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Photosynthetic CO_2-use efficiency in lichens and their isolated photobionts: the possible role of a CO_2-concentrating mechanism

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Abstract. The CO_2 dependence of net CO_2 assimilation was examined in a number of green algal and cyanobacterial lichens with the aim of screening for the algal/cyanobacterial CO_2-concentrating mechanism (CCM) in these symbiotic organisms. For the lichens *Peltigera aphthosa* (L.) Willd., *P. canina* (L.) Willd. and *P. neopolydactyla* (Gyeln.) Gyeln., the photosynthetic performance was also compared between intact thalli and their respective photobionts, the green alga *Coccomyxa* PA, isolated from *Peltigera aphthosa* and the cyanobacterium *Nostoc* PC, isolated from *Peltigera canina*. More direct evidence for the operation of a CCM was obtained by monitoring the effects of the carbonic-anhydrase inhibitors acetazolamide and ethoxyzolamide on the photosynthetic CO_2-use efficiency of the photobionts. The results strongly indicate the operation of a CCM in all cyanobacterial lichens investigated and in cultured cells of *Nostoc* PC, similar to that described for free-living species of cyanobacteria. The green algal lichens were divided into two groups, one with a low and the other with a higher CO_2-use efficiency, indicative of the absence of a CCM in the former. The absence of a CCM in the low-affinity lichens was related to the photobiont, because free-living cells of *Coccomyxa* PA also apparently lacked a CCM. As a result of the postulated CCM, cyanobacterial *Peltigera* lichens have higher rates of net photosynthesis at normal CO_2 compared with *Peltigera aphthosa*. It is proposed that this increased photosynthetic capacity may result in a higher production potential, provided that photosynthesis is limited by CO_2 under natural conditions.

Key words: Alga(green) – Carbon dioxide concentrating mechanism – Carbonic anhydrase – Cyanobacterium – Lichen – Photosynthesis (lichen)

Introduction

Lichens are the result of a successful symbiosis between a heterotrophic fungal partner (mycobiont) and a photosynthetic autotroph (photobiont), that may be either a green alga, a cyanobacterium or in some cases both (Honegger 1991). The initiation and maintenance of the metabolic activity of lichens require that water is taken up and stored (Lange and Ziegler 1986; Lange et al. 1986). Surplus water may, however, limit the photosynthetic activity of the lichen if this causes swelling of the fungal hyphae, which may block the gaseous pores within the hyphal matrix and thus impede the diffusion of CO_2 to the photobiont (Lange and Tenhunen 1981; Sneglar et al. 1981; Lange et al. 1988; Cowan et al. 1992).

Free-living algae and cyanobacteria have evolved a strategy to overcome CO_2 limitation of photosynthesis as they have acquired a mechanism for the active uptake of inorganic carbon, reffered to as a "CO_2 concentrating mechanism" (CCM; Badger 1987). This mechanism, driven by photosynthetic electron transport (Spalding and Ogren 1982), functions to increase the CO_2 supply to ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco), and hence, the photosynthetic CO_2-use efficiency (i.e. the photosynthetic affinity for CO_2 of intact cells) is increased as photorespiration is suppressed (Badger 1987; Coleman 1991; Kaplan et al. 1991; Badger and Price 1992). In cyanobacteria, carbon is actively transported as CO_2 and HCO_3^-, via a pump for dissolved inorganic carbon (DIC) located in the cell membrane, and accumulates within the cell in the form of HCO_3^- (Price and Badger 1989c; Kaplan et al. 1991; Badger and Price 1992). It has been proposed that the accumulated HCO_3^- is dehydrated to CO_2 and fixed by Rubisco within

Abbreviations and symbols: AZ = acetazolamide (5-acetamido-1,3-thiadiazole-2-sulphonamide); BTP = 1,3-bis[tris(hydroxymethyl) methylamino]propane; CA = carbonic anhydrase (EC 4.2.1.1); CCM = CO_2-concentrating mechanism; Chl = chlorophyll; DIC = dissolved inorganic carbon ($CO_2 + HCO_3^-$); EZ = ethoxyzolamide (6-ethoxy-2-benzo-thiazole-2-sulfonamide); $K_{0.5}$ = concentration required for half-maximal response; Rubisco = ribulose-1,5-bisphosphate carboxylase-oxygenase (EC 4.1.1.39)

a subcellular compartment, the carboxysomes. These are small polyhedral-shaped protein bodies containing both Rubisco and the enzyme carbonic anhydrase (CA) which catalyzes the dehydration of HCO_3^- to CO_2 (Kaplan et al. 1991; Badger and Price 1992; Price et al. 1993). In green microalgae the details of the CCM are less clear, although it has been shown that both CO_2 and HCO_3^- are actively transported and that CA activity is important for its function (Moroney et al. 1985; Coleman 1991; Badger and Price 1992). It has also been suggested that the pyrenoid, a starch-coated proteinaceous structure present in the chloroplast of many eucaryotic algae, may play a similar role in algae as the carboxysome in cyanobacteria (Kuchitsu et al. 1988, 1991).

For two major reasons, one might also expect the CCM to be operating in lichens: first, because it is such a widespread mechanism among free-living algae and cyanobacteria (Badger 1987) and second because it may confer a particular advantage to lichens, as CO_2 diffusion may be slow in these symbionts (Lange and Tenhunen 1981; Sneglar et al. 1981; Lange et al. 1988; Cowan et al. 1992). The possibility of a CCM in cyanobacterial lichens was earlier suggested by Green et al. (1985), and in this paper the possibility was investigated in a number of lichen species by measurements of the photosynthetic CO_2-use efficiency in intact lichen thalli as well as in their isolated photobionts. To obtain more direct proof for the presence of the CCM, acetazolamide (AZ) and ethoxyzolamide (EZ) were used as inhibitors of CA. The data presented here indicate the presence of the CCM in lichens having a cyanobacterial photobiont (*Nostoc* spp.), and in the lichen *Cetraria islandica,* having a green algal photobiont (*Trebouxia* sp.). However, the mechanism appeared to be absent in those green algal lichens having a photobiont lacking the pyrenoid. It is also shown that, as a result of the CCM, cyanobacterial *Peltigera* lichens have higher rates of net photosynthesis at normal CO_2 compared with *Peltigera aphthosa,* which has a green algal photobiont lacking the mechanism.

