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# PHOTOSYNTHETIC INORGANIC CARBON ACQUISITION IN AN ACID-TOLERANT, FREE-LIVING SPECIES OF *COCCOMYXA* (CHLOROPHYTA)[1]

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**The processes of $CO_2$ acquisition were characterized for the acid-tolerant, free-living chlorophyte alga, CPCC 508. rDNA data indicate an affiliation to the genus *Coccomyxa*, but distinct from other known members of the genus. The alga grows over a wide range of pH from 3.0 to 9.0. External carbonic anhydrase (CA) was detected in cells grown above pH 5, with the activity increasing marginally from pH 7 to 9, but most of the CA activity was internal. The capacity for $HCO_3^-$ uptake of cells treated with the CA inhibitor acetazolamide (AZA), was investigated by comparing the calculated rate of uncatalyzed $CO_2$ formation with the rate of photosynthesis. Active bicarbonate transport occurred in cells grown in media above pH 7.0. Monitoring $CO_2$ uptake and $O_2$ evolution by membrane-inlet mass spectrometry demonstrated that air-grown cells reduced the $CO_2$ concentration in the medium to an equilibrium concentration of 15 μM, but AZA-treated cells caused a drop in extracellular $CO_2$ concentration to a compensation concentration of 27 μM at pH 8.0. $CO_2$-pulsing experiments with cells in the light indicated that the cells do not actively take up $CO_2$. An internal pool of unfixed inorganic carbon was not detected at the $CO_2$ compensation concentration, probably because of the lack of active $CO_2$ uptake, but was detectable at times before compensation point was reached. These results indicate that this free-living *Coccomyxa* possesses a $CO_2$-concentrating mechanism (CCM) due to an active bicarbonate-uptake system, unlike the *Coccomyxa* sp. occurring in symbiotic association with lichens.**

*Key index words:* **bicarbonate transport; carbonic anhydrase; $CO_2$-concentrating mechanism; dissolved inorganic carbon; photosynthesis**

*Abbreviations:* **AZA, acetazolamide; CA, carbonic anhydrase; CCM, $CO_2$-concentrating mechanism; Ci, inorganic carbon; DIC, dissolved inorganic carbon; WA, Wilbur-Anderson**

Algae of the genus *Coccomyxa* occur as free-living forms or as phycobionts of asco- and basidiolichens (Peveling and Galun 1976). Recently, a *Coccomyxa* sp. was isolated from in vitro cell cultures of *Ginkgo biloba* (Trémouillaux-Guiller et al. 2002, Trémouillaux-Guiller and Huss 2007). Phylogenetic studies of *Coccomyxa* have revealed three main lineages within this genus, corresponding to free-living *Coccomyxa,* those isolated from basidiolichens *Omphalina*, and *Coccomyxa* isolated from ascolichens belonging to the Peltigerales (Zoller and Lutzoni 2003). There have been studies on the $CO_2$-acquisition strategy used by *Coccomyxa* isolated from lichens, which appears to take up $CO_2$ simply by diffusion (Palmqvist 1993, Palmqvist et al. 1994), but little information is available on inorganic carbon (Ci) acquisition of the free-living forms of this genus.

Many free-living algae that occur in alkaline waters possess a photosynthetic CCM, which functions to accumulate dissolved inorganic carbon (DIC) within the cells, thus increasing the $CO_2$ concentration in the vicinity of the carboxylating enzyme RUBISCO (Badger et al. 1998, Colman et al. 2002). The accumulation of DIC is achieved by the active uptake of $HCO_3^-$ and/or $CO_2$ and the action of an external CA (Colman et al. 2002). The increase in intracellular $CO_2$ concentration favors the carboxylation reaction and inhibits the oxygenase activity of RUBISCO and thereby reduces photorespiration (Raven 1997).

A limited number of eukaryotic microalgae do not possess a CCM, and these species appear either to take up $CO_2$ directly from the air or to occur in waters of neutral to acid pH. For example, *Coccomyxa* sp., the phycobiont in lichen symbioses, relies on the diffusive uptake of $CO_2$ from the air and has a RUBISCO with a $K_m$ ($CO_2$) of 12 μM (Palmqvist et al. 1995), and thus the whole-cell

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affinity for Ci is determined by the low $K_m(CO_2)$ of RUBISCO. Acid-tolerant algae display a variety of different Ci-acquisition mechanisms. The acid-tolerant alga *Chlamydomonas* sp. (UTCC 121) does not possess a CCM, takes up $CO_2$ by diffusion, expresses an external CA when grown at neutral pH, and possesses RUBISCO with $K_m$ ($CO_2$) of 16.3 μM, which indicates that it determines the whole-cell affinity for $CO_2$ (Balkos and Colman 2007). *Euglena gracilis*, which grows over a relatively wide range of pH, has no capacity for active $CO_2$ uptake and does not express an external CA, but a capacity for bicarbonate transport is induced when the alga is grown at pH 7.0 (Colman and Balkos 2005). In contrast, the acid-tolerant alga *Eremosphaera viridis* has the capacity for active $CO_2$ uptake but none for $HCO_3^-$ transport (Rotatore et al. 1992).

*Coccomyxa* sp. (CPCC 508) is an acid-tolerant alga that grows over a wide range of pH from 3.0 to 9.0. In the present study, the mode of Ci uptake in this *Coccomyxa* strain has been investigated.

