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A New Screening Method for Algal Photosynthetic Mutants[1]

CO_2-Insensitive Mutants of the Green Alga *Chlorella ellipsoidea*

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A new method has been developed for screening algal photosynthetic mutants. This method uses autoradiography to assess gross photosynthetic ^{14}C fixation by green algal colonies on agar plates and allows the identification of clones that differ in photosynthetic characteristics from wild-type cells. Three wild-type cells, high-CO_2-grown *Chlorella ellipsoidea*, air-grown *C. ellipsoidea*, and air-grown *Chlorella saccharophila*, had $K_{0.5}$ values for dissolved inorganic carbon (DIC) of 1083, 250, and 50 μM, respectively, and as plaques on agar plates at Chl densities greater than 25 $\mu g\ cm^{-2}$ exhibited relative amounts of ^{14}C fixation of 15, 55, and 100%, respectively. Cells of *C. ellipsoidea* were mutagenized with x-rays and screened by this method. Growth of *C. ellipsoidea* in high CO_2 represses DIC transport and thus lowers its affinity for DIC. Five of the mutants detected by this method showed high-affinity photosynthesis similar to air-grown wild-type cells even when grown in high CO_2. Seven other mutants when grown in high CO_2 showed affinities for DIC intermediate between air-grown and high-CO_2-grown wild-type cells. The affinities of high-CO_2-grown mutants were reflected precisely in their capacities to accumulate DIC intracellularly. These results indicate that the mutants are fully or partially insensitive to the repressive effect of ambient CO_2 concentration on DIC transport.

Mutants of photoautotrophic microorganisms have usually been obtained with species that can be grown heterotrophically on an organic carbon source. A number of photosynthetic mutants, for example, have been isolated from *Chlamydomonas reinhardtii* and have been used for studies on Psis and photorespiration (Bennoun and Levine, 1967; Goodenough et al., 1969; Boynton et al., 1972; Spreitzer and Mets, 1981; Lemaire and Wollman 1989a, 1989b; Suzuki et al., 1990; Spreitzer, 1993; Bennoun, 1994; Suzuki, 1995). In all of these studies, mutants of *C. reinhardtii* were screened as strains incapable of photoautotrophic growth by utilization of the characteristic that this green alga can grow heterotrophically with supplementary acetate (Bernice and Levine, 1970).

This method has been modified when screening for mutations in the CCM or the photorespiratory pathway by selecting cells that will grow in high CO_2 but not in air. A number of such high-CO_2-requiring mutants have been isolated from *C. reinhardtii* (Spalding et al., 1983; Moroney et al., 1986, 1989; Suzuki et al., 1990) and cyanobacteria (Marcus et al., 1986; Price and Badger, 1989; Schwarz et al., 1992). However, this method selects only for mutants that have a dependence on high CO_2 for photoautotrophic growth and does not give any assessment of the rates of Psis of any other mutants that may be present. Of particular interest are mutants that do not respond to the repressive effect of high CO_2 on the induction of DIC transport systems; mutants with genetic lesions in the regulation of DIC transport would not be detectable by this method. These considerations prompted us to investigate a new screening method by which a large number of putative mutants could be directly screened on the basis of gross photosynthetic characteristics.

The CCM has been found to be the major process for the supply of CO_2 to Rubisco in microalgae under carbon limitation (Berry et al., 1976; Kaplan et al., 1980; Colman, 1989) and, therefore, a critical determinant of both the rate of photosynthetic carbon fixation and the rate of carbon flow into the photorespiratory pathway (Kaplan and Berry, 1981). However, the CCM is induced only under low-CO_2 conditions, when carbon flow through the photorespiratory pathway would be expected to increase. It has been shown that the rate of induction of the CCM in *Anabaena variabilis* during adaptation to air can be changed by altering the flow of carbon into the photorespiratory pathway by manipulating the external O_2/CO_2 ratio (Marcus et al., 1983). Furthermore, changes in some photorespiratory enzyme activities upon adaptation to air have been shown to be correlated with CCM induction in *C. reinhardtii* (Marek and Spalding, 1991). It has therefore been postulated that the induction of CCMs in *C. reinhardtii* and in *A. variabilis* occurs in response to the accumulation of photorespiratory metabolites. In contrast, it has recently been shown that the critical criterion for the induction of DIC transport in the green alga *Chlorella ellipsoidea* is the $[CO_2]$ at the cell surface (Matsuda and Colman, 1995b). This suggests that a direct sensing mechanism for external CO_2 may operate in this alga, and high $[CO_2]$ may repress gene expression for DIC transport. Mutational inactivation of the CO_2-sensing system or subsequent signal transduction may therefore cause

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Abbreviations: CA, carbonic anhydrase; CCM, inorganic carbon-concentrating mechanism; DIC, dissolved inorganic carbon; $K_{0.5}$, substrate concentration at half-maximum rate of photosynthesis; P_{max}, maximum rate of photosynthesis; Psis, photosynthesis.

partial or full derepression of DIC transport under high-CO_2 conditions. However, it would be difficult to detect mutants of this type by the usual screening method of conditional lethality, since such mutants would never be lethal under photoautotrophic conditions.

In this report, we describe a new and effective screening method for photosynthetic mutants of microalgae and the results of partial characterization of newly isolated mutants of *C. ellipsoidea*.