Materials and methods

Lichen material and their photobionts. The lichen species investigated are listed in Table 1. All lichens were collected from their typical habitats in the county of Västerbotten, Sweden, in September 1991. They were air-dried at 10° C and then stored at −18° C for up to seven months, except for *Peltigera canina* which was collected fresh from the field in August 1992. Upon removal from the freezer, the lichens were lightly sprayed with water and reactivated for 3–4 d at 15° C, 90–95% relative humidity (RH) and an irradiance of 30–35 μmol photons · m^{-2} · s^{-1} (14 h photoperiod), provided by a bank of fluorescent tubes (Luxline-ES; Sylvania, Danvers, Mass., USA; cool-white F36W/184). Before enclosure in the CO_2-exchange cuvette, the thalli were re-wetted with liquid water and mounted on a wire tray. Excess water was removed by blotting the underside with tissue paper. After the measurements, the lichen thalli were oven-dried overnight at 80° C for dry-weight determinations.

The green alga *Coccomyxa* PA was isolated from *Peltigera aphthosa.* Activated and healthy looking thalli were cleaned in distilled water, cut into pieces and gently ground and squeezed in a mortar. Small quantities of assay medium, standard cyanobacterial growth medium (BG11; Rippka et al. 1981) buffered at pH 7.7 with 15 mM bis-tris-propane (BTP), were added and the homogenate was filtered through a nylon net (170 μm diameter pore size). Intact algal cells were isolated from the filtrate by differential centrifugation at room temperature (Drew and Smith 1967) and washed three times in CO_2-free assay medium prior to immediate experimental use. The isolation procedure took 40–60 min.

For culture purpose, cells of *Coccomyxa* were isolated from *Peltigera aphthosa* by the micropipette method (Ahmadjian 1967) and transferred to BG11 agar plates, supplemented with ampicillin (50 μg · ml^{-1}) to avoid bacterial growth. Single cell colonies were purified by replating three to four times, excluding ampicillin after the third plating. These purified *Coccomyxa* PA colonies were transferred to sterile liquid medium (BG11 with 10 mM BTP; pH 6.8) and grown in batch cultures (test-tubes, 95 mm long, 35 mm diameter), kept in a clear water-bath and maintained at 20° C. The cells were bubbled with air and continuously illuminated with 80 μmol photons · m^{-2} · s^{-1} by a bank of fluorescent tubes (TL 20W/55; Philips, Eindhoven, The Netherlands). The cultures were diluted daily to a chlorophyll (Chl) concentration of 5–10 μg · ml^{-1}.

The cyanobacterium *Nostoc* PC, isolated from *Peltigera canina* (Bergman and Hällbom 1982), a kind gift from Prof. Birgitta Bergman (Department of Botany, University of Stockholm, Sweden) was grown at 25° C in BG11 medium without BTP (pH 6.8–7.2), but otherwise as for *Coccomyxa.*

The unicellular green alga *Chlamydomonas reinhardtii* was grown as described by Palmqvist et al. (1990) and the cyanobacterium *Synechococcus* sp. strain PCC7942 *(Anacystis nidulans)* was grown as described by Krupa et al. (1990). The CO_2-concentrating mechanism was fully induced in *C. reinhardtii* by bubbling with 20 Pa CO_2 in air and in *Synechococcus* by bubbling with 3 Pa CO_2 in air as described previously (Badger and Price 1989).

Chlorophyll was quantitatively determined by extraction in $MgCO_3$-saturated dimethyl sulfoxide (DMSO; Ronen and Galun 1984), using five to six discs per intact thallus (0.87 cm^2) and pelleted algal/cyanobacterial cells.

Table 1. List of investigated lichens, their primary and, where applicable, secondary photobionts (in cephalodia). C, cyanobacterium; G(P), Green alga with pyrenoid in the chloroplast; G, green alga without pyrenoid in the chloroplast

Lichen species	Primary photobiont	Secondary photobiont
Lobaria scrobiculata (Scop.) DC	*Nostoc* sp. (C)[a]	
Peltigera canina (L.) Willd.	*Nostoc* sp. (C)[b]	
Peltigera malacea (Ach.) Funck	*Nostoc* sp. (C)[b]	
Peltigera neopolydactyla (Gyeln.) Gyeln	*Nostoc* sp. (C)[c]	
Cetraria islandica (L.) Ach.	*Trebouxia* sp. (G(P))[c]	
Lobaria pulmonaria (L.) Hoffm.	*Disctyochloris reticulata* (G)[b]	*Nostoc* sp. (C)[b]
Nephroma arcticum (L.) Torss.	*Coccomyxa* sp. (G)[b]	*Nostoc* sp. (C)[b]
Peltigera aphthosa (L.) Willd.	*Coccomyxa* sp. (G)[b]	*Nostoc* sp. (C)[b]

[a] Hallingbäck 1989; [b] Tschermak-Woess 1988; [c] Henssen and Jahns 1974

Measurement of CO_2 exchange. Exchange of CO_2 by individual lichen thalli was measured in an open system (Compact Minicuvette System 400, gas mixing unit GMA1 and cuvette GK-022; H. Walz, Effeltrich, FRG at 15° C and at 90–99% RH. The CO_2 analyzer was calibrated against a range of CO_2 standards. Water vapour was removed before entering the CO_2 analyzer using a cool-trap. Carbon-dioxide concentrations below 15 Pa were obtained by further dilution with CO_2-free air, supplied by a mass-flow controller. When desired, the O_2 concentration was reduced to 3% by dilution with N_2, supplied by a gas cylinder and a mass-flow controller, and the resulting O_2 concentration was measured in an oxygen electrode (Hansatech, King's Lynn, Norfolk, UK). For all gas mixtures the CO_2 concentration in the cuvette was measured by an absolute CO_2 analyzer (LCA-3; ADC, Hoddesdon, Herts., UK). Photosynthesis was measured at light saturation and light was provided by a slide projector (250W, 24V), measured at the surface of the lichen thallus by the cuvette light sensor, which had been calibrated against a quantum sensor (Li-189; Li-Cor Inc., Lincoln, Neb., USA). To cause a minimum of stress to each individual thallus the exposure time in the cuvette was kept at a minimum (< 1 h). To obtain a complete CO_2-response curve, data were pooled from several thalli, each of which was typically used for four to five CO_2 concentrations. The procedure was justified by measuring the CO_2-saturated photosynthetic rate for all of the samples (Table 2), which gave an indication of the variation between individual thalli. Rates were calculated according to von Caemmerer and Farquhar (1981).