## MATERIALS AND METHODS

*Growth conditions.* An axenic culture of *Coccomyxa* sp. (CPCC 508) was obtained as UTCC 508 from the University of Toronto Culture Collection (now the Canadian Phycological Culture Centre at the University of Toronto). The cells were grown in aerated cultures in modified acid medium (MAM; Olaveson and Stokes 1989) over a pH range of 2.0 to 5.0, buffered with 10 mM, 2-*N*-morpholino ethane-sulfonic acid, sodium salt (MES), or in Bold's basal medium (BBM; Stein 1973) buffered with 10 mM bis-tris-propane (BTP) over the range of pH 6.0 to 9.0, at 20°C–22°C, under continuous illumination at a photon flux density of 45 μmol photons $\cdot m^{-2} \cdot s^{-1}$. Cultures were bubbled at a rate of 28 $L \cdot h^{-1}$ with air (0.0376% $CO_2$).

*Measurement of CA activity.* External carbonic anhydrase ($CA_{ext}$) activity was determined by the modified potentiometric technique described by Williams and Colman (1993). Cells were harvested by centrifugation (Sorval RC2B, Norwalk, CT, USA) at 5,000*g* for 15 min, washed with 20 mmol $\cdot L^{-1}$ sodium-barbital buffer, pH 8.3, and resuspended at a concentration of 30 μg chl $\cdot mL^{-1}$ in 1.5 mL of the same buffer. To determine total CA activity, the algae were homogenized in liquid nitrogen, the extract suspended in 50 mM bicine, and an aliquot of the homogenate was taken for CA measurement. Ice-cold, $CO_2$-saturated distilled water (0.5 mL) was injected into the cell suspension or cell homogenate at 4°C, and the time taken for the pH of the suspension to drop from pH 8.3 to 8.0 was measured. $CA_{ext}$ activity was calculated as Wilbur-Anderson (WA) units using the formula $T_c/T_s - 1$, where $T_c$ and $T_s$ represent the time taken for the pH change, in the presence and absence of cells, respectively. The final chl concentration of the sample was 30 μg $\cdot mL^{-1}$. Chl was quantified by extraction with $MgCO_3$-saturated dimethyl sulphoxide (DMSO; Ronen and Galun 1984).

*Measurement of photosynthetic rates.* Photosynthetic rates were measured as $O_2$ evolution in a Clark-type oxygen electrode (Hansatech Inc., Kings Lynn, Norfolk, UK). Cells were harvested at the mid-log phase by centrifugation at 5,000*g* for 15 min, washed, and resuspended to a final chl concentration of 15–30 μg $\cdot mL^{-1}$ in Ci-depleted photosynthetic medium containing 10 μM $MgSO_4$, 45 μM $K_2SO_4$, 85 μM $CaCl_2$, and 1.5 mM NaCl buffered over the range pH 7.0 to 9.0 with 10 mmol $\cdot L^{-1}$ BTP. Cell suspensions (1.0 mL) were placed in a sealed electrode chamber at a photon flux density of 500 μmol photons $\cdot m^{-1} \cdot s^{-1}$ at 25°C and bubbled with $N_2$ to reduce the $O_2$ concentration. The cells were allowed to consume the residual Ci until the $CO_2$-compensation concentration was reached before known amounts of Ci were added to reinitiate photosynthesis. Photosynthetic rates were calculated as μmol $O_2 \cdot mg^{-1}$ chl $\cdot h^{-1}$.

The capacity of cells to take up $HCO_3^-$ actively was evaluated by comparing the photosynthetic rate at a defined Ci concentration with the theoretical $O_2$-evolution rate that can be supported by the maximum rate of uncatalyzed breakdown of $HCO_3^-$ in the medium to $CO_2$ under the same conditions. Cells were treated with 40 μM AZA for 10 min prior to the assays, to inhibit external CA. The uncatalyzed rates of $CO_2$ formation in freshwater were calculated according to Miller and Colman (1980).

*Mass spectrometry.* $CO_2$ ($m/z = 44$) uptake and $O_2$ evolution ($m/z = 32$) by algal cell suspensions were measured using aqueous inlet mass spectrometry as described by Miller et al. (1988). Washed cells (6.0 mL), untreated or treated with inhibitor, corresponding to 30–40 μg $\cdot mL^{-1}$ chl, were placed in a glass reaction vessel with magnetic stirrer, and the chamber closed with a stopper. The chamber was connected to the ion source of the mass spectrometer by a sampling port covered with a dimethyl silicone membrane, which allowed the passage of gases but not that of ionic species. The mass spectrometer was calibrated for $CO_2$ and $O_2$ as described by Espie et al. (1989). Rates of $O_2$ evolution or consumption were derived from the slopes of the $m/z = 32$ signal.

The cells, illuminated at a photon flux density of 250 μmol $\cdot m^{-1} \cdot s^{-1}$ and at 25°C, were supplied with 500 μmol Ci. The ability of cells to take up $CO_2$ actively was tested by measuring the depletion of $^{12}CO_2$ in the medium in light to determine whether a chemical disequilibrium was created at the $CO_2$-compensation concentration as indicated by a rise in $CO_2$ concentration on the subsequent addition of 40 μg $\cdot mL^{-1}$ bovine CA. A second method used the disappearance of $CO_2$ from untreated and AZA-treated cell suspension after the addition of a pulse of pure $CO_2$. Any increase in the rate of $CO_2$ disappearance from suspensions of AZA-treated cells in the light compared to that of the same cells in the dark was taken as a measure of active $CO_2$ uptake (Espie et al. 1989). Aqueous $CO_2$ was prepared by bubbling 100 mL ice-cold acidified water (pH 2.0) with 5% (v/v) $CO_2$ in $N_2$.

