried out with antibody directed toward the β-CA of *Phaeodactylum tricornutum* (PtCA; upper panel) and the β-CA of *Coccomyxa* sp. (CoCA; middle panel), and antibody raised against the form II RUBISCO of *Gonyaulax* sp. (GpRUB; lower panel) as a reference.

RUBISCO. Figure 1 shows a typical Western blot obtained with *P. reticulatum* crude extract hybridized with an antibody raised against RUBISCO type II of *Gonyaulax* sp. This analysis confirmed the presence of type II RUBISCO in *P. reticulatum*, as was expected on the basis of the presence of peridinin as an accessory pigment (Morse et al. 1995, Whitney and Yellowlees 1995, Whitney and Andrews 1998). The densitometric quantification of the hybridization bands afforded very similar results for cells grown at either air or elevated CO_2 concentration ($P > 0.05$). No immunological reaction was detected when *P. reticulatum* polypeptides were exposed to anti-*Chlamydomonas* type I RUBISCO serum.

Photosynthesis and respiration. Table 5 summarizes the main photosynthetic parameters for cells cultured in media equilibrated with either 370 or 5000 ppm CO_2. P_{max} remained essentially unaffected by CO_2 availability during growth ($P > 0.05$). The affinity of photosynthesis for CO_2, instead, was significantly ($P < 0.05$) higher in cells grown in low CO_2, whether expressed as $K_{1/2}$ (~7-fold lower) or as CO_2 conductance (4.5-fold higher). Furthermore, in cells grown in low CO_2, photosynthesis was saturated at a CO_2 concentration of 40 μmol · L^{-1}, which was almost 20% lower than the CO_2 concentration necessary to attain saturation of photosynthesis in cells grown at high CO_2 ($P < 0.05$). The CO_2 compensation points, however, were similar for cells cultured in the two CO_2 growth regimes (~0.2 μmol · L^{-1}; $P > 0.05$). Oxygen consumption rates were not influenced by the CO_2 available during growth (Table 5; $P > 0.005$).

pH-drift experiments. Cells grown in low CO_2 were able to increase the pH of their medium up to a maximum value of 8.32 ± 0.03 (Fig. 2). Alkalinity did not change significantly during the incubation and was equal to ~1.6 mEq · L^{-1}. On the contrary, the CO_2 concentration declined by 2.5-fold over the duration of the experiment, reaching a final concentration of 4.5 ± 3.1 μmol · L^{-1}. In addition, HCO_3^- (final concentration = 1.24 ± 0.44 mmol · L^{-1}) and DIC (final concentration = 1.40 ± 0.52 mmol · L^{-1}) decreased by about 2-fold (Fig. 2; $P < 0.05$). Cells grown in high CO_2 showed

TABLE 5. Photosynthetic parameters of *Protoceratium reticulatum* cells in response to changes in pCO_2 in the gas phase.

	P_{max}	R	$K_{1/2(DIC)}$	$K_{1/2(CO_2)}$	$\Gamma_{(DIC)}$	$\Gamma_{(CO_2)}$	$S_{(DIC)}$	$S_{(CO_2)}$	$\alpha_{(CO_2)}$	$\alpha_{(DIC)}$
pCO (ppm)	(pmol O_2 · h^{-1} · cell^{-1})		(μmol · L^{-1})						(pmol O_2 · h^{-1} · cell^{-1} · mmol^{-1} · DIC)	
370	3.14 ± 0.54	−2.59 ± 0.31	236 ± 198	1.66 ± 1.42	37.0 ± 17.1	0.20 ± 0.08	6000 ± 707	40.0 ± 7.07	2.10 ± 0.43	3.99 ± 126
5000	2.70 ± 0.14	−2.90 ± 0.46	2046 ± 680	11.5 ± 3.88	32.6 ± 21.1	0.17 ± 0.11	7500 ± 577	47.5 ± 5.00	0.46 ± 0.02	81.7 ± 3.71

R, dark respiration; Γ, CO_2/HCO_3^- compensation point of photosynthesis; α, CO_2/HCO_3^- conductance of photosynthesis; S, CO_2/HCO_3^- saturation point of photosynthesis; pCO_2, partial pressure of CO_2. Values are means ± SD (n = 4).