Measurement of O_2 evolution. Exchange of O_2 by algae and cyanobacteria was measured in an oxygen electrode (Hansatech). Newly isolated cells of *Coccomyxa* were measured at 15° C while free-living algae and cyanobacteria were measured at their respective growth temperature, i.e. 20° C *(Coccomyxa)*, 25° C (*Nostoc* and *Chlamydomonas reinhardtii*) and 37° C *(Synechococcus)*. Prior to measurements, the cells were harvested from their growth medium by low-speed centrifugation at room temperature ($1000 \cdot g$ for 5 min), washed twice in CO_2-free assay medium (BG11 with 15 mM BTP, pH 7.7) and resuspended in the assay medium to a chlorophyll concentration of $10 \pm 5\ \mu g \cdot ml^{-1}$. The sample was exposed to an irradiance of 300 µmol photons $\cdot m^{-2} \cdot s^{-1}$ provided by a slide projector (described above) and allowed to consume any residing CO_2 in the medium until no further evolution of O_2 was observed. Thereafter, the inorganic-carbon concentration was increased stepwise by adding known concentrations of $NaHCO_3$. The actual concentration of CO_2 at the beginning of each trace was estimated from the equilibrium reaction (Eq. 1) using equilibrium constants (K_1) corrected for the ionic strength and temperature of the medium ($-\log K_1 = 6.05$ (15° C); 6.02 (20° C); 6.00 (25° C); 5.97 (37° C); Stumm and Morgan 1981).

$$CO_2 + H_2O \leftrightarrow HCO_3^- + H^+;$$

$$\text{where } K_1 = [H^+][HCO_3^-]/[CO_2(aq)] \qquad \text{Eq. (1)}$$

At the lowest concentrations of inorganic carbon the maximum rate of O_2 evolution was read within 1–2 min, while at higher concentrations the steady-state rate could be observed for 4–5 min. Acetazolamide and EZ were added after the carbon-depletion period and allowed to equilibrate with the cells for 5 min under weak illumination (30 µmol photons $\cdot m^{-2} \cdot s^{-1}$).

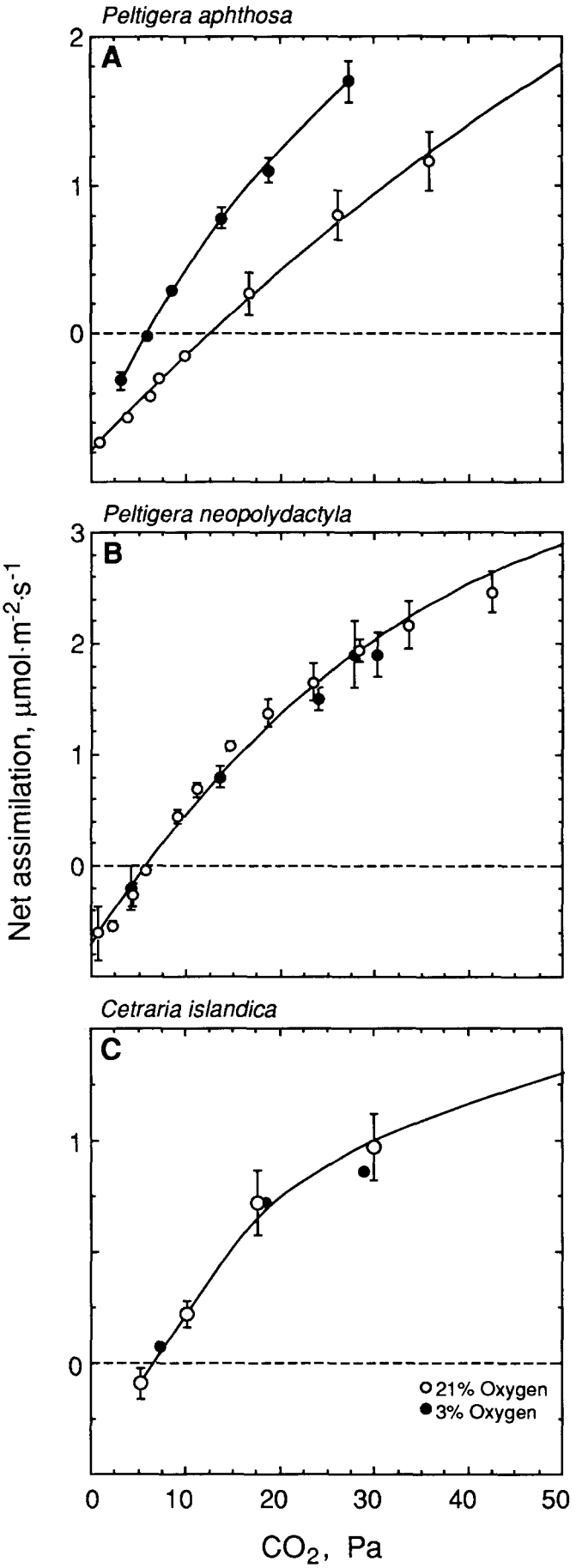
Results

From the photosynthetic characteristics summarized in Table 2, two groups of lichens are clearly distinguished: one with a low and another with a high photosynthetic affinity for CO_2. For the former, the half-maximum rate of gross photosynthesis [$K_{0.5}$ (CO_2)] was reached at CO_2 concentrations within 35–48 Pa, while for the latter group it was reached within 15–24 Pa. All three lichens