*Phylogenetic analyses.* DNA of cultured algae was isolated and purified as described (Huss et al. 1986). Nuclear 18S rRNA genes were amplified from total genomic DNA by the PCR with eukaryote specific amplification primers and completely sequenced in an ABI Prism 310 Genetic Analyzer (Perkin-Elmer, Foster City, CA, USA) using oligonucleotide primers complementary to conserved regions of the 18S rRNA (Huss et al. 1999). The obtained sequence was manually aligned with several trebouxiophycean reference sequences and two chlorophycean outgroup taxa using the sequence editor program SeqEdit (Olsen 1990). External primer sequences and highly variable regions that could not be aligned unambiguously were excluded from the analyses, resulting in a total of 1,700 positions. Phylogenetic trees were inferred by the neighbor-joining (NJ), the maximum-parsimony (MP), and the maximum-likelihood (ML) methods using PAUP 4.0b10 (Swofford 2002). For NJ and MP, heuristic bootstrap analyses with 1,000 replicates were calculated. For the NJ analysis, the HKY85 correction was used to convert pair-wise sequence similarities into evolutionary distances, starting trees were obtained via NJ, and the tree-bisection-reconnection (TBR) branch-swapping algorithm was selected. In the MP analysis, starting trees were obtained via random stepwise addition of taxa repeated 10 times, gaps were treated as ''fifth base,'' and TBR was selected. In the ML bootstrap analysis, empirical base frequencies were

used, the transition/transversion (ti/tv) ratio was set to 2, a gamma distribution of 0.5 was assumed for variable sites, and addition of taxa was by NJ. These settings correspond to the HKY85+G model of evolution. The 18S rRNA gene sequence of *Coccomyxa* sp. CPCC 508 was submitted to the European Molecular Biology Laboratory (EMBL) database under the accession number AM981206.

## RESULTS

*Coccomyxa* sp. could grow over the range of pH 3.0–9.0. Rates of growth were determined by measuring the change in $OD_{730}$, and the highest cell densities were obtained over the range pH 5.0 to pH 8.0 (Fig. 1).

*Extracellular CA.* Intact cells of *Coccomyxa*, grown in aerated cultures over the pH range 3.0 to 9.0, were assayed for $CA_{ext}$ activity using potentiometric assay. Cells grown between pH 5.0 and 9.0 exhibited the presence of $CA_{ext}$: measurable activity was detected in cells grown at pH 5.0, but none in those grown at pH 3.0 or 4.0 (Table 1). The activity increased with the increase in growth pH, the maximum being exhibited by cultures at pH 9.0. $CA_{ext}$ activity was completely inhibited by AZA, when used at a final concentration of 40 μM, in cells grown over the whole pH range (Table 1). The total CA activity in cells grown at pH 7.0 was calculated to be 486 WA units · $mg^{-1}$ chl, and consequently, intracellular CA constituted 97.6% of the total CA activity.

*Kinetics of photosynthesis.* The kinetics of photosynthesis were determined for cells grown at pH 7.0, 8.0, and 9.0. Cells grown at pH 7.0 had the highest $V_{max}$ and the highest $K_{1/2}$ ($CO_2$). With an increase in the growth pH, the value of the $V_{max}$ as well as $K_{1/2}$ ($CO_2$) gradually decreased (Table 2). The highest measured rates of photosynthesis under non-Ci-limiting conditions were 73.13 μmol $O_2 \cdot mg^{-1}$ chl · $h^{-1}$ for cells grown at pH 7.0 and 66.2 μmol $O_2 \cdot mg^{-1}$ chl · $h^{-1}$ for cells grown at pH 9.0. The $K_{1/2}$ ($CO_2$) at pH 7.0 was 15.5 μM and 6.1 μM at pH 9.0. *Coccomyxa* had a highest apparent affinity for $CO_2$ when grown at alkaline pH.

To determine the effect of the inhibition of external CA on photosynthesis, cells grown over the range pH 7.0–9.0 were incubated with 40 μM AZA upon reaching compensation point. AZA inhibited $CA_{ext}$ activity (Table 1) but also reduced the $V_{max}$ of photosynthesis (Fig. 2) and increased the $K_{1/2}$

TABLE 1. The effect of growth pH on the external carbonic anhydrase ($CA_{ext}$) activity of *Coccomyxa* cells.

| Growth pH | $CA_{ext}$ activity WA units · $mg^{-1}$ chl | $CA_{ext}$ activity + AZA WA units · $mg^{-1}$ chl |
|---|---|---|
| 3 | 0.08 ± 0.2 (9) | 0.8 ± 0.2 (6) |
| 4 | 0.9 ± 0.9 (9) | 0.5 ± 0.5 (6) |
| 5 | 4.0 ± 3.7 (8) | 1.0 ± 1.4 (6) |
| 6 | 7.1 ± 2.1 (7) | 4.5 ± 3.4 (6) |
| 7 | 11.6 ± 2.3 (11) | 3.6 ± 2.1 (6) |
| 8 | 11.7 ± 1.7 (9) | 4.0 ± 3.7 (6) |
| 9 | 12.4 ± 1.8 (7) | 2.3 ± 2.2 (6) |

AZA, acetazolamide; WA, Wilbur-Anderson.
Values given as means ± SD (*n* replicates).

