

## REWINDING THE TAPE: SELECTION OF ALGAE ADAPTED TO HIGH CO<sub>2</sub> AT CURRENT AND PLEISTOCENE LEVELS OF CO<sub>2</sub>

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**Abstract.**—Selective history is thought to constrain the extent and direction of future adaptation by limiting access to genotypes that are advantageous in a novel environment. Populations of *Chlamydomonas* previously selected at high CO<sub>2</sub> were either backselected at ambient levels of CO<sub>2</sub>, or selected at levels of CO<sub>2</sub> that last occurred during glaciation in the Pleistocene. There was no effect of selective history on adaptation to either level of CO<sub>2</sub>, and the high CO<sub>2</sub> phenotypes were evolutionarily reversible such that fitness in ambient CO<sub>2</sub> returned to values seen in controls. CO<sub>2</sub> uptake affinity improved relative to the ancestor in both ambient and glacial CO<sub>2</sub>, although wild-type regulation of CO<sub>2</sub> uptake, which deteriorated during previous selection at high CO<sub>2</sub>, was not restored by selection at lower levels of CO<sub>2</sub>. Trade-offs in both CO<sub>2</sub> uptake affinity and growth were seen after selection at any given level of CO<sub>2</sub>. Adaptation to ambient and glacial-era levels of CO<sub>2</sub> produced a range of phenotypes, suggesting that chance rather than selective history contributes to the divergence of replicate populations in this system.

**Key words.**—Adaptation, CO<sub>2</sub>, chance, divergence, history, reverse selection.

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One of the most influential ideas in evolutionary biology is that of the importance of historical contingency on adaptive outcomes, which is often articulated as the thought experiment of “replaying life’s tape” (Gould 1989, pp. 320–321). Several experiments have demonstrated that replicate populations tend to become convergently adapted while diverging in terms of underlying characters such as specific mutations, physiology, or morphology (Travisano and Lenski 1996; Travisano 1997; Nakatsu et al. 1998; Riley et al. 2001; Teotónio et al. 2002; Yedid and Bell 2002; MacLean and Bell 2003). These have confirmed experimentally that adaptive outcomes tend to be unique at some level, even over relatively short timescales. History contributes to this when an ancestral state constrains future adaptation by limiting the range of beneficial changes that can evolve from some particular starting point. The most fundamental way that this has been shown to occur is by limiting access to beneficial mutations by creating a mutational neighborhood defined by the set of mutants one or a few mutational steps away from the ancestral state (Burch and Chao 2000). The consequence of this sort of constraint is that populations subjected to the same selection regime do not all end up with the same genotype, so that genotypes and phenotypes differ between replicate populations, and some populations may even fail to adapt to subsequent environmental changes. Since lineages are subjected to many different environments over time, adaptation to one environment may affect the chances of adapting to a subsequent environment by limiting or biasing the range of genotypes available for selection.

A second reason that similar populations subjected to the same selection pressure may diverge is that the appearance and spread of novel beneficial mutations is a stochastic process. As a result of finite population sizes and relatively large genomes, only a small fraction of possible combinations of mutations occur every generation. In addition, beneficial mu-

tations are often lost to chance when rare. This creates, by chance, early differences between replicate populations. These differences constrain further adaptation since epistatic interactions now limit the beneficial states accessible by unit genetic change in a given population. The result is that the order of beneficial mutations that fix in a particular population is idiosyncratic (Muller 1939; Mani and Clarke 1990; Korona 1996). In contrast, a higher degree of convergent genetic evolution has been seen in retroviruses, where genome size is small and population size large, such that the order of fixation of mutations becomes less stochastic (Cuevas et al. 2002). The divergence between replicate populations initially selected in one environment may increase the range of adaptive outcomes seen in subsequent environments, in that populations with similar phenotypes may have access to very different beneficial mutations in the novel environment. Alternately, selective history may systematically bias subsequent adaptive outcomes if convergent adaptation occurred in the first environment. For example, it has been suggested that the ability of contemporary plants to adapt to a high CO<sub>2</sub> world is constrained by past adaptation to lower CO<sub>2</sub>, as shown by constitutively high levels of Rubisco, Rubisco activase, and carbonic anhydrase across taxa (Coleman 2000; Sage and Coleman 2001). The relative contributions of adaptation, history, and chance to microevolutionary outcomes can be quantified in selection experiments using several different ancestors, each used to found several replicate populations. All three factors have been found to contribute to outcomes with chance and history playing relatively small roles in the evolution of fitness and characters closely correlated with fitness (Travisano et al. 1995).