Table 2. Some characteristics of the investigated lichen species. Numbers in brackets refer to total number of replicate thalli. The initial slope of the CO_2-reponse curve, the carboxylation efficiency (CE), was calculated for three to four samples, except for *Lobaria scrobiculata*, where $n = 2$. The $K_{0.5}(CO_2)$ of gross photosynthesis was determined from a CO_2-response curve made up of all replicates. SE is given when $n \geq 3$

Species		Initial slope (CE) ($\mu mol \cdot (mg\ Chl)^{-1} \cdot h^{-1} \cdot Pa^{-1}$)	$K_{0.5}(CO_2)$ (Pa)	P_{max} ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Dark respiration ($\mu mol \cdot m^{-2} \cdot s^{-1}$)	Chlorophyll content ($mg\ Chl \cdot m^{-2}$)	Water content ($g \cdot g\ DW^{-1}$)
Lobaria scrobiculata	[2]	2.9	16	3.9	1.1	191	2.7–3.0
Peltigera canina	[4]	7.6 ± 0.5	13	5.4 ± 0.4	1.7 ± 0.1	106 ± 13	4.0–7.5
P. malacea	[3]	2.8 ± 0.1	24	2.7 ± 0.3	0.82 ± 0.05	145 ± 6	4.0–7.2
P. neopolydactyla	[12]	4.6 ± 0.7	20	4.0 ± 0.2	0.9 ± 0.07 (0.7 ± 0.1)[a]	99 ± 3	2.0–7.0
Cetraria islandica	[5]	2 ± 0.3	15	1.9 ± 0.4	0.4 ± 0.3	102 ± 7	1.0–1.7
Lobaria pulmonaria	[5]	1.2 ± 0.4	48	3.5 ± 0.5	0.65 ± 0.1	213 ± 22	1.0–1.5
Nephroma arcticum	[3]	1.1 ± 0.4	45	1.6 ± 0.6	0.56 ± 0.2	110 ± 15	4.0–6.0
Peltigera aphthosa	[12]	1.2 ± 0.3	35	3.9 ± 0.4	0.84 ± 0.08 (0.7–0.2)[a]	211 ± 24	2.0–7.0

[a] Measured in 3% oxygen



with the low affinity for CO_2 are tripartite lichens and have a green alga as primary photobiont, while all lichens with the higher affinity have cyanobacteria as photobionts, except for *Cetraria islandica* which has a green alga (Table 1). The high affinity for CO_2 of the cyanobacterial lichens was also manifested by a three to four times steeper initial slope of the CO_2-response curve, compared with those of the low-affinity, green algal lichens (Table 2). However, the initial slope of the CO_2-response curve of *Cetraria islandica* was only about two times steeper compared with those of the other green algal lichens.

In higher-plant leaves, a low affinity for CO_2 is usually related to photorespiration, which occurs because O_2 competes with CO_2 at the catalytic site of the carboxylating enzyme, Rubisco. This is also likely to be the explanation for the low CO_2-use efficiency in *Peltigera aphthosa*, because this species showed a 50–60% increase in net photosynthesis when the oxygen concentration was decreased to 3% (Fig. 1A). However, the increase in net photosynthesis may instead reflect a decreased mitochondrial respiration, imposed by the low O_2, which was as high as 20% of gross photosynthesis at 21% O_2 (Table 2), but this possibility could be excluded because the rate of dark respiration was not significantly affected by the reduced O_2 (Table 2). It can thus be concluded that the stimulation of net photosynthesis in *Peltigera aphthosa* at low oxygen is most probably due to the suppression of Rubisco oxygenase activity. In contrast to this, in *Peltigera neopolydactyla* and *Cetraria islandica*, a decreased oxygen concentration had little or no effect on net photosynthesis (Fig. 1B, C). This lack of O_2 sensitivity is similar to that which can be found in plants, algae and cyanobacteria that possess some kind of a CO_2-concentrating mechanism, which suppresses photorespiration.

It is reasonable to assume that the photobionts are responsible for the observed differences in CO_2-use efficiency. This hypothesis was tested by comparing intact thalli with isolated photobionts. Indeed, intact thalli of *Peltigera aphthosa* and newly isolated cells of its primary photobiont, *Coccomyxa* PA, had the same CO_2-response curve with an initial slope in the range of 2–3 µmol · (mg Chl)$^{-1}$ · h^{-1} · µM^{-1} (Table 3) and a $K_{0.5}$ (CO_2) of 16 µM (Fig. 2A). The isolation of *Coccomyxa* resulted in a 30% loss of the photosynthetic activity on a chlorophyll basis, compared with inatct thalli, which may account for the apparently higher initial slope value of the lichen (Table 3). To allow the comparison between inatct thalli and isolated algae on the same CO_2 basis, the concentration of CO_2 dissolved in the water-film [CO_2(aq)] surrounding the photobionts within lichens was calculated from Eq.

Fig. 1A–C. Net rate of CO_2 uptake as a function of ambient CO_2 concentration for the tripartite lichen *Peltigera aphthosa* (**A**) the cyanobacterial lichen *P. neopolydactyla* (**B**) and the green algal lichen *Cetraria islandica* (**C**) in 21% (○) and 3% O_2 (●). Error bars represent ± SE for at least five replicate thalli assayed in 21% O_2 and three replicate thalli assayed in 3% O_2