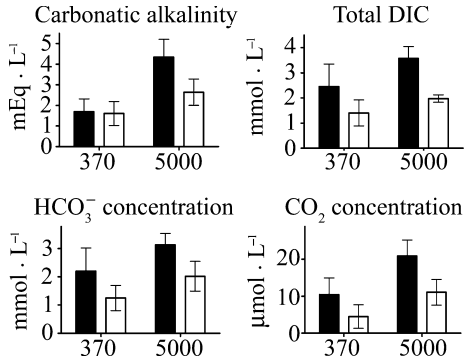

FIG. 2. Results of end-point pH-drift experiments conducted on *Protoceratium reticulatum* cultivated in media equilibrated with gas phases containing either 370 or 5000 ppm of CO_2. The black bars show the mean value at the beginning of the experiment; the white bars show the mean value at the end of the experiment. The error bars show the standard deviations ($n = 3$).

a pH compensation point of 8.33 ± 0.01 (Fig. 2), with a 1.6-fold decrease in alkalinity (final alkalinity = 2.6 ± 0.6 mEq·L^{-1}). The CO_2 concentration in the incubation medium declined from 21.0 ± 4.31 µmol·L^{-1} to 11.1 ± 3.49 µmol·L^{-1}; HCO_3^- (final concentration = 2.0 ± 0.5 mmol·L^{-1}) and DIC (final concentration = 2.0 ± 0.1 mmol·L^{-1}) concentrations decreased by 60% and 50%, respectively (Fig. 2; $P < 0.05$).

Internal inorganic carbon concentration. The silicone oil centrifugation data showed that cells cultivated at 5000 ppm CO_2 contained the amount of inorganic carbon expected if they were in equilibrium with the medium, while the cells cultured at atmospheric CO_2 concentration showed a consistent but rather limited (1.5-fold) accumulation of inorganic carbon (Table 6; $P < 0.05$).

DISCUSSION

When compared to the work on the CCMs of green microalgae and, to some extent, diatoms, the investigations of dinoflagellate CCMs are scant and usually not performed with a classical comparison between cells grown in low and high CO_2. Our data were collected to fill in these gaps.

Protoceratium reticulatum is a peridinin-containing ''red'' dinoflagellate expected to contain form II RUBISCO. This expectation was confirmed by the specific immunological reaction of a protein extract of *P. reticulatum* with an anti-form-II RUBISCO (Fig. 1). Since the selectivity factor of RUBISCO II for CO_2, measured in different species of dinoflagellates, is unable to support a positive photosynthetic budget at atmospheric CO_2 levels (Raven and Johnston 1991, Whitney and Andrews 1998), algae with this type of RUBISCO require a CCM to support growth (Badger and Price 1994, Leggat et al. 1999, Nimer et al. 1999a, Clark and Flynn 2000, Colman et al. 2002, Dason et al. 2004). Our results are compatible with this idea: cells grown in low CO_2 are appreciably more efficient at using subsaturating concentrations of DIC than cells grown in high CO_2, as shown by the almost 10-fold lower $K_{1/2}$(CO_2, DIC) and 5-fold lower CO_2 conductivity in cells grown in low CO_2 than in cells cultured at high CO_2 (Table 5). The extent of this variation is similar to that observed in organisms whose CCMs are usually considered very efficient (Giordano and Bowes 1997, Rost et al. 2003). This not withstanding, we were only able to observe a very limited accumulation of CO_2 inside cells grown in low CO_2, while no accumulation was detected in cells cultured at 5000 ppm (Table 6). The low amount of CO_2 accumulated in *P. reticulatum* cells grown in low CO_2 might be thought inconsistent with the presence of a CCM. However, the fact that similar results were obtained for algae with obviously active CCMs (Berman-Frank et al. 1994, 1998, Raven 1997b, Leggat et al. 1999, Nimer et al. 1999a) and the disequilibrium theory proposed by Raven (1997b) suggest that a large CO_2 accumulation is not necessary for a CCM to work effectively. While this theory is most easily understood for algae with a pyrenoid, Morita et al. (1998) also used it to explain the function of a CCM in some pyrenoidless microalgae with low intracellular CO_2 accumulation. Pyrenoids are often present in dinoflagellates (Dodge 1975, Schnepf and Elbrächter 1999), and their formation and disassembly, at least in *Gonyaulax* sp., has been found to correlate with CO_2 fixation rates (Nassoury et al. 2001, 2005). The *P. reticulatum* cells used for this study, however, did not contain pyrenoids, regardless of the CO_2 concentration available or entrainment to L:D cycles (Fig. S1, see supplementary material). This finding is a further indication that the presence of a pyrenoid is not essential for CCM activity (Morita et al. 1998, Giordano et al. 2005, Raven et al. 2005).