MATERIALS AND METHODS

Algal Cells and Mutagenesis

Chlorella ellipsoidea (UTEX 20) and *Chlorella saccharophila* (UTEX 2496) were grown axenically in batch culture as described previously (Gehl et al., 1990). High-CO_2-grown cells of *C. ellipsoidea* were obtained by aerating cultures with 6% (v/v) CO_2, harvested at mid-logarithmic phase (about 4×10^6 cells mL^{-1}), and mutagenized with x-ray irradiation of 2, 5, or 10 krad. Mutagenized cells were plated on minimal agar plates (9.0 cm i.d.), the medium of which was composed of 1.1% (w/v) purified agar with Bold's basal medium (pH 6.6). The plates, containing 0.8 to 2.0×10^3 cells per each, were kept in 6% (v/v) CO_2 under a continuous PPFD of 100 μmol m^{-2} s^{-1}. After colonies had developed, each plate was replicated to two minimal agar plates, which were then placed in the light under either 6% (v/v) CO_2 or air, and the colonies were allowed to grow for 3 to 4 d.

Screening of Mutants

Mutants were screened as follows. The replicated colonies were transferred from agar plates onto Whatman No. 1 filter paper by blotting the surface of the plate with an 8.2-cm-diameter filter paper. The filter paper disc was then placed, algal colonies facing upward, in a Petri dish on two layers of Whatman No. 1 filter paper wetted with 2.5 mL of 50 mM Bicine-NaOH, pH 8.0, which had previously been equilibrated with N_2 for 30 min at 100°C and contained less than 2.0 μM DIC. The inoculated filter papers, contained in a Petri plate, were then placed in a reclosable plastic bag (24 × 14 cm; RELOC, Adelco Inc., Oakville, Ontario, Canada), which was equipped with an injection port closed with a rubber serum stopper. The air in the plastic bag was removed by vacuum, the algal colonies were illuminated for 3 min at a PPFD of 200 μmol m^{-2} s^{-1}, and 14.8 kBq of $^{14}CO_2$ were then released from $NaH^{14}CO_3$ (2.10 GBq $mmol^{-1}$, ICN) in 1 mL of 25% (v/v) H_3PO_4 in a 20-mL gas-tight syringe and injected into the bag. The final gaseous [$^{14}CO_2$] in the bag was estimated at less than 50 nmol L^{-1}. Algal colonies were allowed to photosynthesize in the light for 30 min, after which the inoculated filter papers were dried for 4 h at 80°C. The filter papers containing the dried algal colonies were placed on x-ray film for 12 h at −80°C. The resultant autoradiographs were used to detect colonies that showed gross differences in ^{14}C incorporation. Each of the selected clones was removed from the agar plate, resuspended individually in H_2O, plated on minimal agar plates at a cell density of about 4.0×10^3 per plate, and screened again as described above. This screening protocol will be termed the ^{14}C-screening method throughout this report.

Characterization of the ^{14}C-Screening Method

Wild-type cells of *C. ellipsoidea*, grown in high CO_2 or air, and of *C. saccharophila*, grown in air, were harvested and resuspended in H_2O at various [Chl]. Twenty-microliter samples of each cell suspension were placed as circular plaques on minimal agar plates and incubated in 6% (v/v) CO_2 in the light for 1 h so that [DIC] would not vary among algal plaques. One duplicated plate was prepared for determination of Chl density as described below. The algal plaques preincubated in high CO_2 were blotted to filter paper and allowed to take up $^{14}CO_2$, and the filter paper was dried as described above. Each plaque was cut from the dried filter paper with a paper punch (6.5 mm i.d.) and suspended in 100 μL of CO_2-absorbing solution (80% [v/v] oxi-amine [RIC Co., Addison, IL]; 20% [v/v] ethanolamine [Packard, Downers Grove, IL]) and 5 mL of scintillation fluid. To measure the amount of acid-stable carbon, the punched disc of the algal plaque was incubated in 0.5 mL of 1 N HCl at 80°C overnight, after which the dried disc was resuspended in scintillation fluid. Radioactivity on the filter paper was measured by liquid scintillation counting. Chl density was measured as follows. The duplicated algal plaques were transferred to filter paper and allowed to dry. Discs were punched out of the algal plaques and soaked in 100 μL of DMSO for 1 h to extract the Chl from the dried algae, 1 mL of methanol was added, and the [Chl] was determined spectrophotometrically as described previously (Gehl and Colman, 1985).

Inoculated filter papers with 1.5 to 4 times higher colony densities than that in the primary screening were illuminated at a PPFD of 200 μmol m^{-2} s^{-1} for 3 min in a sealed bag as described above. Five microliters of buffer were then taken out of the side of the filter paper, and the [DIC] was measured by GC (Birmingham and Colman, 1979). Changes in [DIC] in algal plaques during screening were estimated as follows. Filter paper discs of 6.5 mm in diameter without and with cells of *C. ellipsoidea* or *C. saccharophila*, both grown in air at a Chl density of 20 to 30 μg cm^{-2}, were placed on filter paper wetted with 50 mM Bicine-NaOH, pH 8.0, containing 200 μM DIC in a Petri plate and kept under N_2. The discs were illuminated at a PPFD of 200 μmol m^{-2} s^{-1} for 3, 13, 23, and 33 min, taken out of the Petri plate, and soaked in 200 μL of N_2-equilibrated H_2O in microcentrifuge tubes. Fifty microliters of H_2O were immediately taken out of each microcentrifuge tube, and the [DIC] was measured by GC. The water contents of the filter paper discs were estimated at 5.8 ± 0.26 (n = 8) and 6.0 ± 0.13 μL (n = 15) without and with algal cells, respectively.

Characterization of Isolated Mutants

Twenty microliters of cell suspension of each mutant were placed as circular plaques on minimal agar plates and

allowed to grow under high CO_2 or air. The relative levels of ^{14}C fixation among the mutants were estimated using the ^{14}C-screening method followed by the measurement of radioactivity on the surface of the algal plaques with an Instant Imager (Packard Instrument Co., Meriden, CT). Isolated mutants were transferred to liquid culture and aerated with 6% (v/v) CO_2 in Bold's basal medium. Air-adapted cells were obtained by transferring high-CO_2 cells to air for 2 d. Cells at mid-logarithmic phase (A_{730} 0.2–0.4) were harvested by centrifugation at 4500*g* for 3 min, washed with H_2O and N_2-equilibrated 50 mM Na^+/K^+-phosphate buffer, pH 7.8 (containing less than 2 μM DIC), and resuspended in the same buffer at a [Chl] of 40 μg mL^{-1}.