TABLE 2. The effect of pH on the photosynthetic rates and inorganic carbon (Ci) affinities measured at 25°C, 1,000 μmol photons · $m^{-2}$ · $min^{-1}$, and a chl concentration of 30 μg · $mL^{-1}$.

| Growth conditions pH | $V_{max}$ (μmol $O_2$·mg chl$^{-1}$ · $h^{-1}$) | $K_{1/2}$(Ci) (μmol · $L^{-1}$) | $K_{1/2}$($CO_2$) (μmol · $L^{-1}$) |
|---|---|---|---|
| pH 7 | 73.13 ± 4.3 (4) | 84.5 ± 12.2 | 15.5 ± 2.23 |
| pH 7 + AZA | 53.6 ± 17.4 (3) | 225.4 ± 14.1 | 41.4 ± 4.89 |
| pH 8 | 68.16 ± 12.5(4) | 514.6 ± 20.6 | 11.3 ± 1.91 |
| pH 8 + AZA | 52.27 ± 17.7 (4) | 1881.1 ± 26.9 | 41.3 ± 8.2 |
| pH 9 | 66.2 ± 16.27 (4) | 2774.7 ± 19.3 | 6.1 ± 1.44 |
| pH 9 + AZA | 50.7 ± 12.1(3) | 4258.2 ± 18.7 | 9.4 ± 1.91 |

AZA, acetazolamide.
Cells, grown at different pHs, were incubated with 40 μM AZA after reaching compensation. Values are means ± SD with the number of replicates in parentheses.

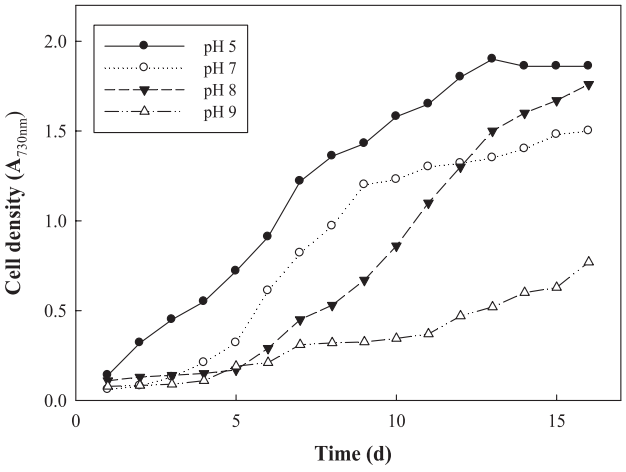


FIG. 1. Rates of growth of *Coccomyxa* sp. in aerated culture in media of different pHs.

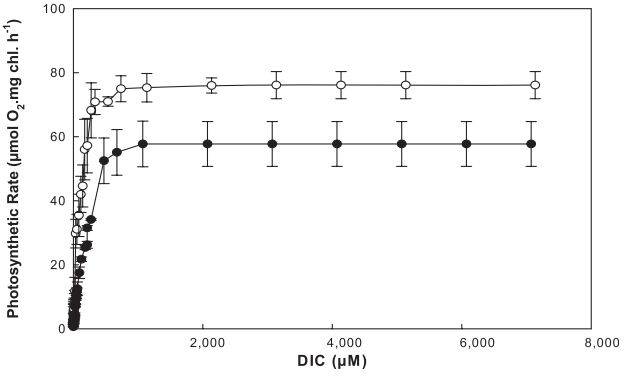


FIG. 2. The effect of dissolved inorganic carbon (DIC) concentration on the rate of $O_2$ evolution by cells of *Coccomyxa* grown at pH 7 (●). Control (○), cells treated with 40 μM acetazolamide (AZA). Rates measured at pH 7, at 25°C. Values given as means ± SD.

($CO_2$) (Table 2). This finding probably indicates that these cells are permeable to the inhibitor and that the reduction in the $V_{max}$ is due to the inhibition of internal CA.

*Active bicarbonate transport. Coccomyxa* grown at various pHs were assayed for their capacity to transport bicarbonate. $CA_{ext}$ was inhibited by preincubation of cell suspensions for 10 min with 40 μM AZA and then washed to remove excess inhibitor. Cells were then incubated in light and allowed to reach $CO_2$ compensation concentration, and the initial rate of $O_2$ evolution was then measured upon the addition of 100 μM $NaHCO_3$. The rate of $O_2$ evolution was compared with the calculated rate of spontaneous $CO_2$ formation from bicarbonate over the range of pH 7.0–9.0 at 25°C. The measured photosynthetic rates were equal to the spontaneous rates of uncatalyzed $CO_2$ formation at pH 7.0 and exceeded them at pH 8.0 and 9.0 (Table 3). These rates of $O_2$ evolution that exceed the supply rate of $CO_2$ are unequivocal evidence for the capacity for bicarbonate uptake. Therefore, these cells appear to take up Ci by direct bicarbonate uptake when grown at alkaline pH. Addition of bovine CA to the cell suspensions did not stimulate the photosynthetic rate (data not shown), indicating a lack of active $CO_2$ uptake.