One of the consequences of a given population evolving idiosyncratically is that evolution should be irreversible, given that the probability of retracing a specific series of mutational events by back mutation is small relative to the chance of adapting by compensatory mutation. However improbable the reversal of evolution is at a genetic level or over very long timescales, several examples of reversible phe-

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notypic evolution have been seen in microevolution experiments (Travisano et al. 1995; Teotónio et al. 2002; Estes and Lynch 2003). Back-selection experiments can examine whether and how phenotypes are evolutionarily reversible; they can also give important insights into the current biology of a system and allow us to study how fitness is recovered. The reversibility of phenotypes over evolutionary time is of particular interest in environments that fluctuate very slowly relative to the life cycle of the organism, such as variations in CO<sub>2</sub> and temperature during glacial-interglacial cycles. This forces populations to become repeatedly adapted to two recurring environments over evolutionary time. Although environmental fluctuation at shorter timescales has been shown to cause the evolution of generalist types (Reboud and Bell 1996), it is unclear whether fluctuations over hundreds or thousands of generations systematically affect adaptive outcomes or current biology. As with short-term environmental fluctuations, any adaptation to current conditions that completely inhibited adaptation to future conditions would not persist through more than half a cycle of environmental change. Over several cycles, this could result in a system that was selected for long-term evolvability. Adaptation to a fluctuating environment over microevolutionary timescales has been shown to result in a "bet-hedging" strategy, where the lineage that persists over time may not be the one that performs best in the average environment, but rather the one that is able to avoid performing poorly (or going extinct) in the most hostile environment encountered (Gillespie 1973, 1974). This strategy results through repeated reversals of adaptive trends over shorter timescales (Simons 2002). Reverse evolution may also be of interest in practical questions such as the evolution of antibiotic resistant bacteria or in environmental cleanups, involving reductions in concentrations of nutrients or toxins for bacterial, algal, and plant populations that have become adapted to pollutants. Reverse evolution and fitness recovery are also important in the conservation of bottlenecked populations where adaptedness may have been degraded by mutation accumulation.

An interesting case of long-term environmental fluctuations is that of changes in atmospheric CO<sub>2</sub>. Estimates of past CO<sub>2</sub> levels based on CO<sub>2</sub> concentrations in gas bubbles in ice cores (Barnola et al. 1991) and other methods (for examples see Shackleton and Pisias 1985; Beerling et al. 1995) indicate that atmospheric CO<sub>2</sub> levels have fluctuated by at least 80 ppm over glacial-interglacial cycles. During the last Pleistocene glaciation that ended about 10,000 years ago, CO<sub>2</sub> levels are thought to have been about 200 ppm. CO<sub>2</sub> levels then rose to a preindustrial level of about 280 ppm. Since the beginning of the industrial revolution CO<sub>2</sub> levels have continued to rise, largely due to anthropogenic activity, and are expected to roughly double in the next century from present-day levels of about 400 ppm (Watson et al. 2001). Although it is obviously necessary to drastically increase the per-generation change in CO<sub>2</sub> in order to conduct a laboratory experiment, it is still possible to investigate how CO<sub>2</sub> experienced in the past may constrain future adaptation in photosynthetic organisms, provided that one with a suitably short generation time is used.

Previously, we reported that replicate populations of the unicellular green alga *Chlamydomonas reinhardtii* selected

for 1000 generations at high CO<sub>2</sub> failed to show any adaptive change in response to the high CO<sub>2</sub> environment. In addition, the among-population variance of fitness and most related characters increased, suggesting relaxed selection. Each of the populations showed reduced fitness in the ancestral environment, measured as a reduction in at least one of growth rate, maximum population size, or competitive fitness (Collins and Bell 2004). A subset of the High selection lines were studied and found to have a decreased ability to induce high-affinity CO<sub>2</sub> uptake, a basic and well-characterized process in *Chlamydomonas* and many other microalgae (Collins et al. 2006). Most microalgal species respond to CO<sub>2</sub> limitation by the induction of a carbon concentrating mechanism (CCM; Badger et al. 1998; Sültemeyer 1998; Badger and Spalding 2000). The CCM is an inducible system that enables microalgae to respond to extracellular changes in inorganic carbon, and it occurs in most microalgal species studied to date (Colman et al. 2002). The CCM elevates CO<sub>2</sub> concentration in the vicinity of Rubisco, the main carboxylating enzyme in carbon fixation, when carbon is scarce (Moroney and Somanchi 1999).

In this study, a subset of the High selected populations were backselected down to ambient CO<sub>2</sub> levels and low (Pleistocene glacial) levels of CO<sub>2</sub>. Neither of these environments is completely novel, in that the high-CO<sub>2</sub>-selected *Chlamydomonas reinhardtii* (or its predecessor) has experienced them in either the recent or very distant past, but both are environments that the High selected lines were poorly adapted to at the beginning of the experiment. This design allows us to address two questions. First, how does selective history constrain adaptation and the reversibility of evolution? Second, and more specifically, how does natural selection reverse loss-of-function phenotypes, such as the high-CO<sub>2</sub>-requiring types that resulted from selection at high CO<sub>2</sub>?

## MATERIALS AND METHODS

### *Selection Experiment*

The experimental design is shown in Figure 1. We founded 10 replicate lines from a single clone of M566B (lab isolate), and 10 replicate lines from a single clone of CC-2344 (*Chlamydomonas* Genetics Center, Duke University, Durham, NC). Five replicates from each clone were grown in a high CO<sub>2</sub> environment and five replicates from each clone were grown in an ambient CO<sub>2</sub> environment. The ambient CO<sub>2</sub> environment consisted of flasks being bubbled with air containing 430 ppm CO<sub>2</sub> for the entire experiment. Lines in the high CO<sub>2</sub> treatment were initially grown in flasks being bubbled with air containing 430 ppm carbon dioxide, and CO<sub>2</sub> levels were raised steadily to 1050 ppm over the first 600 generations of the experiment. These lines were then grown at 1050 ppm CO<sub>2</sub> for a further 400 generations. Lines were propagated by batch culture grown in bubbled flasks containing 300 mL of Suoka high salt medium, pH 7 (HSM; Harris 1989) in a chamber in the McGill University phytotron under constant light at 25°C. We transferred 1 mL of culture (about 10<sup>5</sup> cells) every three to four days for approximately 1000 generations for each replicate line. The populations from this High selection phase form the ancestor populations for this study.