The rate of Psis was measured in a Clark-type O_2 electrode with or without the addition of bovine CA as described previously (Matsuda and Colman, 1995a). The apparent $K_{0.5}$[DIC] was determined as described by Rotatore and Colman (1991). The CO_2-compensation concentration was determined by a GC method. The intracellular [DIC] was determined using the silicone-oil centrifugation technique (Matsuda and Colman, 1995a). Chl recovery after the silicone-oil centrifugation was monitored by centrifuging cells through the silicone-oil layer into DMSO, followed by spectrophotometric measurement of the recovered Chl as described by Gehl and Colman (1985).

RESULTS

Characterization of the ^{14}C-Screening Method

The effectiveness of the ^{14}C-screening method was tested on two algal species grown under conditions known to provide cells with distinct physiological characteristics: high-CO_2- and air-grown *C. ellipsoidea* and air-grown *C. saccharophila*. *C. ellipsoidea* grown in air had a much lower $K_{0.5}$[DIC] than that of *C. ellipsoidea* grown in high CO_2 (Fig. 1). This difference in photosynthetic affinity for DIC has been attributed to differences in the activities of DIC transport between these cells (Matsuda and Colman, 1995a). Furthermore, the lower $K_{0.5}$[DIC] value observed in air-grown *C. saccharophila* as compared to that of air-grown *C. ellipsoidea* (Fig. 1) has been shown to be due to the occurrence of external CA in *C. saccharophila* (Gehl et al., 1990; Williams and Colman, 1995). Cells of air- and high-CO_2-grown *C. ellipsoidea* showed maximal ^{14}C incorporations of about 55 and 15%, respectively, of that in air-grown *C. saccharophila* (Fig. 1). The ratios of ^{14}C incorporation among these cells were constant at Chl densities of greater than 25 μg cm^{-2} and reflected the difference in photosynthetic affinity for DIC among these cells (Fig. 1). At a Chl density of 25 μg cm^{-2}, algal plaques were pale green on the agar plate.

The acid-stable components of incorporated ^{14}C with this method amounted to 88.2 ± 2.5, 89.8 ± 4.3, and 89.0 ± 1.3% in air- and high-CO_2-grown *C. ellipsoidea* and air-grown *C. saccharophila*, respectively.

The initial [DIC] in inoculated filter papers immediately before the ^{14}C-fixation reaction varied depending on the cell density on the filter paper. When plates (9.0 cm i.d.) with 0 or more than 30,000 well-developed colonies were used, both of which were incubated in 6% (v/v) CO_2, [DIC]s in the inoculated filter papers were 55 ± 5 (n = 4) or 487 ± 40 μM (n = 6), respectively. With colony densities of 1.5 to 4 times that in the primary screening, the initial [DIC]s were estimated at 145.2 ± 13.0 (n = 3) and 204.0 ± 57.4 μM (n = 4) when screening air- and high-CO_2-grown colonies, respectively. The [DIC] in filter paper discs with *C. ellipsoidea* and *C. saccharophila*, both grown in air, was shown to be stable during screening at 295 ± 22 and 351 ± 23 μM (n = 16), respectively, whereas the [DIC] in filter paper discs without algal cells was constant at 196 ± 29 μM (n = 16) throughout the screening reaction.