*Measurement of $CO_2$ and $O_2$ fluxes.* The uptake of $CO_2$ and the evolution of $O_2$ were monitored by mass spectrometry in suspensions of *Coccomyxa* grown at pH 8. Suspensions of untreated *Coccomyxa* cells were incubated in the dark, and respiration caused a steady release of $CO_2$ and a decrease in $O_2$ concentration (Fig. 3). Cold Ci was then added to a final concentration of 500 $\mu mol \cdot L^{-1}$. Illumination of the cells caused a sharp drop in $CO_2$ concentration at an initial rate of 2.85 ± 0.03 (5 replicates) $\mu mol \cdot mg^{-1}$ chl $\cdot min^{-1}$ and evolution of $O_2$ at

TABLE 3. Comparison of the rates of $O_2$ evolution by AZA-treated *Coccomyxa* grown at different pHs with the calculated rate of spontaneous $CO_2$ formation at 25°C.

| Growth pH | Chl concentration $\mu g \cdot mL^{-1}$ | Photosynthetic rates (nmol $O_2 \cdot mL^{-1} \cdot min^{-1}$) | $O_2$-evolution rate/ $CO_2$-supply rate |
|---|---|---|---|
| 7 | 30 | 52.28 ± 5.6 (5) | 0.998 |
| 7 | 40 | 57.0 ± 0.86 (5) | 1.09 |
| 8 | 30 | 11.48 ± 3.15 (5) | 1.57 |
| 8 | 40 | 15.6 ± 1.8 (4) | 2.14 |
| 9 | 30 | 5.57 ± 0.21 (5) | 3.19 |
| 9 | 40 | 8.58 ± 0.25 (4) | 4.91 |
| 9 | 50 | 11.24 ± 0.31 (3) | 6.43 |

AZA, acetazolamide.

Cells were treated with 40 μM AZA for 10 min prior to assay. Cell suspensions at chl concentrations of 30 to 50 $\mu g \cdot mL^{-1}$ were allowed to take up the available inorganic carbon in the medium, and the photosynthesis rate determined on the addition of $NaHCO_3$ to a final concentration of 100 $\mu mol \cdot L^{-1}$. The calculated uncatalyzed rate of $CO_2$ formation from 100 μM $NaHCO_3$ at pH 7 and 25°C is 52.38 $nmol \cdot mL^{-1} \cdot min^{-1}$, 7.303 at pH 8.0, and 1.748 $nmol \cdot mL^{-1} \cdot min^{-1}$ pH 9.0. Values are given means ± SD (replicates).

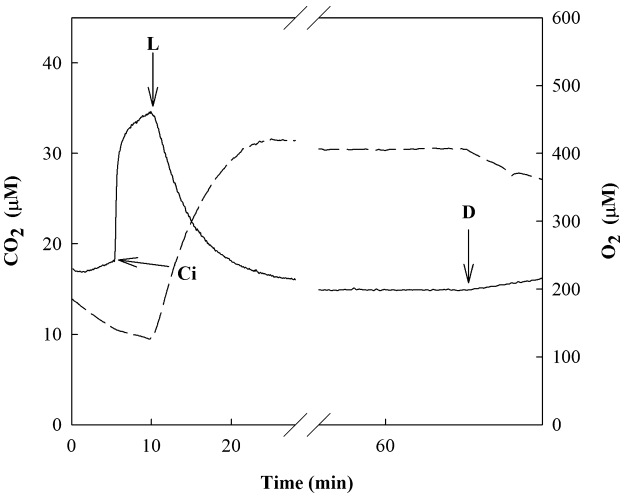


FIG. 3. Changes in $CO_2$ (——) and $O_2$ (- - -) concentrations during the dark (D) and illumination (L) cycles in suspensions of untreated *Coccomyxa* cells grown and maintained at pH 8. Cells were illuminated and allowed to take up the available $CO_2$ and provided with 500 $\mu mol \cdot L^{-1}$ Ci before the light was turned on. Once the cells had drawn down the $CO_2$, they were provided with 40 ug bovine carbonic anhydrase. Ci, inorganic carbon.

40.14 ± 0.68 (5) $\mu mol \cdot mg^{-1}$ chl $\cdot min^{-1}$. This step was followed by a reduction in the rate of $CO_2$ depletion, although there was a continued evolution of $O_2$ (Fig. 3). Since the rates of $O_2$ evolution were greater than the rates of $CO_2$ depletion, it indicated that the cells were either using $HCO_3^-$ directly or $CO_2$ derived from $HCO_3^-$ due to the action of $CA_{ext}$. After prolonged incubation of the cells in light, the $CO_2$ concentration fell to a constant value of 15.05 ± 0.28 (20) $\mu mol \cdot L^{-1}$, a concentration equal to the equilibrium $CO_2$ concentration at this pH and temperature. When the light was turned off, there was a linear increase in $CO_2$ concentration due to dark respiration (Fig. 3).