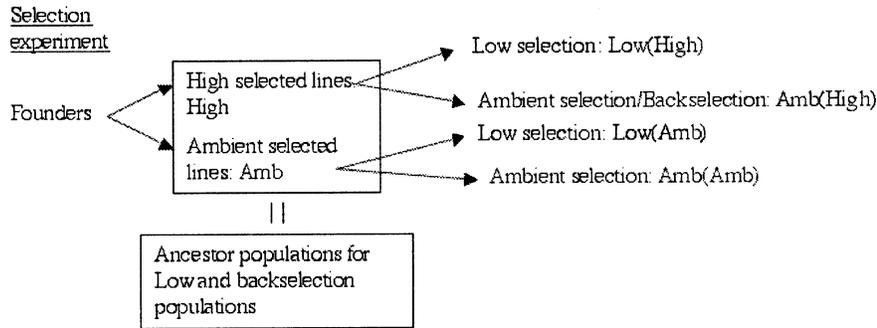


FIG. 1. Design of selection experiment.

Five of the High selection lines and five of the Ambient selection lines were chosen for selection at decreasing  $\text{CO}_2$ , such that a broad range of variation in growth and other characters (such as photosynthesis rate) was represented. The populations from the end of our previous high  $\text{CO}_2$  selection experiment are thus the ancestor lines for the work described here (please refer to Figure 1). All High selection lines chosen for this work showed evidence of decreased fitness at ambient  $\text{CO}_2$  and have been previously described (Collins and Bell 2004). The High selection lines used in this experiment had a significantly lower growth rate at ambient  $\text{CO}_2$  than did the Ambient lines (from data in Collins and Bell [2004]: for growth rate,  $t = 2.35$ ,  $df = 8$ ,  $P = 0.05$ ; maximum cell density  $t = 1.95$ ,  $df = 8$ ,  $P = 0.09$ ). In addition, the High lines chosen included those with the most marked differences relative to the Ambient lines in terms of growth rates, photosynthesis rates, and respiration rates. Each line was split into two replicates for each treatment. The replicate lines were grown in separate phytotron chambers at either a low  $\text{CO}_2$  environment (180 ppm) or an ambient  $\text{CO}_2$  environment (430 ppm).  $\text{CO}_2$  levels were controlled as described in Romer (2001). Lines in the low  $\text{CO}_2$  treatment were initially grown in flasks being bubbled with air containing 430 ppm  $\text{CO}_2$ , which was then decreased steadily to 180 ppm over 17–26 transfers, or about 150–225 generations. Lines that grew more slowly were transferred less frequently. Lines in the ambient  $\text{CO}_2$  treatment were grown at 430 ppm  $\text{CO}_2$  for the duration of the experiment if they were previously selected at ambient  $\text{CO}_2$ , and grown at decreasing  $\text{CO}_2$  (from 1050 ppm to 430 ppm) if they were previously selected at high  $\text{CO}_2$ . Several of the High selected lines initially failed to grow at lower  $\text{CO}_2$ . These lines were re-inoculated several times from the most recent ancestor. In all cases, lines were propagated by serial transfer, and were transferred when they reached a density of approximately  $10^5$  cells/mL. In all cases, the ancestral population for each Low or Ambient selection line was stored on agar slants for the duration of the selection experiment. Hereafter, the term “ancestor” is used to refer to these lines that were either selected at high or ambient  $\text{CO}_2$ . The ancestor lines used here correspond to the High and Ambient selection lines described in Collins and Bell (2004). The format used to refer to each line is Selection(History): for example, a line initially selected at high  $\text{CO}_2$  that was then selected at ambient  $\text{CO}_2$  is Amb(High).

### Statistical Analysis

See Figure 1 for terminology. Adaptation to low  $\text{CO}_2$  was tested as a difference in grand mean between populations selected at low  $\text{CO}_2$  versus those selected at ambient  $\text{CO}_2$ . To assess the contributions of selective history and ancestry after selection at low  $\text{CO}_2$ , fitness data were analyzed using a nested ANOVA. The effects in the model are: selective history, founder, history  $\times$  founder, ancestor(history, founder).