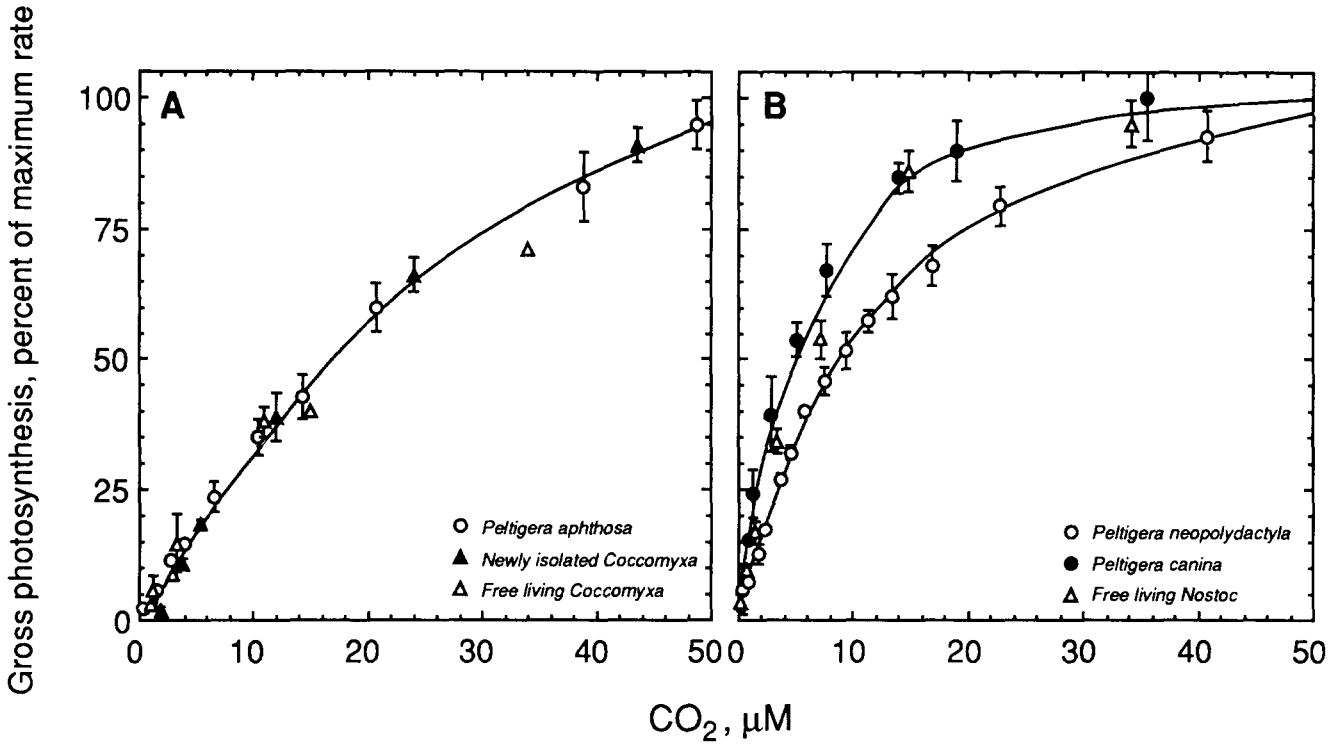


Fig 2A, B. Gross rate of photosynthesis as a function of the estimated concentration of dissolved CO_2 for intact lichen thalli and their newly isolated or cultured photobionts, in **A** for *Peltigera aphthosa* (○), isolated (▲) and cultured *Coccomyxa* PA (△), and in **B** for *Peltigera canina* (●), *P. neopolydactyla* (○) and cultured *Nostoc* PC (△). Photosynthesis of intact thalli was measured as CO_2 gas exchange and the concentration of dissolved CO_2 [CO_2(aq); µM) present in the water phase surrounding the photobiont was calculated from Eq. 2. Photosynthesis of cells was measured as O_2 evolution in BG11-BTP medium at pH 7.7, with controlled additions of HCO_3^-. Error bars (± SE) are indicated for at least four replicate thalli, three to four independent samples of cultured cells and three separate isolations of *Coccomyxa* PA

Table 3. Some characteristics of the investigated lichen species and their photobionts. Photosynthesis was measured as CO_2 gas exchange in the intact lichens and as O_2 evolution in photobiont cells. The concentration of dissolved CO_2 present in the water phase surrounding the photobionts was calculated from Eq. 2. The CO_2 concentration of the liquid medium was calculated according to Eq. 1, assuming equilibrium between the inorganic carbon species. SE is given when $n \geq 3$. $K_{0.5}(CO_2)$ was estimated from the data presented in Figs. 2 and 3

Material	Assay temperature (°C)	P_{max} (net) (µmol · (mg Chl)$^{-1}$ · h^{-1})	Dark respiration (µmol · (mg Chl)$^{-1}$ · h^{-1})	$K_{0.5}(CO_2)$ (µM)	Initial slope (CE) (µmol · (mg Chl)$^{-1}$ · h^{-1} · µM^{-1})
Peltigera aphthosa	15	66 ± 8	14.3 ± 1.4	16	2.8 ± 0.7
Coccomyxa, isolated	15	44.8 ± 1.3	8.1 ± 0.7	16	1.8 ± 0.2
Coccomyxa, isolated	20	68.9 ± 1.5	13.5 ± 2.4	16	2.6 ± 0.4
Coccomyxa, free-living	20	130 ± 6	21.7 ± 2.6	16	3.2 ± 0.3
P. neopolydactyla	15	145 ± 11	33 ± 3	9	12 ± 1.8
P. canina	15	183 ± 14	58 ± 4	5	19 ± 0.9
Nostoc PC, free-living	25	169 ± 7	60 ± 5	5	16.4 ± 1.2
Chlamydomonas reinhardtii	25	130 ± 3	11 ± 2	5	9.2 ± 0.6
Synechococcus	37	221 ± 12	51.6 ± 8	1	41

2, assuming equilibrium with the CO_2 external to the lichen [CO_2 (g)].

$$CO_2(g) \leftrightarrow CO_2(aq); \text{ where } K_H = [CO_2(aq)]/pCO_2 \quad \text{Eq. (2)}$$

($-\log K_H = 1.41$, at 15° C; Stumm and Morgan 1981; K_H is the Henry's law constant for solubility in water, pCO_2 the pressure of CO_2 in atmosphere).

It should be noticed, though, that this calculated internal concentration of CO_2(aq) may have been overestimated, due to a lower than ambient CO_2 concentration within the lichen thallus as a result of slow diffusion (Cowan et al. 1992).