Similar experiments monitoring the depletion of $CO_2$ with cells pretreated with 40 μM AZA to inhibit $CA_{ext}$ were conducted as described above. Treatment with AZA did not have any effect on the rates of $O_2$ evolution and $CO_2$ depletion, and $O_2$ evolution greatly exceeded $CO_2$ depletion (e.g., Fig. 4). Prolonged incubation in the light was required to reduce the $CO_2$ concentration to the compensation concentration, 27.03 ± 2.48 (20) $\mu mol \cdot L^{-1}$ $CO_2$. Furthermore, when the light was turned off, there was no release of $CO_2$ from the cells, which would be expected in the absence of CA, if the cells had accumulated an internal pool of unfixed Ci (data not shown). However, in experiments where suspensions of AZA-treated *Coccomyxa* cells were allowed to take up $CO_2$ and then placed in the dark before they approached $CO_2$ compensation concentration, there was an immediate rise in $CO_2$-concentration in the medium (Fig. 4). This rise in $CO_2$ concentration lasted <1 min before the original rate of dark respiration was attained (Fig. 4). The rapid efflux of

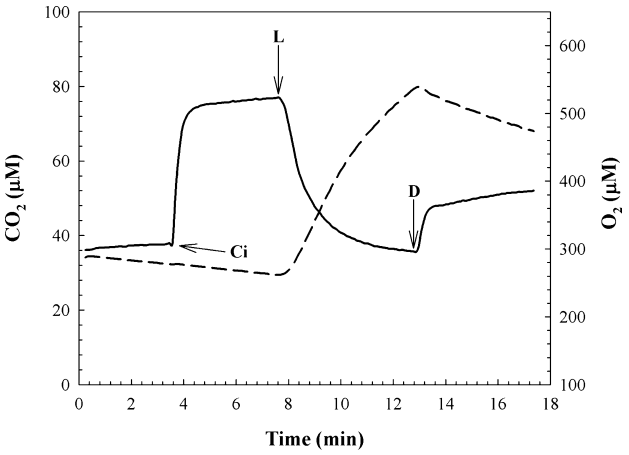


FIG. 4. Changes in $CO_2$ (——) and $O_2$ (- - -) concentrations during the dark (D) and illumination (L) cycles in cell suspensions of *Coccomyxa* grown and maintained at pH 8 and pretreated for 10 min with 40 μM acetazolamide (AZA) and then washed free of the inhibitor. Cells were illuminated and allowed to take up the available $CO_2$ and provided with 500 μmol · $L^{-1}$ Ci before the light was turned on. Ci, inorganic carbon.

$CO_2$ from these cells indicates the presence of an unfixed internal pool of Ci prior to reaching compensation concentration.

The capacity of *Coccomyxa* cells to take up $CO_2$ actively was tested using the $CO_2$-pulsing method (Espie et al. 1989). Transient changes in $CO_2$ concentration were monitored after the addition of 10 μL of $CO_2$-saturated water to suspensions of cells, in the light and the dark, and compared with that occurring in buffer at the same pH. In buffer alone, changes in $CO_2$ concentration are due solely to the uncatalyzed hydration to $HCO_3^-$, and therefore, any change in the rate of $CO_2$ disappearance indicates either active uptake by the cells or the presence of an $CA_{ext}$. The rate of $CO_2$ disappearance was accelerated by cells in the light and the dark due to the presence of $CA_{ext}$ (Fig. 5). In contrast, the rate of

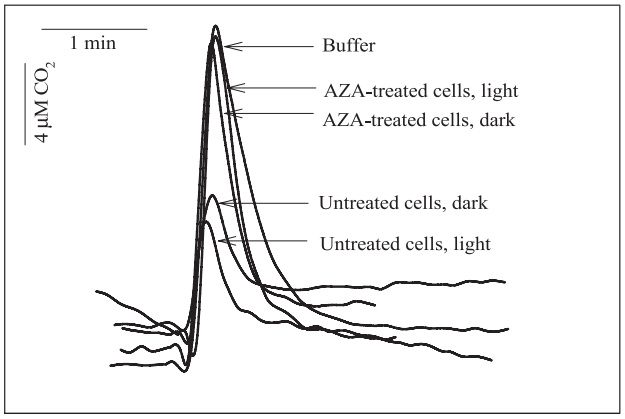


FIG. 5. Transient changes in $CO_2$ concentration in buffer solution at pH 8.0 and in suspensions of AZA-treated and untreated cells of *Coccomyxa* sp., in light and dark, after the supply of 10 μL $CO_2$-saturated water. AZA, acetazolamide.

$CO_2$ disappearance in suspensions of cells treated with AZA, either in the light or the dark, was not significantly different from that in buffer alone (Fig. 5.), indicating that $CO_2$ was not actively taken up by these cells.

*Phylogenetic position.* The 18S rRNA gene sequence of *Coccomyxa* sp. CPCC 508 has a length of 1,797 bp and contains no group I intron. The phylogenetic tree shown in Figure 6 clearly indicates that this alga belongs to the genus *Coccomyxa* (Chlorophyta, Trebouxiophyceae), although it is distinct from species for which reference data are available. It is most closely related to the lichen-forming *C. glaronensis* and to an intracellular symbiont of *Ginkgo biloba* (Trémouillaux-Guiller and Huss 2007), with eight and nine differences in the 18S rRNA sequence, respectively.

## DISCUSSION

It is well known for green algae that the ability to tolerate highly acidic environments of pH 2–3 is not restricted to a distinct evolutionary lineage but was developed independently several times by adaptation (Huss et al. 2002). In phylogenetic trees, acid-tolerant strains may be found in close proximity to strains that are less tolerant or even sensitive to conditions of high acidity as demonstrated in Figure 6 for the alga under study, *Coccomyxa* sp. CPCC 508, and several other examples.