TABLE 6. Accumulation of inorganic carbon in cells of *Protoceratium reticulatum* cultivated at either ambient (370 ppm) or elevated (5000 ppm) pCO_2.

pCO_2 (ppm)	DIC_{out}	DIC_{in}	DIC_{in}/DIC_{out}	CO_{2out}	CO_{2in}	CO_{2in}/CO_{2out}	pH_{out}	pH_{in}
370	533 ± 67.6	796 ± 184	1.51 ± 0.39	1.97 ± 0.25	1.19 ± 0.27	0.60 ± 0.16	8.28 ± 0.00	8.61 ± 0.00
5000	419 ± 25.0	118 ± 79.5	0.28 ± 0.17	3.15 ± 0.18	0.31 ± 0.23	0.11 ± 0.07	8.00 ± 0.00	8.38 ± 0.00

DIC_{out}, DIC_{in}, CO_{2out} and CO_{2in} values are expressed in µmol · L^{-1}.
The subscripts ''in'' and ''out'' identify the compartment (either intracellular or extracellular) to which the data refer. The values are the means ± SD ($n = 3$). pCO_2, partial pressure of CO_2.

The distribution and role of CAs in dinoflagellates is still not entirely clear. Most of the dinoflagellates studied so far show very little CA activity on their surface (Colman et al. 2002, Dason et al. 2004, Rost et al. 2006), although external CA activity was detected in some studies (Berman-Frank et al. 1994, 1995, Nimer et al. 1997, 1999a,b). In contrast, internal CA activity was found in all dinoflagellates assayed (Berman-Frank et al. 1994, Leggat et al. 1999, Dason et al. 2004, Rost et al. 2006). *Protoceratium reticulatum* seems to be a rather typical dinoflagellate with respect to CA activity, with no activity at the surface of the cells and appreciable activity within the cell (Table 3). While the involvement and role of CAs in the CCM of *P. reticulatum* remain to be clarified, our results indicate that this species contains a soluble, intracellular CA. The fact that protein extracts from *P. reticulatum* cross-react only with antibodies directed against β-CA, specifically those from *Ph. tricornutum* (Satoh et al. 2001) and from *Coccomyxa* sp. (Hiltonen et al. 1995, Hiltonen et al. 1998) and not with antibodies against α-CA is highly suggestive, albeit not conclusive, of the presence of a β-CA in *P. reticulatum*. The intracellular CA activity in *P. reticulatum,* as in *Ph. tricornutum* (Satoh et al. 2001) but not in the obligately lichenized *Coccomyxa* sp., which lacks a CCM (Hiltonen et al. 1998, Raven et al. 2005), is induced by low CO_2 (Table 3). In addition, the abundance of the protein recognized by anti-PtCA and anti-CoCA is increased in the low-CO_2 treatment (Fig. 1), pointing to its implication in a biophysical CCM. It is not currently known how many CAs are present in dinoflagellates; BLAST searches of dinoflagellate expressed sequence tag (EST) libraries retrieve sequences with modest similarity to α, β, and γ CAs (expected values typically in the range 10^{-7} to 10^{-9}).