### Growth Assays

Pure culture growth rates were measured in 384-well plates containing 90  $\mu\text{L}$  HSM per well. Cultures were first acclimated for three to six days, then diluted and transferred to assay plates. For High or Ambient selection lines that often failed to grow at lower concentrations of  $\text{CO}_2$ , several extra acclimation cultures were inoculated, and the surviving cultures were used for growth assays at lower concentrations of  $\text{CO}_2$ . This may have allowed some back selection of the lines, so that the results presented here are conservative. The plates were grown in the same phytotron chamber as above at either 180 ppm, 430 ppm or 1050 ppm  $\text{CO}_2$ . Absorbance of each culture was measured every 24 h. Relative maximum densities were calculated from the maximum absorbance maintained by a culture. The average maximum growth rate or density attained by the Amb(Amb) lines growing under ambient  $\text{CO}_2$  was arbitrarily given a value of one in all assays so that the growth rates are comparable across the entire experiment. Because all growth rates and population densities are relative to the mean value of the Amb(Amb) populations, some of the ancestral lines may have values below 1.0. The direct response to selection was calculated as an increase in either growth rate or carrying capacity of a given population relative to the ancestor of that particular population in the selected environment.

### Sorting Response to Row $\text{CO}_2$ in Ancestral Populations

During acclimation to different levels of  $\text{CO}_2$  for the growth assays, we noted that some of the High and Ambient selection lines failed to grow consistently when suddenly placed in lower concentrations of  $\text{CO}_2$ . Based on this, we hypothesized that they contained at least two types, at least one of which was incapable of growing at lower concentra-

tions of CO<sub>2</sub>. If this were true, acclimation cultures inoculated with only high-CO<sub>2</sub>-requiring cells would not grow, whereas those inoculated with either low-CO<sub>2</sub>-tolerant cells or a mix of the two types would grow. In practice, this would lead to an overestimation of growth rates of these populations in CO<sub>2</sub> environments lower than the one they were selected in. To test whether some of the High and Ambient selection lines (the ancestral populations for the low CO<sub>2</sub> and back-selection experiments) contained a mix of low-CO<sub>2</sub>-tolerant and high-CO<sub>2</sub>-requiring types, the ancestral populations for the Low selected lines were inoculated into HSM and acclimated to the environment in which they were originally selected (either high or ambient CO<sub>2</sub>). Serial dilutions of the growing cultures were transferred to 384-well microplates, placed under low CO<sub>2</sub> and allowed to grow for 10 days, until all surviving cultures reached maximum population densities. The frequency of high or ambient CO<sub>2</sub>-requiring cells in the ancestral populations was estimated by plotting the number of cells initially present in each well against the proportion of wells that grew at low CO<sub>2</sub> over 10 twofold dilutions for each ancestral population. The largest population size in which no cultures were able to grow was taken as the maximum frequency of types that could grow at low CO<sub>2</sub>. For example, if no cultures grew at an initial inoculum of 45 cells but a subset grew with an initial inoculum of 90 cells, then the maximum frequency of cells capable of growth at low CO<sub>2</sub> is 1/45. The limit of detection of this assay is a frequency of 0.5 because cultures of *Chlamydomonas* do not grow reliably from an average inoculum of fewer than two cells in this assay. Replicate plates of the two lowest dilutions were grown at ambient and high CO<sub>2</sub> to test whether the diluted populations contained enough viable cells to grow in the ancestral environment.

#### Carbon Flux Measurements

CO<sub>2</sub> exchange rates in whole cells were measured by mass spectrometry as described by Amoroso et al. (1998), with the following modifications. Cells were acclimated to either 1050 ppm CO<sub>2</sub> (high CO<sub>2</sub>), to air (ambient CO<sub>2</sub>), or to 180 ppm CO<sub>2</sub> (low CO<sub>2</sub>) for 24 h. Washed cells were resuspended in HSM buffered with 50 mM bistrisphosphate at pH 7.0 at 25°C. Because it is necessary to inhibit the extracellular carbonic anhydrase to use this method, 20 μM acetazolamide (AZA) was added to the cells. Acetazolamide inhibits cell surface carbonic anhydrase (CA), but cannot enter the cell. We determined that this concentration of AZA had no effect on intracellular CA. All our control lines had evolved insensitivity to AZA and autolysis, presumably due to changes in cell wall composition. Because the method above could not be used on them, the well-characterized wild-type strain 11-32b (culture collection of algae, University of Göttingen, Germany; described in Amoroso et al. 1998), was used for comparison. Control lines were similar to wild type in terms of growth and photosynthesis rates (data not shown). CO<sub>2</sub> fluxes were calculated using previously published formulas (Badger et al. 1994).  $K_{0.5}$  (half-saturation constant) and  $V_{max}$  (maximum rate of uptake) were calculated by least-squares nonlinear regression using Prism 4.0 (GraphPad; <http://www.graphpad.com>). Bicarbonate uptake was found to be

TABLE 1. Estimated maximum frequencies of low CO<sub>2</sub>-tolerant types in ancestors of low CO<sub>2</sub> selection lines. Each frequency represents a mean value ± standard error of the mean from two independent replicates.

Selection line	Selected environment	Estimated frequency of types able to grow at low CO <sub>2</sub>
4	high CO <sub>2</sub>	>0.5
11	ambient CO <sub>2</sub>	0.045 ± 0.022
12	ambient CO <sub>2</sub>	0.012 ± 0.004
20	ambient CO <sub>2</sub>	>0.5

low at pH 7.0 (data not shown), so only CO<sub>2</sub> uptake is reported here. We made two or three independent measurements for each estimate.