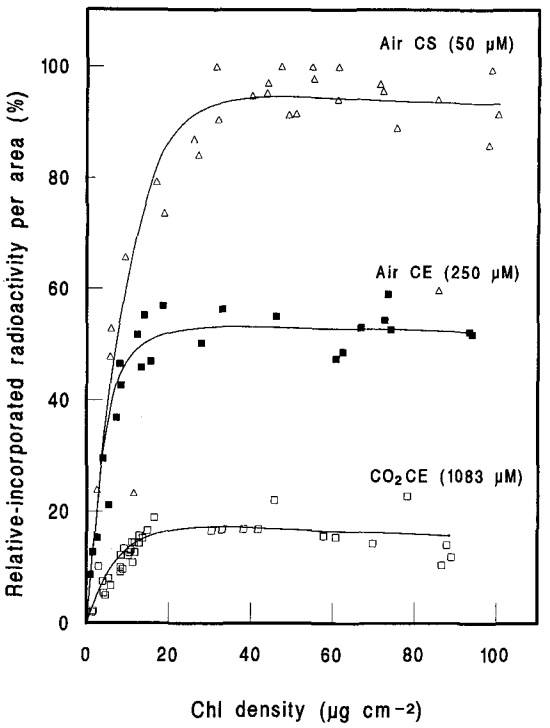
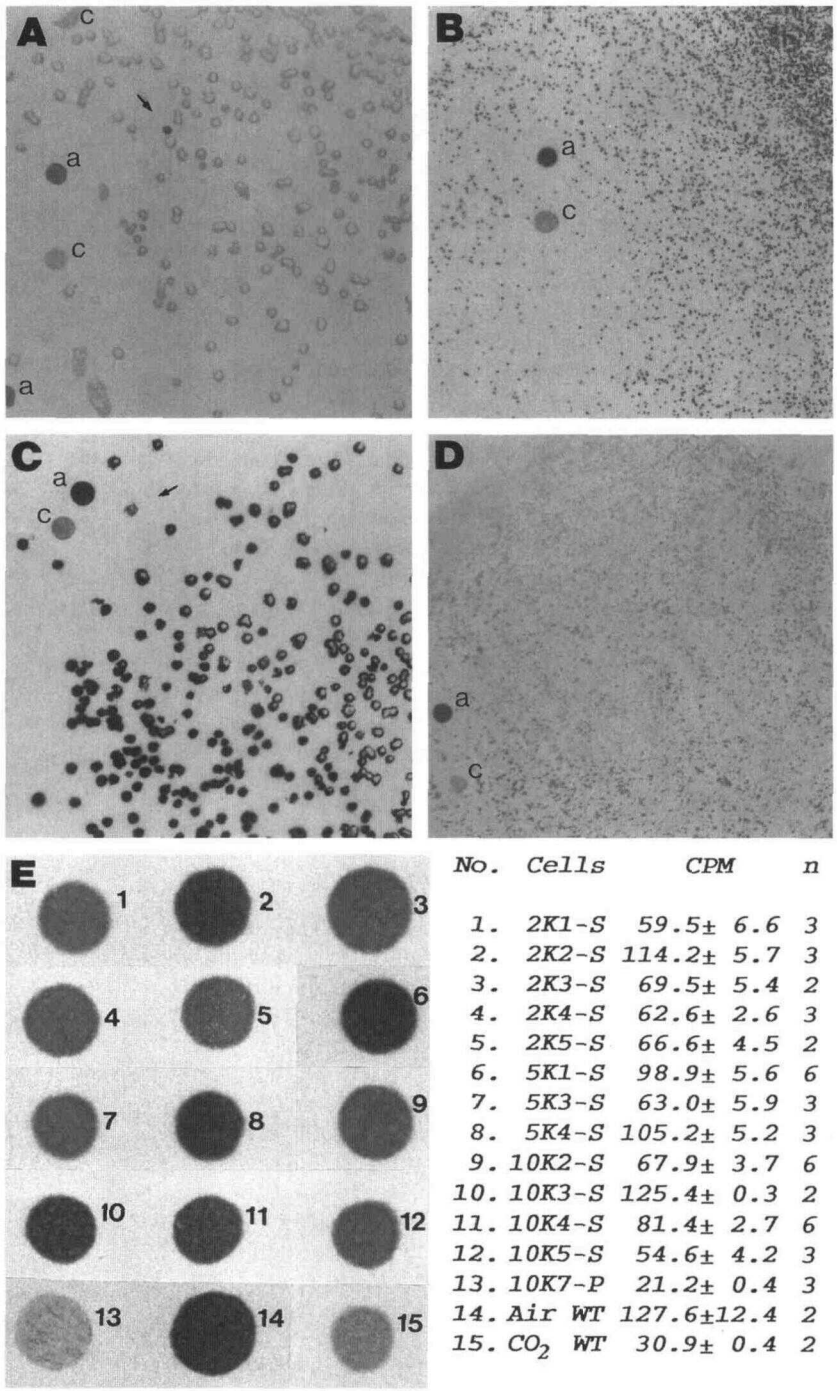


Figure 1. Relationship between relative ^{14}C incorporation and Chl density in the ^{14}C-screening method in three different wild-type *Chlorella* cells. △, Air-grown *C. saccharophila* (CS); ■, air-grown *C. ellipsoidea* (CE); □, high-CO_2-grown *C. ellipsoidea* (CE). Incorporated ^{14}C radioactivity is indicated as a percentage of the maximum ^{14}C fixation in air-grown *C. saccharophila* (100%). $K_{0.5}$[DIC] values of *C. saccharophila* and *C. ellipsoidea* measured at pH 8.0 and 7.8, respectively, are given in parentheses. These data are from four experiments.

Isolation of Mutants

The autoradiographic patterns from primary and secondary screening of putative *C. ellipsoidea* mutants (Fig. 2, A and B) indicated the occurrence of 12 mutants that, when grown in high CO_2, showed ^{14}C incorporation higher than high-CO_2-grown wild-type cells (type 1). Mutation frequencies were 6.7×10^{-4}, 3.3×10^{-4}, and 2.5×10^{-4} with x-ray irradiations of 2, 5, and 10 krad, respectively. On the



No.	Cells	CPM	n
1.	2K1-S	59.5± 6.6	3
2.	2K2-S	114.2± 5.7	3
3.	2K3-S	69.5± 5.4	2
4.	2K4-S	62.6± 2.6	3
5.	2K5-S	66.6± 4.5	2
6.	5K1-S	98.9± 5.6	6
7.	5K3-S	63.0± 5.9	3
8.	5K4-S	105.2± 5.2	3
9.	10K2-S	67.9± 3.7	6
10.	10K3-S	125.4± 0.3	2
11.	10K4-S	81.4± 2.7	6
12.	10K5-S	54.6± 4.2	3
13.	10K7-P	21.2± 0.4	3
14.	Air WT	127.6±12.4	2
15.	CO_2 WT	30.9± 0.4	2

Figure 2. Autoradiographs of *C. ellipsoidea* colonies or plaques on agar plates. A and B, Primary and secondary screening of 5K1-S, respectively (the arrow indicates a 5K1-S colony); C and D, primary and secondary screening, respectively, of 10K7-P (the arrow indicates a 10K7-P colony); E, comparison of ^{14}C radioactivity incorporated by high-CO_2-grown type-1 (1-12) and air-grown type-2 (13) mutants and wild-type cells (14 and 15) with the surface cpm of each algal plaque. Values are means ± SE of *n* replicates. For all screenings, positive and negative markers of air-grown (a) and high-CO_2-grown (c) *C. ellipsoidea* were placed on plates as circular plaques (A-D).

other hand, only one mutant showed low ^{14}C incorporation (type 2) when grown in air (Fig. 2, C and D).

Comparisons of ^{14}C incorporation using the ^{14}C-screening method showed that the type-1 mutants grown in high CO_2 yielded a wide range of surface radioactivity from about 55 to 125 cpm per plaque, whereas wild-type cells grown in high CO_2 yielded about 30 cpm (Fig. 2E). In contrast, the single type-2 mutant grown in air yielded about 20 cpm, whereas wild-type cells grown in air showed about 130 cpm (Fig. 2E).