To estimate the potential contribution of the iCA of *P. reticulatum* to the CCM, we compared the CA-catalyzed rate of HCO_3^- conversion to CO_2 in cells grown at high- and low-CO_2 concentrations (Table 4) with their photosynthetic $K_{1/2(DIC)}$ (Table 5). These calculations suggest that the one order of magnitude difference in photosynthetic affinity for DIC between the cells grown in high CO_2 and those grown in low CO_2 may be explained by the difference (one order of magnitude) in iCA activity, supporting a role for this CA in the CCM of *P. reticulatum*.

The extent to which the presence of a CCM relieves cells from CO_2 limitation is not easy to determine. Recently, Rost et al. (2006) showed that photosynthesis and growth of three species of marine dinoflagellates were not limited by inorganic carbon, even at high pH. These authors manipulated DIC availability by changing growth pH. Our study differs from that described by Rost and colleagues because our intention was to investigate the effect of CO_2 concentration only, separating it from the effect of pH, which was kept constant (although other parameters of the inorganic carbon system in solution were allowed to vary). Our approach afforded results that indicate that *P. reticulatum* photosynthesis and growth are appreciably limited by DIC availability. Obviously, the impossibility of manipulating just one component of the DIC system does not exclude the possibility that the factors that vary in concert with CO_2 availability in the two approaches were responsible for the observed differences. For example, pH variations can influence the availability of essential nutrients, membrane transport processes, and some metabolic functions (Smith and Raven 1979, Raven 1993, Dason et al. 2004), and changes in total DIC and alkalinity may also have some additional uncharacterized effect. It should also be mentioned that because of the aim and design of our work, which was that of describing in classical terms the CCM of *P. reticulatum*, we subjected our cells to a much larger variation in CO_2 concentrations than did Rost et al. The CCMs of dinoflagellates may require large variations of external CO_2 to show an appreciable repression, and it could be essentially constitutive under natural conditions. If this is the case, the present-day ecological and, possibly, evolutionary significance of the high-CO_2 repression of these mechanisms will become rather obscure. Moreover, a response not dissimilar to that reported here was observed in the same species in an unbuffered medium equilibrated with a gas phase containing 1000 ppm CO_2 (A. Norici, personal communication), a concentration that in some scenarios may occur at the end of this century (Beardall et al. 1998).

At this preliminary stage in the study of dinoflagellate CCMs, very few mechanistic details are known. The DIC species that is acquired appears to be CO_2 in species like *Amphidinium carterae* Hulburt and *Heterocapsa oceanica* F. Stein (Colman et al. 2002, Dason et al. 2004), and HCO_3^- in species like *Prorocentrum minimum* (Pavill.) J. Schiller, *Heterocapsa triquetra* Iwataki, G. Hansen et Fukuyo, and *Ceratium lineatum* (Ehrenb.) Cleve (Rost et al. 2006). Bicarbonate also appears to be acquired in some zooxanthellae (Goiran et al. 1996), although some symbiotic dinoflagellates are able to switch from HCO_3^- uptake to CO_2 use (Leggat et al. 1999, 2002). The pH values attained at end of the pH-drift experiment (~8.3; Fig. 2) suggest that *P. reticulatum* is a CO_2 user (Maberly 1990).

In conclusion, the data reported here are suggestive of a decisive involvement of CA in *P. reticulatum* CCM and show that for the activity of this CCM, pyrenoids are not required. More in-depth molecular and physiological studies will be required to understand fully the mechanisms and regulation of dinoflagellate CCMs.

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Supplementary Material

The following supplementary material is available for this article

Figure S1. Plastid ultrastructure in *Protoceratium reticulatum* grown under different [CO_2] and light cycles.

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1529-8817.2007.00368.x

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