Algae that grow in waters over a range from acid to alkaline pH must be able to maintain a relatively constant internal pH and acclimate to the changing proportions of Ci species in the surrounding medium. *E. gracilis,* for example, takes up $CO_2$ by diffusion when grown at acid pH, but bicarbonate transport is induced when cells are grown at pH 7.0 where a large proportion of the available Ci is bicarbonate (Colman and Balkos 2005). However, *E. gracilis* does not maintain a constant internal pH above 7.0, and this appears to limit its growth range. In contrast, *Coccomyxa* sp. grows well over a pH range of 3.0 to 9.0, indicating that it maintains a constant internal pH and maintains photosynthesis over this pH range.

*Coccomyxa* sp. expressed $CA_{ext}$ when grown above pH 6.0 (Table 1), and its presence increased the whole-cell affinity for Ci at pH 7.0 (Table 2). $CA_{ext}$ was found to be completely inhibited by AZA treatment, which reduced the cell affinity ($K_{1/2}$ Ci) of the cells (Table 2, Fig. 2) but also reduced the $V_{max}$ (Fig. 2), suggesting that the compound permeated the cell membrane and inhibited internal CA. Although AZA is generally considered impermeable to algal cells (Moroney et al. 1985), a similar inhibition of both external and internal CA by AZA has been observed in an acid-tolerant *Chlamydomonas* (Balkos and Colman 2007).

The occurrence of $CA_{ext}$ in a microalga is usually associated with active $CO_2$ uptake by the alga

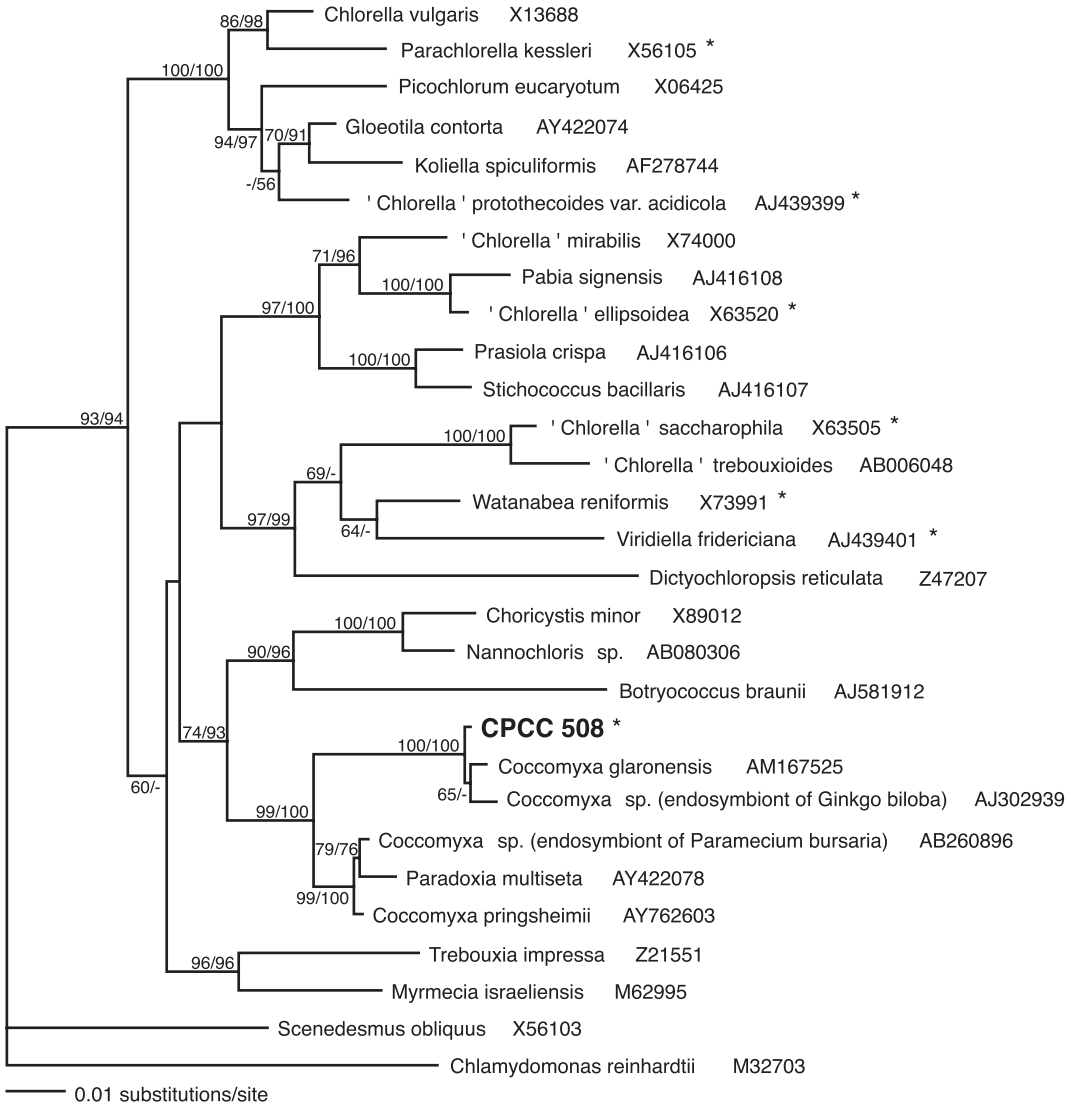


FIG. 6. Maximum-likelihood tree inferred from complete 18S rRNA gene sequences of representative trebouxiophycean green algae showing the phylogenetic position of *Coccomyxa* sp. CPCC 508. The chlorophytes *Scenedesmus obliquus* and *Chlamydomonas reinhardtii* were used as outgroup. Bootstrap support of maximum-parsimony and neighbor-joining analyses is shown along nodes if >50%. Branch lengths are proportional to the number of substitutions/site (note scale bar). Reference sequences were taken from GenBank and are identified by their accession number. Asterisks indicate strains with reported acid tolerance in the range of pH 2–3.