## RESULTS

### *Tempo of Adaptation to Low CO<sub>2</sub>*

Several of the ancestral lines often failed to grow at low CO<sub>2</sub>, although if enough cultures were inoculated, a subset would grow. This suggested that these ancestral populations were made of several phenotypes, at least one of which was unable to grow at low CO<sub>2</sub>. Acclimation to low CO<sub>2</sub> for the growth assays described above lasts for several generations, and may present an opportunity for an initial rapid response to low CO<sub>2</sub> by sorting of the base population. Of 10 ancestor populations, four showed evidence of an initial rapid adaptation to low CO<sub>2</sub> by sorting. The presence of evidence of initial adaptation did not systematically correspond to a particular founding genotype or selection history. The estimated frequencies of the phenotypes able to grow at low CO<sub>2</sub> in these cultures is listed in Table 1. For some ancestor populations, the frequency of types able to grow at low CO<sub>2</sub> is listed as >0.5; this is because, at or below an average inoculum of two cells, it is not possible to tell whether a culture fails to grow because it contains high-CO<sub>2</sub>-requiring cells or because it contains no viable cells at all. Practically, populations were not exposed to sudden drops in CO<sub>2</sub> during the selection experiment itself; thus, the presence of this fast sorting response indicates that the growth rates reported in the following sections represent a conservative estimate of the amount of change that has occurred between the ancestral and derived populations, but should not be used directly to draw conclusions about how rapidly sorting occurred during the actual selection experiments.

### *Direct Response to Selection*

Responses to selection are summarized in Table 2. Figures 2A and B show the maximum growth rates and maximum population densities of lines selected at low CO<sub>2</sub>. Two different clones were used as founders at the beginning of the experiment (see Fig. 1), and an ANOVA shows a significant founder effect on growth rate (founder  $F_{1,4} = 9.84$ ,  $P = 0.02$ ), although there is no significant founder effect on population density. There is no significant effect of particular ancestor on growth rate (ancestor(history,founder)  $F_{4,6} = 1.09$ ,  $P = 0.44$ ) or population density ( $F_{4,6} = 0.53$ ,  $P = 0.72$ ). There is no significant effect of selective history on the mean growth rates (history  $F_{1,1} = 2.62$ ,  $P = 0.16$ ) or mean population

TABLE 2. Summary of responses to selection. Genetic responses relative to ancestor are bold. Physiological responses due to environment are italic.

Selection (history)	Environment		
	Low	Ambient	High
Amb(Amb)	<i>Reduced growth</i>	Control for Amb(High) back-selection growth and regulation	Control for Amb(High) <i>Increased growth</i>
Amb(High)	<i>Reduced growth</i>	<b>Increased growth, higher affinity CO<sub>2</sub> uptake; same phenotypes as Amb(Amb)</b>	<b>Same growth and CO<sub>2</sub> uptake as Amb(Amb)</b> <i>Increased growth</i>
Low(Amb)	<b>Increased growth, higher affinity CO<sub>2</sub> uptake</b>	<b>Range of responses, including no growth</b> <i>Higher affinity CO<sub>2</sub> uptake</i>	<b>Range of growth rates including zero</b> <b>Lower affinity CO<sub>2</sub> uptake</b>
Low(High)	<b>Increased growth, higher affinity CO<sub>2</sub> uptake</b>	Range of responses <i>Higher affinity CO<sub>2</sub> uptake</i>	<b>Range of growth rates</b> <b>Lower affinity CO<sub>2</sub> uptake</b>

densities (history  $F_{1,1} = 0.038, P = 0.85$ ) of the Low selection lines. The interaction between founder and history is not significant for either growth rate or mean population density. In addition, the Low selection lines have the same range of

growth rates and population sizes regardless of selective history.

Figure 3 shows the direct response to selection in terms of change relative to the particular ancestor of each line at low CO<sub>2</sub> in lines that were previously selected at either high or ambient CO<sub>2</sub>. An ANOVA did not detect a significant effect of selection history in the Low selection lines on either growth rate ( $F_{1,1} = 2.11, P = 0.20$ ) or maximum population density ( $F_{1,1} = 0.22, P = 0.66$ ). The mean direct response to selection is positive for maximum population sizes ( $t = 5.17, df = 13, P < 0.001$ ), and for maximum growth rate ( $t = 2.04, df = 13, P = 0.03$ ). On average, there is a 2.1-fold improvement over the ancestral population density and a 1.6-fold increase over the ancestral growth rate. Each line had a greater maximum growth rate, maximum cell density, or both, than its ancestor. Several of the lines with High ancestors failed to show any increase in maximum population size. In cases where there was little or no response, the ancestor already had a high maximum cell density. There is a significant effect of founder ( $F_{1,4} = 12.40, P = 0.01$ ) and particular ancestor (ancestor (history,founder)  $F_{4,6} = 6.49, P = 0.02$ ) on growth rate, but not on population size (founder  $F_{1,4} = 0.58, P = 0.48$ ; ancestor (history,founder)  $F_{4,6} = 0.83, P = 0.55$ ).