Partial Characterization of the Isolated Mutants

The mutants of *C. ellipsoidea* were grown in liquid culture in high CO_2 and their apparent affinities for DIC were determined by O_2 electrode analysis. Type-1 mutants were able to be classified into two major subclasses, 1a and 1b. Five mutants designated as 2K2-S, 5K1-S, 5K4-S, 10K3-S, and 10K4-S (type 1a), when grown in high CO_2, showed levels of photosynthetic affinity for DIC comparable to those of air-grown wild-type cells with and without added

CA (Table I). These high affinities did not change markedly upon transfer of cells to air (Table I). Plots of photosynthetic rate against [DIC] indicated that high-CO_2-grown 5K1-S had photosynthetic kinetics nearly identical with those of 5K1-S grown in air with or without added CA (Fig. 3A). Among these mutants, 2K2-S and 10K3-S, grown in high CO_2, exhibited significantly lower P_{max} than wild-type cells (Table I). All type-1a mutants, irrespective of growth conditions, had $K_{0.5}$[DIC] values similar to those of air-grown wild-type cells (Table I) and the addition of bovine CA to mutant cells decreased their $K_{0.5}$[DIC] to that of air-adapted cells in the presence of added CA (Table I). In contrast, seven mutants, 2K1-S, 2K3-S, 2K4-S, 2K5-S, 5K3-S, 10K2-S, and 10K5-S (designated as type 1b), when grown in high CO_2, exhibited $K_{0.5}$[DIC] values without added CA of between 350 and 530 μM in contrast to values of 250 and 1083 μM, respectively, for air- and high-CO_2-grown wild-type cells (Table I). The addition of bovine CA to the high-CO_2-grown type-1b mutants decreased $K_{0.5}$[DIC] values to 43 to 63% of those without added CA, and CA addition to air- and high-CO_2-grown wild-type cells lowered $K_{0.5}$[DIC] values to 24 and 74% of those without added CA, respectively (Table I; Fig. 3B). After the cells had adapted to air for 2 d, these intermediate $K_{0.5}$ values decreased and reached the levels observed in air-grown wild-type cells with and without added CA (Table I; Fig. 3B).

Plots of photosynthetic rate against [DIC] for high-CO_2-grown 5K3-S showed clear differences in photosynthetic affinities between high-CO_2- and air-grown cells (Fig. 3B) and between high-CO_2- and air-grown wild-type cells (Fig. 3C). The P_{max} of high-CO_2-grown type-1 mutants, except for 2K2-S and 10K3-S, varied from 87 to 147 μmol O_2 mg^{-1} Chl h^{-1} (Table I), but such variation was also observed in wild-type cells (data not shown).

The type-2 mutant designated 10K7-P had a P_{max} that was about 35 to 50% of that in the wild-type cells independent of growth conditions (Table I). However, unlike the type-1 mutants, high-CO_2-grown 10K7-P had the typical low-affinity Psis observed in high-CO_2-grown wild-type cells but acquired high-affinity Psis after adaptation to air (Table I; Fig. 3D). In both high-CO_2- and air-grown mutants, the high affinity for DIC was reflected in low CO_2-compensation concentrations (Table I).

As a measure of the cells' capacity to take up HCO_3^-, the rates of Psis without added CA at 100 μM DIC and pH 7.8 were compared with the spontaneous dehydration rate of HCO_3^- (10.8 nmol mL^{-1} min^{-1}; Matsuda and Colman, 1995a). The contribution of CO_2 alone to Psis in wild-type *C. ellipsoidea* grown in air under the same conditions was estimated according to a mathematical method described by Williams and Colman (1995). The absolute rates of Psis due to only CO_2 took the form of a rectangular hyperbola when [DIC] was held constant and [Chl] was increased (data not shown) and approached 6.4 ± 0.8 nmol mL^{-1} min^{-1} (n = 3) at [Chl]s greater than 20 mg L^{-1}. These data indicate that the theoretical maximum contribution of CO_2 to Psis, 10.8 nmol mL^{-1} min^{-1}, is a rather conservative estimate and, therefore, valid as the criterion of HCO_3^- uptake. All high-CO_2-grown type-1a mutants and four of the seven type-1b mutants, 2K1-S, 2K5-S, 5K3-S, and 10K5-S, showed the capacity to evolve O_2 at rates exceeding 10.8 nmol mL^{-1} min^{-1} (Table I), indicating that they could use HCO_3^- as a source of substrate for Psis.

Cells of all of the mutant strains, grown in liquid culture, were tested for their capacity to accumulate DIC using the

Table I. *Photosynthetic characteristics of wild-type cells and isolated mutants of C. ellipsoidea grown in high CO_2 or adapted to air*

Values were determined using cell suspensions of 40 μg Chl mL^{-1} at pH 7.8 and 25°C, with and without added CA.