Similar experiments were attempted with *Peltigera neopolydactyla* and *Cetraria islandica*. Unfortunately, isolation of photobionts from either of these two species

proved difficult, probably because these photobionts are more closely associated with fungal hyphae than *Coccomyxa* is in *Peltigera aphthosa* (Honegger 1991). However, I decided to use free-living, rather than newly isolated, photobionts for the comparison with intact thalli. This seems valid as there appears to be little difference between newly isolated and free-living cells of *Coccomyxa* (Fig. 2A, Table 3). Free-living cells of *Nostoc*, previously isolated from *Peltigera canina* (Bergman and Hällbom 1982) were used. Again, a close similarity in photosynthetic characteristics of the lichen and its photobiont was observed: intact thalli of *Peltigera canina* and free-living cells of *Nostoc* PC had almost identical CO_2-response curves with an initial slope in the range of 16–19 μmol · (mg Chl)$^{-1}$ · h^{-1} · μM^{-1} (Table 3) and a $K_{0.5}$ (CO_2) of 5 μM (Fig. 2B). It is interesting to note that *Peltigera neopolydactyla* had a somewhat lower affinity for CO_2, having a $K_{0.5}$ (CO_2) of 9 μM (Fig. 2B) and an initial slope of 12 μmol · (mg Chl)$^{-1}$ · h^{-1} · μM^{-1} (Table 3) compared with *Peltigera canina*, indicating that the two lichens may have different types of *Nostoc*.

As suggested above, lichens with a high affinity for CO_2 most probably possess the CCM, found in several species of free-living cyanobacteria and microalgae. It was therefore of interest to compare the photosynthetic DIC-use efficiency (i.e. the photosynthetic affinity for $CO_2 + HCO_3^-$ of intact algal/cyanobacterial cells assayed in liquid media), of the two photobionts *Coccomyxa* and *Nostoc*, with that of the green alga *Chlamydomonas reinhardtii* and the cyanobacterium *Synechococcus* PCC7942 which are both known to possess the CCM. Owing to the different growth temperatures of these cells, the measurements were performed at different temperatures. However, as there appeared to be only minor, if any, differences in the initial slope and the $K_{0.5}$ of intact thalli of *Peltigera aphthosa*, assayed at 15° C, compared with free-living cells of *Coccomyxa*, assayed at 20° C (Fig. 2A, Table 3), this comparison would still seem to be valid for

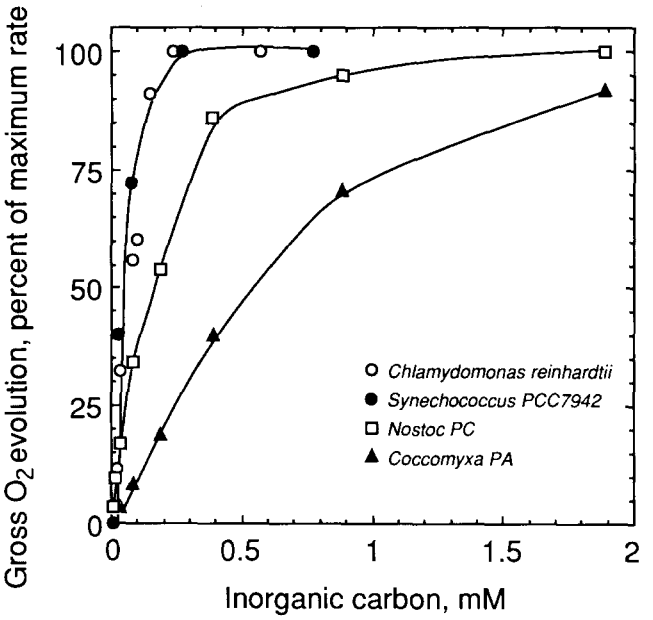


Fig. 3. Gross rate of O_2 evolution as a function of total concentration of inorganic carbon in the green microalga *Chlamydomonas reinhardtii* (○) and the cyanobacterium *Synechococcus* sp. PCC 7942, both having a fully induced CCM (●); and the two lichen photobionts *Nostoc* PC (□) and *Coccomyxa* PA (▲). The measurements were carried out in BG11-BTP medium at pH 7.7 with controlled additions of HCO_3^-