Figure 4 shows the growth rates and maximum population

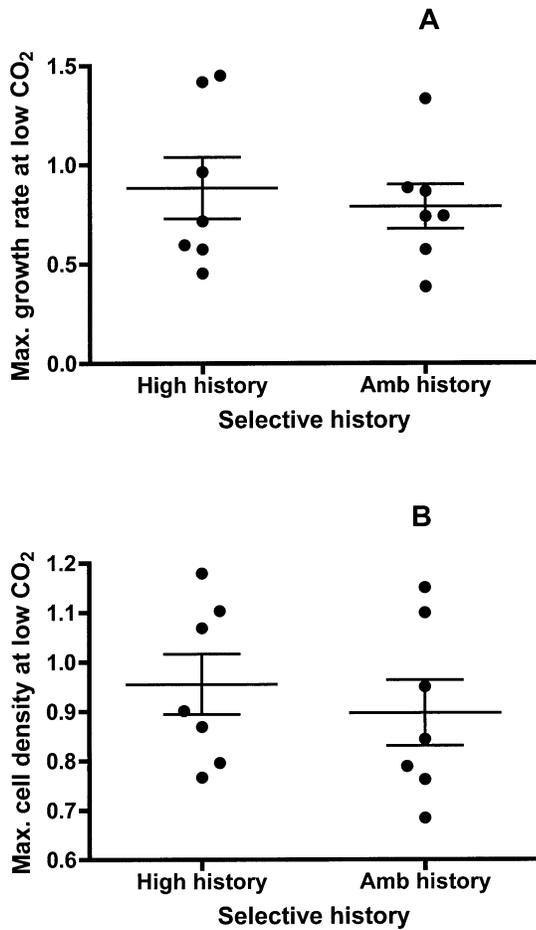


FIG. 2. (A) Maximum growth rate of Low(Amb) and Low(High) lines. (B) Maximum sustainable cell densities in Low(Amb) and Low(High) lines. In both cases, each point represents a single selection line. Horizontal lines show mean  $\pm$  SE across all lines. Values are relative to Amb(Amb) lines growing at ambient CO<sub>2</sub>. Each point represents a mean value from at least 30 replicate cultures in two independent assays.

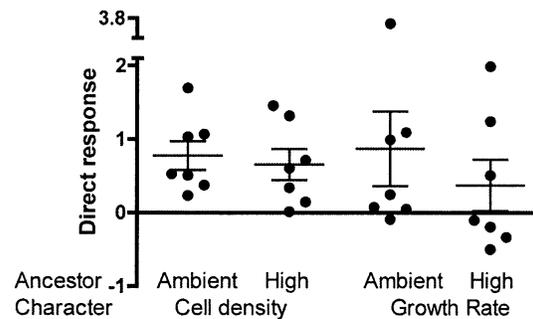


FIG. 3. Direct response to selection at low CO<sub>2</sub> in Low(Amb) and Low(High) lines, shown either as change in maximum sustainable cell density or maximum growth rate. The direct response to selection is calculated from average absolute values of characters as follows: (derived - ancestor)/ancestor. Each point represents a single selection line. Horizontal lines show mean  $\pm$  SE across all lines.