Cells	High CO_2 Grown						Air Adapted				
	Comp pt[a]	$^-$CA			$^+$CA		Comp pt[a]	$^-$CA		$^+$CA	
		PS rate[b]	$K_{0.5}$[c]	P_{max}[d]	$K_{0.5}$[c]	P_{max}[d]		$K_{0.5}$[c]	P_{max}[d]	$K_{0.5}$[c]	P_{max}[d]
WT[e]	26.0 (0.8)[f]	3.6 (0.9)[f]	1083	95	800	95	3.4 (0.8)[f]	250	98	59	98
Type 1a											
5K1-S	3.5 (0.2)[f]	15.5 (1.0)[f]	201	97	71	96	1.9	226	94	56	96
5K4-S	1.9	16.6	188	87	78	95	4.6	313	110	69	110
10K4-S	2.9	16.6	213	104	74	101	2.4	230	125	71	115
2K2-S	4.9	12.2	206	66	81	72	2.2	219	90	67	88
10K3-S	4.4	13.5	128	53	60	60	2.8	211	85	58	82
Type 1b											
2K1-S	6.9 (0.7)[f]	11.7 (0.4)[f]	447	144	226	137	2.7	249	124	65	132
2K3-S	9.4	9.3	531	135	341	130	5.5	325	115	67	118
2K4-S	8.8	10.7	497	147	288	142	4.0	279	120	64	120
2K5-S	5.1	12.7	369	124	158	115	3.5	286	133	58	138
5K3-S	7.3 (0.6)[f]	11.4 (0.4)[f]	500	125	287	135	1.9	207	100	57	105
10K2-S	6.8	10.7	425	113	219	102	2.1	187	89	57	89
10K5-S	4.6	14.3	350	138	165	143	2.3	285	135	55	133
Type 2											
10K7-P	30.4	2.2	543	49	475	49	2.4	89	37	30	35

[a] CO_2-compensation concentration (μL CO_2 L^{-1}) measured with added CA. [b] Rate of Psis (nmol mL^{-1} min^{-1}) at 100 μM DIC. [c] $K_{0.5}$[DIC] in micromolar. [d] Expressed as μmol O_2 mg^{-1} Chl h^{-1}. [e] Wild-type *C. ellipsoidea*. [f] Values are the means (SE) of three replicated experiments.

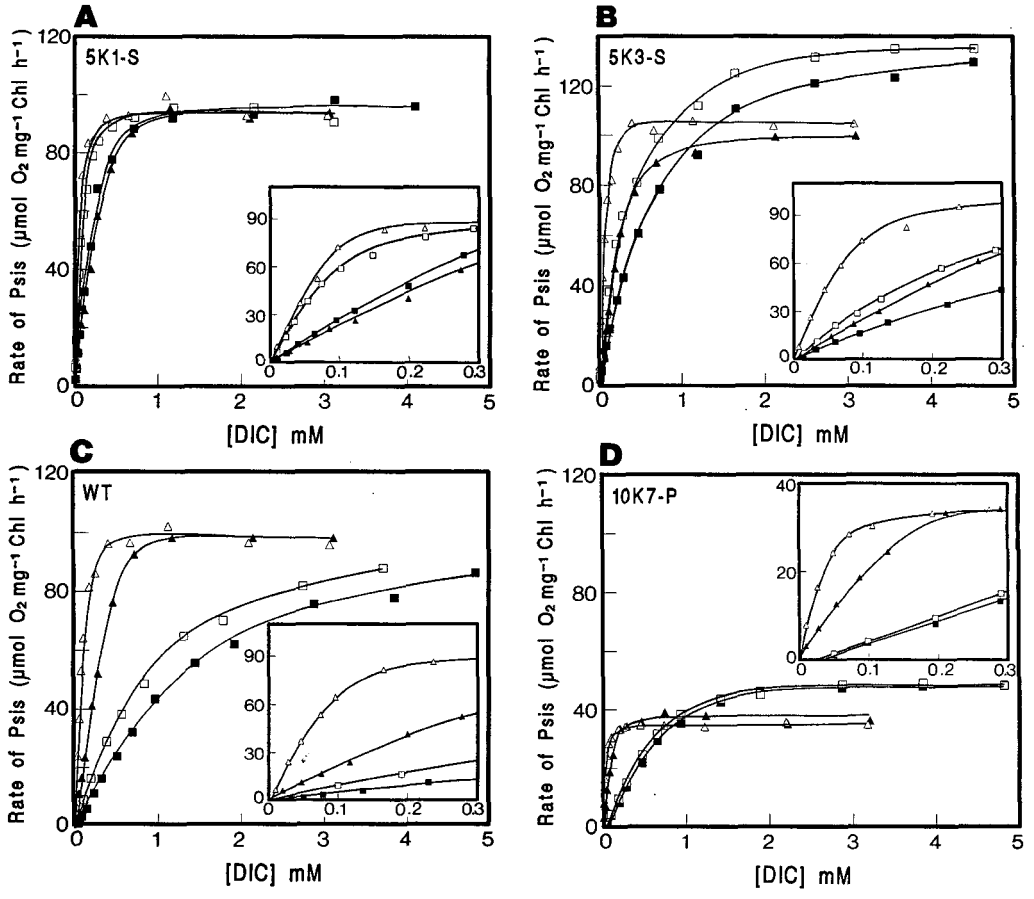


Figure 3. Rates of Psis at various [DIC] of wild-type or mutant cells of *C. ellipsoidea* at pH 7.8 and 25°C. A, 5K1-S mutant cells; B, 5K3-S mutant cells; C, wild-type cells; D, 10K7-P mutant cells. Shown are curves for high-CO_2-grown cells without CA (■) and with added CA (□) and air-grown cells without CA (▲) and with added CA (△). Insets, Rates of Psis at [DIC] of less than 0.3 mM.

silicone-oil centrifugation technique. High-CO_2-grown 5K1-S was found to accumulate intracellular DIC at levels similar to those in air-grown wild-type cells; intracellular [DIC]s at an initial DIC of 100 μM and pH 7.8 were 294 and 490 nmol mg^{-1} Chl and those in air-grown wild-type cells were 367 and 517 nmol mg^{-1} Chl, without and with added CA, respectively (Fig. 4). Two high-CO_2-grown mutants of type 1b, 2K1-S and 5K3-S, accumulated 218 and 205 nmol DIC mg^{-1} Chl, respectively, without added CA (Fig. 4). With added CA, 2K1-S and 5K3-S accumulated 360 and 304 nmol DIC mg^{-1} Chl, respectively. These levels of accumulation were about 55 to 70% of those in air-grown wild-type cells but 6- to 8-fold higher than those in high-CO_2-grown wild-type cells (Fig. 4) both with and without added CA. These levels of accumulation resembled those in wild-type cells adapted to air for 3 to 4 h (Matsuda and Colman, 1995a). The internal cell volume of these mutants, estimated by the silicone-oil centrifugation method, ranged between 50 and 85 μL mg^{-1} Chl, values that are not significantly different from those observed in wild-type cells in logarithmic phase (77.1 ± 2.5 μL mg^{-1} Chl, n = 33) (Matsuda and Colman, 1995a). The calculated intracellular [DIC]s based on internal cell volume were 6.3 and 9.6 mM in high-CO_2-grown 5K1-S and those in air-grown wild-type cells averaged 6.3 and 8.1 mM without and with added CA, respectively.