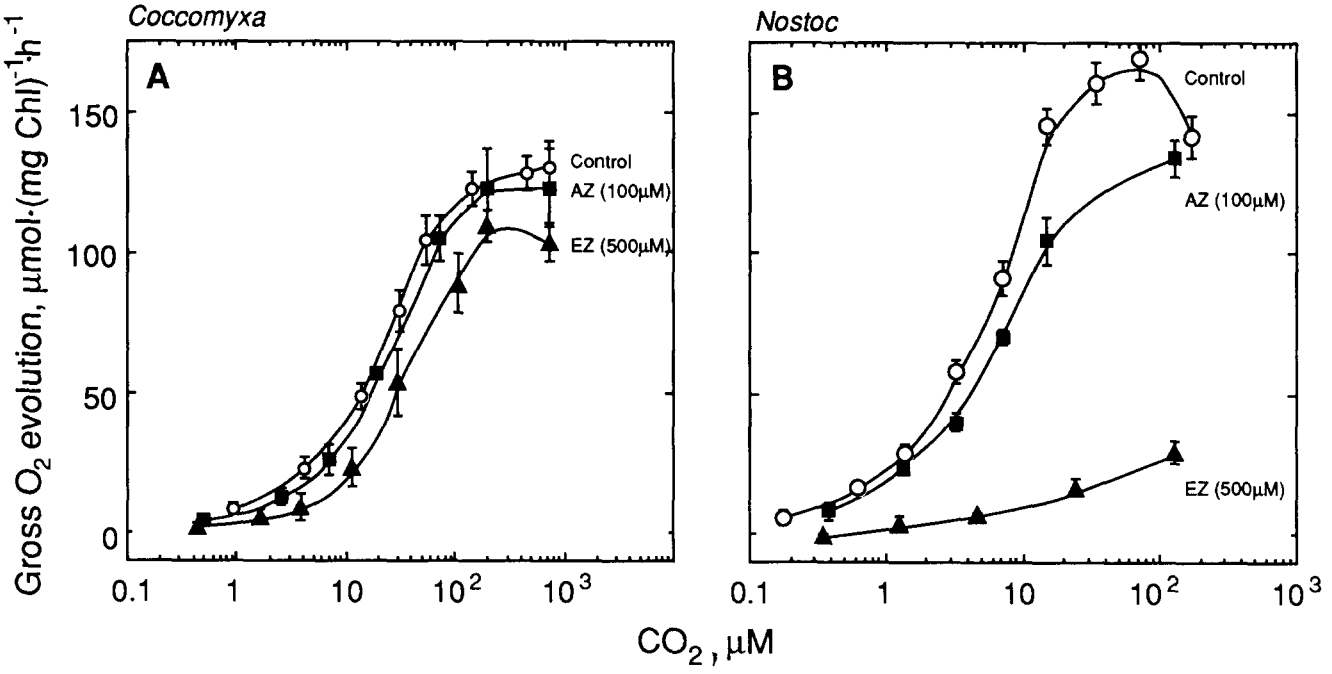


Fig. 4A, B. The effects of the inhibitors acetazolamide (*AZ*; ■) and ethoxyzolamide (*EZ*; ▲) on the relationship between the rate of O_2 evolution and the concentration of dissolved CO_2 in cell cultures of *Coccomyxa* PA (**A**) and *Nostoc* PC (**B**) compared with uninhibited cells (*control*; ○). Measurements were carried out in BG11-BTP medium at pH 7.7 with controlled additions of HCO_3^-. Error bars (± SE) are indicated for at least three independent cell samples

the intentions of this investigation. Indeed, *Nostoc* had almost as high a DIC-use efficiency as the two species having the CCM, and all these three species were far more efficient than *Coccomyxa* (Fig. 3, Table 3).

There are also more direct ways to demonstrate the presence of the CCM. One is focused on the enzyme CA which plays a key role in the CCM of both cyanobacteria and green algae (Badger and Price 1992). The activity of this enzyme can be inhibited by AZ, a hydrophilic compound which therefore predominantly inhibits extracellular CA, and EZ which penetrates biological membranes and inhibits intracellular forms of CA (Moroney et al. 1985). Ethoxyzolamide probably also inhibits another component of the CCM, the DIC pump that transports inorganic carbon (Price and Badger 1989b). Acetazolamide had no effect on photosynthesis in *Coccomyxa,* while EZ caused approximately 20% inhibition of maximum O_2 evolution (P_{max}; Fig. 4A). A moderate inhibition of photosynthesis by EZ was also observed for barley protoplasts (data not shown) and is probably due to the inhibition of a CA located in the chloroplast (Cowan 1986). In *Nostoc,* AZ caused a minor inhibition of P_{max}, whereas EZ produced a profound inhibition of photosynthesis at all CO_2 levels (Fig. 4B).

Discussion

Photosynthesis of the *Nostoc*-lichens studied here showed high efficiency at low CO_2 (Table 2, Fig. 2B) and was not affected by O_2 (Fig. 1B), indicating that these lichens possess a CCM. The same observations have been made before in cyanobacterial lichens (Sneglar and Green, 1980; Bauer 1984) and the possible existance of a CCM in cyanobacterial lichens was also put forward by Green et al. (1985). The high affinity for CO_2 of the cyanobacterial lichens was probably a property of *Nostoc,* as free-living *Nostoc* had the same CO_2-use efficiency as the intact thalli (Table 3, Fig. 2B), and it can thus also be concluded that the CCM is probably unnaffected by the lichenization of the photobiont. Also, the CO_2-use efficiency of *Nostoc* was similar to that observed in the cyanobacterium *Synechococcus* PCC7942 (Fig. 3) and highly sensitive to EZ (Fig. 4B), indicating that *Nostoc* has a CCM similar to that of other cyanobacteria (Badger 1987; Coleman 1991; Kaplan et al. 1991; Badger and Price 1992). This conclusion is further supported by the observation that lichenized *Nostoc* have most of their Rubisco in carboxysomes (Bergman and Rai 1989), which appears to be an important prerequisite for the functioning of the CCM in cyanobacteria in general (Kaplan et al. 1991; Badger and Price 1992). It has been speculated that cyanobacteria require a CCM because their Rubisco has a low inherent affinity for CO_2 compared with that of higher plants and green algae (Badger and Andrews 1987). The very strong depression of photosynthesis in *Nostoc,* when the CCM was inhibited by EZ (Fig. 4B) thus indicates that this mechanism is also necessary for photosynthesis in this cyanobacterium.

The indications for the operation of a CCM in *Nostoc* lichens, as presented in this and other studies, finds strong support in the results presented by Badger et al. (1993), where it was established that cyanobacterial lichens are able to accumulate an internal pool of DIC in the range 10–20 mM. However, even though there is thus strong evidence for the operation of the CCM in *Nostoc,* this mechanism appears to be somewhat less efficient than is the case for *Synechococcus* (Fig. 3). It has been shown that *Synechococcus,* as a part of the CCM, can actively transport both CO_2 and HCO_3^- via a DIC pump. Although the ability to transport CO_2 is present in both high- and low DIC-acclimated cells, HCO_3^- transport is dependent on the particular growth conditions and is enhanced when the cells are acclimated to extremely low DIC concentrations (bubbling with ≤ 3 Pa CO_2; Badger and Price 1992). Thus, it can be hypothesized that *Nostoc* corresponds to the high DIC-grown state of *Synechococcus* where HCO_3^- transport has not yet been induced. This is also supported by the resemblance between EZ inhibition of photosynthesis in *Nostoc* (Fig. 4B) and that occurring in high-DIC-grown *Synechococcus* (Price and Badger 1989a). In aquatic environments, HCO_3^- uptake would confer an advantage when the pH is high (>7), where this carbon species is the predominant one. In cyanobacterial lichens, liquid water is required for photosynthetic activity (Lange et al. 1986, 1988) and the photobiont is thus normally exposed to an aquatic environment. However, very little is known about the pH of this environment and it is therefore an open question whether the proposed lack of HCO_3^- uptake in *Nostoc* discussed above is related to a low pH within the lichen thallus.