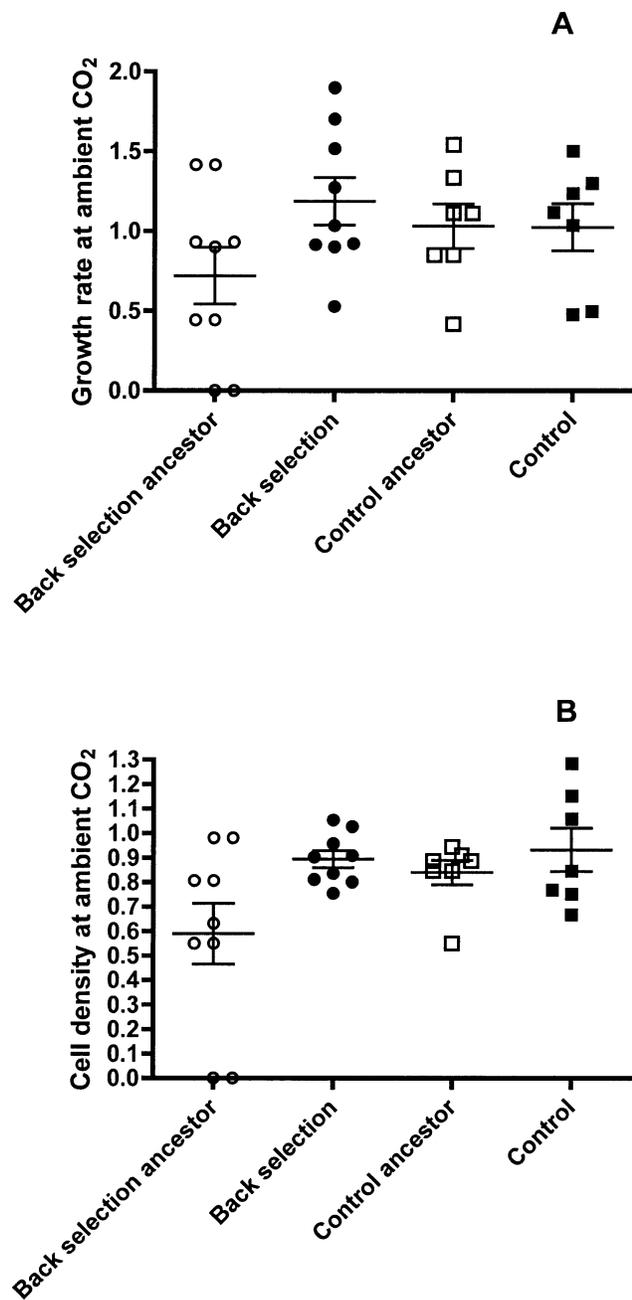


FIG. 4. (A) Response to back selection in Amb(High) (circles) and control lines (squares), measured as maximum growth rate at ambient CO<sub>2</sub>. (B) Response to back selection in Amb(High) (circles) and control lines (squares), measured as maximum sustainable population size at ambient CO<sub>2</sub>. Values for derived populations (filled symbols) are shown directly next to the growth rates for ancestor populations (open symbols). Control lines are Amb(Amb) lines, which were grown at ambient CO<sub>2</sub> for the duration of the selection experiment. The Amb(Amb) lines are thus unselected lines with respect to CO<sub>2</sub>. In both cases, each point represents a single selection line. Horizontal lines show mean ± SE across all lines. Values are relative to Amb(Amb) lines growing at ambient CO<sub>2</sub>. Each point represents a single selection line mean value from at least 30 replicate cultures in two independent assays.

densities of Amb(High) and Amb(Amb) lines grown at ambient CO<sub>2</sub>. Amb(High) lines have the same mean growth rates ( $t = 0.91$ ,  $df = 15$ ,  $P = 0.37$ ) and maximum cell densities ( $t = 0.92$ ,  $df = 14$ ,  $P = 0.37$ ) in their ancestral environment as cultures that were grown at ambient CO<sub>2</sub> for the entire selection experiment, although the between-line variance in maximum cell density in the back-selection lines is lower than in the Ambient selected lines ( $F_{6,8} = 14.85$ ,  $P = 0.001$ ). The High and Ambient lines used for this experiment initially had significantly different growth rates in ambient CO<sub>2</sub> before back selection or continued selection at ambient CO<sub>2</sub>.

#### Indirect Response to Selection

The expected response to an increase in environmental CO<sub>2</sub> is an increase in growth rate. On average, lines selected at low CO<sub>2</sub> responded to an increase in CO<sub>2</sub> by increasing growth rate ( $F_{2,2} = 20.10$ ,  $P < 0.0001$ ) (see Table 2). However, two of the Low selected lines failed to grow reliably at ambient CO<sub>2</sub>, and one failed to consistently grow at high CO<sub>2</sub>. The same average result, an increase in growth rate in response to increases in CO<sub>2</sub>, was previously reported in both High and Ambient ancestor populations (Collins and Bell 2004), with some High lines failing to grow reliably at ambient CO<sub>2</sub>. However, none of the ancestral Ambient lines failed to grow at high CO<sub>2</sub>. The appearance of lines unable to grow at levels of CO<sub>2</sub> greater than those they were selected at was only observed in lines selected at low CO<sub>2</sub>.

#### Changes in CO<sub>2</sub> Uptake Affinity

Figure 5 shows the changes in net CO<sub>2</sub> uptake affinity in Low and backselected lines. Low lines generally evolve higher affinity net CO<sub>2</sub> uptake than their ancestor (paired  $t = 2.56$ ,  $df = 3$ ,  $P < 0.01$ ; mean difference = 9.35  $\mu\text{M}$ ). Amb(High) and Low(High) lines do re-evolve a response for changes between high and ambient CO<sub>2</sub>, though this was done by decreasing affinity at high CO<sub>2</sub> rather than improving affinity at ambient CO<sub>2</sub>. For all three Low selection lines measured, a large decrease relative to the ancestor in net CO<sub>2</sub> uptake affinity at high CO<sub>2</sub> was seen (paired  $t = 8.48$ ,  $df = 2$ ,  $P < 0.01$ ; mean difference = 26.29  $\mu\text{M}$ ).

#### DISCUSSION

Our selection experiment was designed to investigate how selective history constrains adaptation to lower concentrations of CO<sub>2</sub>. In microbial systems used to study adaptation, sorting of genetic variance for fitness does not occur at the beginning of the experiment, since selection experiments are traditionally founded with clonal cultures. In this case, the Low selection lines were founded from genetically diverse populations. Because of this, adaptation to sudden drops in CO<sub>2</sub> experienced by the populations during growth assays had the potential to involve a rapid sorting step, in which high-or ambient-CO<sub>2</sub>-requiring mutants could be replaced by types that are capable of growing at low CO<sub>2</sub>. We estimated that phenotypes requiring high or ambient CO<sub>2</sub> exist in at least four of the 10 populations used as founders of Low selection lines. This sorting resembles changes in population or species composition in response to sudden changes in CO<sub>2</sub>