DISCUSSION

Mutagenesis of photoautotrophic microorganisms may not always result in lethal or conditionally lethal mutations but sometimes merely alter the rate of Psis under limited or saturated [DIC]. It would be useful, therefore, to have a screening method that could be used to assess rapidly the rate of net Psis in a large number of possible mutants. We have developed such a rapid screening method and have evaluated its effectiveness using two closely related species of *Chlorella*.

Three kinds of wild-type cells used for evaluation of the ^{14}C-screening method have previously been shown to be different in photosynthetic affinity for DIC, and these differences are due to either the occurrence of external CA or active DIC transport (Gehl et al., 1990; Matsuda and Colman 1995a; Williams and Colman, 1995). Visible differences on autoradiographs of ^{14}C-labeled algal plaques among these wild-type cells reflected their photosynthetic affinities for DIC (data not shown). This is supported by direct measurements of ^{14}C radioactivity incorporated by

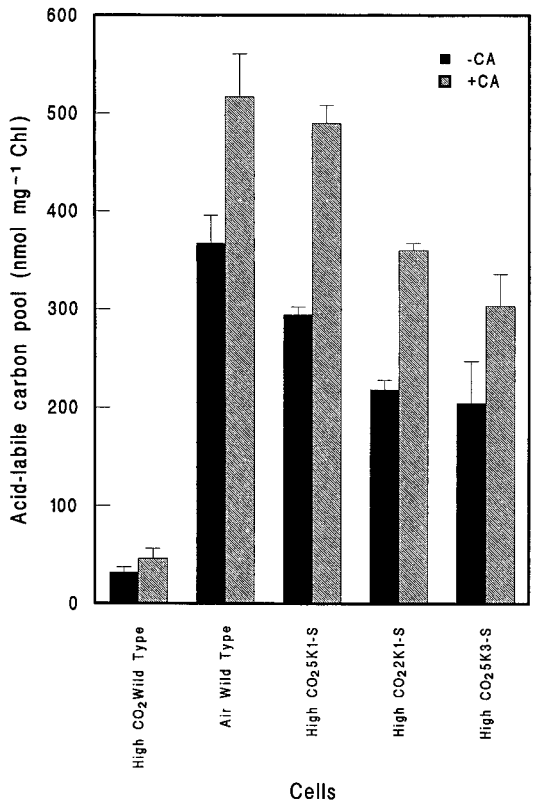


Figure 4. Intracellular accumulation of acid-labile DIC in wild-type cells grown in high CO_2 or air and in three mutant strains, 5K1-S, 2K1-S, and 5K3-S, grown in high CO_2. Values were determined by the silicone-oil centrifugation method at 100 μM DIC, pH 7.8, with or without added CA. Values are the means ± SE of two to six replicate experiments.

these algal plaques and measurement of the Chl density of the plaques (Fig. 1), which therefore validate the ^{14}C-screening method.

Photosynthetic ^{14}C fixation in this screening method was apparently a function of cell density below 25 μg Chl cm^{-2} in all types of cells but remained constant above this cell density (Fig. 1). From the total cell volume of *C. ellipsoidea* of 191.2 ± 5.3 μL mg^{-1} Chl (n = 36), as determined by the silicone-oil centrifugation method, the thickness of a colony of 25 μg Chl cm^{-2} is estimated to be 47.8 μm, which would comprise 10 to 20 layers of cells. Given this, ^{14}C fixation would occur only at the surface of the algal plaque, which was exposed to the gaseous $^{14}CO_2$. Cells below this layer would be light limited or outside the range of $^{14}CO_2$ diffusion. The constant [DIC] in the filter paper discs indicates that little DIC is taken up from the filter paper disc, presumably because the layers of nonphotosynthesizing algae in the plaque constitute a diffusion barrier to CO_2 and HCO_3^-.

Incorporation of $^{14}CO_2$ by this screening method clearly reflects the affinity of the different types of cells for DIC but it is difficult to specify the detailed mechanistic basis for these differences. If we assume that $^{14}CO_2$ is absorbed in the alkaline liquid layer on the cell surface, it would be rapidly hydrated to form HCO_3^-. High-CO_2-grown *C. ellipsoidea* has a limited capacity to take up both CO_2 and HCO_3^- (Matsuda and Colman, 1995a). In contrast, air-adapted cells have the capacity to take up both CO_2 and HCO_3^- by active transport, but the affinity of cells for CO_2 is higher than that for HCO_3^- (Williams and Colman, 1995), so air-adapted cells of *C. ellipsoidea* will be CO_2 limited and take up HCO_3^-. However, *C. saccharophila* has external CA and would not be CO_2 limited but would take up $^{14}CO_2$ rapidly. This would explain the difference in ^{14}C incorporation by the two species (Fig. 1).

During ^{14}C screening, 13 colonies exhibited ^{14}C incorporation distinct from other colonies and the mutants were readily detected on autoradiographs (Fig. 2). Radioactivity measured at the surface of mutant plaques reflected the cells' affinities for DIC (Fig. 2E; Table I), and the degree of ^{14}C incorporation allowed us to characterize them by visual estimation. These results again confirm the quantitative validity of the ^{14}C-screening method.