The relatively low affinity for CO_2 in the green algal lichens having either *Coccomyxa* or *Disctyochloris* as primary photobiont (Table 1) indicates that these lichens are lacking a CCM (Table 2). This conclusion is also supported by the high oxygen sensitivity of photosynthesis in *Peltigera aphthosa* (Fig. 1A). Among others, *Pseudocyphellaria delisea* and *P. colensoi* are also green algal lichens with a low affinity for CO_2. These species are also characterized by a high O_2 sensitivity of photosynthesis and, in addition, a high carbon-isotope discrimination towards ^{13}C (Snelgar and Green 1980; Lange et al. 1988). Since most free-living green algae so far studied, e.g. *Chlorella* spp., *Chlamydomonas* spp., *Dunaliella* spp. and *Scenedesmus* spp. have been found to possess a CCM (Badger 1987; Coleman 1991), it could be argued that the apparent absence of a CCM in green algal lichens may be due to suppression of this mechanism upon lichenization of the photobiont. However, the CCM was also apparently absent in free-living cells of *Coccomyxa* as they had a much lower DIC-use efficiency than either of the species *Chlamydomonas reinhardtii, Nostoc* or *Synechococcus* (Fig. 3, Table 3), and were much less sensitive to EZ (Fig. 4A). The effects of EZ on photosynthesis in *Coccomyxa* were in fact more similar to what was found for barley protoplasts than for green algae having the CCM (data not shown).

In contrast to this, the green algal lichen *Cetraria islandica,* having *Trebouxia* sp. as photobiont (Tab. 1), had a higher CO_2-use efficiency, with a $K_{0.5}$ in the range of that found for the cyanobacterial lichens, and an

initial slope of the CO_2-response curve somwhere between the values for the cyanobacterial lichens and the low-affinity green algal lichens (Table 2). This is thus an indication that the CCM may be present in *Trebouxia*, as it is in the majority of the free-living green algae. Some other green algal lichens, for instance *Ramalina maciformis* (Cowan et al. 1992), *Pseudocyphellaria amphistica, P. billardierii* and *P. psilophylla* (Snelgar and Green 1980) also have a very low CO_2 compensation point, and in the case of *P. billardierii* a low O_2 sensitivity. These species are also characterized by a low carbon-isotope discrimination towards ^{13}C (Lange and Ziegler 1986; Lange et al. 1988). In addition, it was recently found by Badger et al. (1993) that the *Trebouxia* lichen *Hypogymnia physodes* is able to accumulate an internal pool of inorganic carbon, even though not to the same extent as the cyanobacterial lichens. In this context it is interesting to note that the most conspicuous difference between *Trebouxia*, the photobiont of *Cetraria islandica, Ramalina maciformis* and *Hypogymnia physodes* on the one hand, and *Coccomyxa* and *Disctyochloris* on the other hand, is the absence of a pyrenoid in the two latter photobionts (Ahmadjian 1967; Tschermak-Woess 1988), indicating that this structure may in fact explain the differences in CO_2-use efficiency within the group of green algal lichens. As mentioned above, the lichen genus *Pseudocyphellaria* has species containing green algal photobionts having both low and high affinities for CO_2. Possibly, this may reflect whether or not the pyrenoid is present in the photobiont of each lichen. Unfortunately the species of the photobionts were not specified in these investigations (Snelgar and Green 1980; Lange et al. 1988). However, it is known that *Pseudocyphellaria* spp. can associate with three different genera of green algae (Tschermak-Woess 1988), namely *Chlorella*, which has both the pyrenoid and a CCM (Badger 1987), *Trebouxia* which has the pyrenoid, and *Dictyochloropsis* where it is absent. The probable presence of a microalgal CCM in some of the green algal lichens thus needs further investigation, especially in relation to the actual species of the photobiont.

From the data presented here, it is also clear that the cyanobacterial lichens have an increased photosynthetic capacity compared with those green algal lichens which apparently lack a CCM. At the CO_2 concentration of ambient air, *Peltigera neopolydactyla* had a photosynthetic capacity which was twice as high on an area basis compared with that of *Peltigera aphthosa* (Fig. 1A, B), and *Peltigera canina* was found to be even more efficient (Fig. 2B). Whether this increased photosynthetic capacity is also accompanied by increased photosynthetic production is, however, difficult to predict. For instance, there are few data on the CO_2 concentration in the micro-habitats of these lichens, but it may be as high as 100 Pa for lichens growing on the forest ground (Huber 1952; D.S. Coxon, Department of Biology, McMaster University, Hamilton, Ontario, Canada, personal communication). Apart from the external CO_2, the question of whether photosynthesis in lichens is limited by CO_2 also depends on the resistance to CO_2 diffusion within the lichen thallus. One factor of potential importance for the latter is the water content of the lichen, because high water contents may increase the CO_2 diffusion resistance (Lange and Tenhunen 1981; Snelgar et al. 1981; Lange and Ziegler 1986; Cowan et al. 1992). In this context it is interesting to note that cyanobacterial lichens are characterized by a requirement for liquid water as well as high photosynthetic activity at high water content (Lange et al. 1986, 1988). This was also observed in the cyanobacterial *Peltigera* species investigated here, where no depression in photosynthesis was observed even though the water content was quite high (Table 2). It may thus be hypothesized that the cyanobacterial lichens require a liquid environment for the operation of the CCM and that this mechanism facilitates photosynthesis when the lichen thallus is inundated.

Finally, the results presented here also indicate that the contribution to photosynthesis in *Peltigera aphthosa* from the cephalodial *Nostoc* (Table 1) is insignificant. Otherwise, the CCM present in *Nostoc* would have been expressed as an enhanced CO_2-use efficiency in the intact thallus compared with the free-living *Coccomyxa*. This conclusion is consistent with the finding of a high proportion of N_2-fixing cells (heterocysts) relative to vegetative cells in *Nostoc*, when it occurs in cephalodia (Rai 1988), and the finding of a low number of carboxysomes in the few remaining vegetative cells (Bergman and Rai 1989), indicating that cephalodia have a low photosynthetic capacity.

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