(Colman et al. 2002), but no such active $CO_2$ uptake could be detected in *Coccomyxa* sp. (Fig. 5). In an acid-tolerant *Chlamydomonas*, $CA_{ext}$ is expressed above a growth pH of 5.5, and its activity increases linearly up to pH 7.0 (Balkos and Colman 2007). Since the alga uses only $CO_2$ and takes it up by diffusion, the function of $CA_{ext}$ is presumably to maintain an equilibrium $CO_2$ concentration at the cell surface as the proportion of $CO_2$ of the total Ci decreases and the rate of spontaneous bicarbonate dehydration decreases with an increase in pH. The 3-fold increase in $CA_{ext}$ activity in *Coccomyxa* from pH 5.0 to a maximum at 7.0 suggests that its function in this alga is the same as that in the acid-tolerant *Chlamydomonas*.

At alkaline pH, $HCO_3^-$ is the predominant species of inorganic carbon for uptake by *Coccomyxa*, but some $CO_2$ is always present. If an algal cell can use only $CO_2$ from the external medium, the rate of photosynthesis will be limited, in the absence of a catalyst, by the rate of spontaneous $CO_2$ formation from $HCO_3^-$. If the photosynthetic rates exceed the calculated uncatalyzed dehydration rate, it indicates that $HCO_3^-$ is taken up as a source of Ci. These rates in AZA-treated *Coccomyxa*, over the pH range 7.0 to 9.0, were 1.09 to 6.4 times greater than the rates that could be supported by uncatalyzed $CO_2$ formation in the medium (Table 3). This finding clearly shows that active transport of $HCO_3^-$ occurs in this species.

Mass-spectrometric monitoring of $CO_2$ uptake and $O_2$ evolution by *Coccomyxa* cells at pH 8.0 indicated that the rates of $O_2$ evolution greatly exceeded the rates of $CO_2$ depletion from the medium both in untreated cells (Fig. 3) and in AZA-treated cells (Fig. 4), confirming the occurrence of active bicarbonate uptake in this species. In untreated cells, the activity of $CA_{ext}$ could possibly indicate the active uptake of $CO_2$ derived from bicarbonate, but no active $CO_2$ was detected in $CO_2$-spiking experiments (Fig. 5). These results demonstrate that this alga relies solely on active bicarbonate uptake to supply Ci at alkaline pH.

Mass-spectrometric monitoring also demonstrated that the $CO_2$ concentration was reduced by untreated cells to 15 μM, a level equivalent to the equilibrium $CO_2$ concentration at this pH because of the presence of $CA_{ext}$. Similar monitoring of gas exchange by AZA-treated cells showed that the cells reached a final compensation concentration of 27 μM, which is higher than the equilibrium $CO_2$ concentration This species does not have the capacity for active $CO_2$ uptake, as shown by the $CO_2$-pulsing experiments (Fig. 5), and therefore, at the $CO_2$ compensation concentration, there would be a continued light-dependent uptake of bicarbonate, which would equilibrate rapidly inside the cell, catalyzed by internal CA, to produce $CO_2$. This $CO_2$ would then leak out of the cell and in the absence of active $CO_2$ uptake would raise the $CO_2$ concentration of the external medium. In cells with $CA_{ext}$, the $CO_2$ leaking from the cells would be rapidly equilibrated with the bicarbonate in the medium, and thus the equilibrium concentration of $CO_2$ would be maintained. However, in cells where $CA_{ext}$ was inhibited, $CO_2$ leaking into the medium would equilibrate only slowly with bicarbonate since the spontaneous hydration of $CO_2$ is slow at pH 8.0. The sustained uptake of bicarbonate by cells lacking $CA_{ext}$, coupled with a continued leak of $CO_2$ from the cells would therefore maintain a $CO_2$ concentration in the medium above the normal equilibrium. This proposed bicarbonate pump-$CO_2$ leak mechanism would also explain why an internal inorganic carbon pool was not detected when cells at compensation point were darkened as has been observed in other chlorophyte algae (Sültemeyer et al. 1989, Rotatore and Colman 1991).

The results of this study indicate that this free-living *Coccomyxa* species can take up $CO_2$ by diffusion at acid pH and expresses $CA_{ext}$ at neutral pH to maintain an equilibrium $CO_2$ concentration for diffusive uptake, whereas at alkaline pH a bicarbonate transporter is present to supply Ci to support photosynthesis. There is an initial build-up of an intracellular pool of inorganic carbon as a result of active bicarbonate uptake. However, an internal $CO_2$-bicarbonate equilibrium would be maintained because of the high internal CA activity and result in the leakage of $CO_2$ from the cells. Since these cells have no active $CO_2$ uptake, $CO_2$ would be lost to the medium, and, in the absence of $CA_{ext}$, it would cause the cells to exhibit a $CO_2$ compensation concentration above equilibrium values, as has been observed in this study. This species, therefore, exhibits all the characteristics of a CCM when grown at alkaline pH.

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