Most of the type-1a mutants grown in high CO_2 exhibited physiological properties nearly identical with those in wild-type cells grown in air, and these did not change significantly after the mutants had adapted to air (Table I; Fig. 3, A and C). Our previous studies showed that the degree of adaptation of high-CO_2-grown *C. ellipsoidea* to air is closely correlated with the level of acid-labile carbon accumulation and $K_{0.5}$[DIC] values (Matsuda and Colman, 1995a). These values are critical in assessing the degree of induction of DIC transport systems in this green alga. The $K_{0.5}$[DIC] values observed in type-1a mutants grown in high CO_2 and air were comparable to those in air-grown wild-type cells (Table I; Fig. 3, A and C). This suggests that active DIC transport operates constitutively in these mutants grown in high CO_2.

Alternatively, changes in photosynthetic efficiency such as the CO_2/O_2 specificity factor of Rubisco might account for the high-affinity Psis in high-CO_2-grown mutants. This possibility was recently suggested by Suzuki (1995) for revertants of phosphoglycolate phosphatase-deficient mutants of *C. reinhardtii*. These revertants are able to grow photoautotrophically in air, despite the lethality of air to the original strain (18–7F), because of the absence of phosphoglycolate phosphatase. High-affinity Psis exhibited by high-CO_2-grown revertants could not be explained by the operation of a CCM, since periplasmic CA was absent (Suzuki, 1995). It was therefore postulated that these revertants were rather the result of a significant improvement of CO_2/O_2 specificity of Rubisco (Suzuki, 1995). In contrast, all type-1a mutants of *C. ellipsoidea* showed rates of Psis equivalent to that of air-grown wild-type cells at 100 μM DIC without added CA (12.2–16.6 nmol O_2 mL^{-1} min^{-1}, Table I), and these values were significantly higher than the spontaneous supply rate of CO_2 from HCO_3^- (10.8 nmol mL^{-1} min^{-1}). This indicates a direct uptake of HCO_3^- by the cells. The addition of bovine CA caused the same decrease in $K_{0.5}$[DIC] as in air-grown wild-type cells independent of growth CO_2 conditions (Table I), indicating the existence of an active CO_2-transport system that is not saturated by CO_2 at the rate that is maintained by the

spontaneous dehydration of HCO_3^-. Furthermore, there was no significant difference in CO_2-compensation concentrations among high-CO_2- and air-grown type-1a mutants and air-grown wild-type cells (Table I). In addition, the levels of acid-labile carbon accumulation in high-CO_2-grown 5K1-S were similar to those in air-grown wild-type cells and more than 10 times that in high-CO_2-grown wild-type cells with and without added CA (Fig. 4). This tendency was also observed in other type-1a mutants (data not shown). These results clearly indicate that active transport of both CO_2 and HCO_3^- constitutively operates at maximum activity in these type-1a mutants and confirm that they have high-affinity Psis even when grown in high CO_2.

It has been shown that the adaptation of *C. ellipsoidea* from high CO_2 to air occurs in response exclusively to [CO_2] in the medium, and neither intracellular [DIC] nor light-dependent metabolism is involved in this process (Matsuda and Colman, 1995b). It is probable that the sensing of high CO_2 at the surface of *C. ellipsoidea* cells represses the expression of DIC transport. Type-1a mutants may be fully impaired in this repressive signal transduction and therefore exhibit a CO_2-insensitive phenotype. If this is the case, type-1b mutants pose an intriguing problem in that all high-CO_2-grown cells of these mutants showed photosynthetic characteristics that were intermediate between those of high-CO_2-grown wild-type cells and air-grown wild-type cells (Table I). The values of $K_{0.5}$[DIC] and CO_2-compensation concentration of high-CO_2-grown type-1b mutants are equivalent to those in wild-type *C. ellipsoidea* adapted from high CO_2 to air for 2 to 4 h (Matsuda and Colman, 1995a) or those in wild-type cells adapted to intermediate [CO_2] between 40 and 120 μM for 5 h (Matsuda and Colman, 1995b). It is probable, therefore, that these mutants have intermediate activities of CO_2 and HCO_3^- transport. This view is supported by the finding of intermediate levels of accumulation of acid-labile carbon in high-CO_2-grown 2K1-S and 5K3-S cells (Fig. 4). DIC accumulation in these mutants adapted to air was similar to that in air-grown wild-type cells. Similar levels of DIC accumulation were observed in all other type-1b mutants grown in high CO_2 or air (data not shown). Since air-adapted type-1b mutants showed wild-type Psis (Table I), the simplest and most plausible interpretation of this phenotype may be that these mutants are partially impaired in the signal transduction process for CO_2 sensing.

This newly developed screening method has been shown to be effective not only in detecting photosynthetic mutants of green algae on the basis of photosynthetic affinity for DIC but also in comparing these affinities under DIC-limited conditions. With some modification, this method may also be effective for selecting mutants on the basis of other parameters of Psis; for example, 10K7-P was detected on the basis of its low P_{max} (Fig. 2).

Most of the isolated mutants of *C. ellipsoidea* exhibited a distinct phenotype of full or partial insensitivity to high CO_2. These data strongly support our previous finding that a CO_2-sensing mechanism operates at the algal cell surface and regulates gene expression for DIC transport (Matsuda and Colman, 1995b). The CO_2-sensing mechanism probably represses DIC transport under high-CO_2 conditions and, therefore, the abrupt activation of DIC transport upon transfer of high-CO_2-grown *C. ellipsoidea* to air (Matsuda and Colman, 1995a) may be due to the derepression of DIC transport in the absence of a high [CO_2] at the cell surface.

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