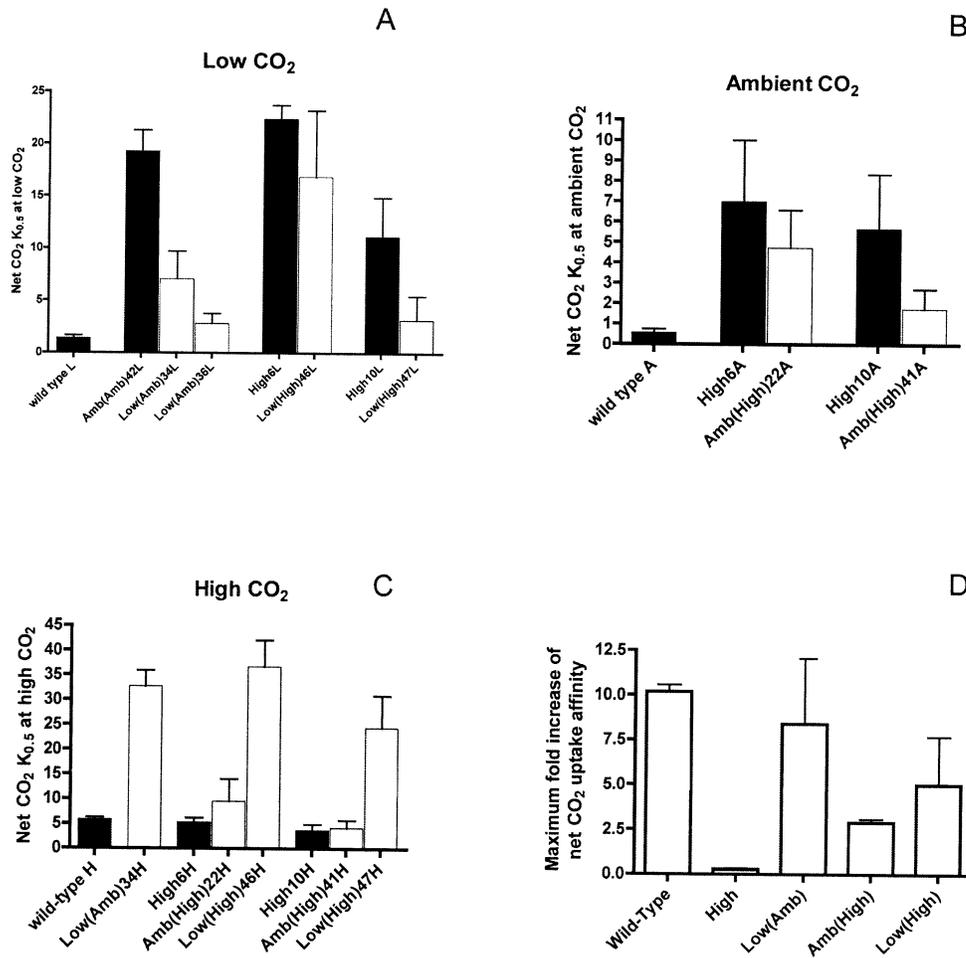


FIG. 5.  $K_{0.5}$  of net CO<sub>2</sub> uptake. In all cases, solid bars represent ancestor or wild-type populations, and open bars immediately following represent lines derived from that ancestor. Bars represent mean  $\pm$  SE from two or three independent replicates. (A) Net CO<sub>2</sub> uptake affinity of Low(Amb) and Low(High) lines at low CO<sub>2</sub>. (B) Net CO<sub>2</sub> uptake affinity at ambient CO<sub>2</sub> in back-selection lines. (C) Net CO<sub>2</sub> uptake affinity of Amb(High), Low(Amb) and Low(High)Low, and ancestor lines at high CO<sub>2</sub>. (D) Maximum increase in net CO<sub>2</sub> uptake affinity ( $K_{0.5}$ ) in wild-type, High (ancestral), Amb(Low), High(Low), and High(Amb) lines. Increase is calculated as ( $K_{0.5}$  at high CO<sub>2</sub>/ $K_{0.5}$  at selected low CO<sub>2</sub>). Each bar represents mean  $\pm$  SE for all lines from a given selection regime.

that have been reported in natural populations (Tortell and Morel 2002). When direct responses to fitness are measured in this experiment, sorting occurs primarily during the acclimation period and is not taken into account in the calculation of the direct response, which measures the improvement over the ancestor that is largely attributable to continued selection. In practice, this means that the evolutionary responses reported here are conservative.

Previously, we have suggested that long-term growth at elevated CO<sub>2</sub> resulted in relaxed stabilizing selection in these populations of *Chlamydomonas*. If so, then any changes in population structure that result from relaxed selection may constitute an important part of the selective history of a population. To take this into account, our Low and back-selection lines were founded from populations rather than from clones. As a result, it is possible that part of the direct response to selection is attributable to changes in frequencies of types present at the onset of the experiment. Using isogenic lines is important to be sure that a response is due entirely to the

substitution of novel mutation, rather than to sorting lineages already present in the base population. This distinction tends to break down for large microbial populations, in which every single-nucleotide mutant may be present within a few generations. Our lines used at the beginning of the Low and back-selection experiments may have contained a range of lineages with little to no effect on growth in the ancestral environment (high or ambient CO<sub>2</sub>). Those with a pronounced reduction in growth rate in the new or changing environment would have been removed rapidly by selection. Those with lesser effects may have persisted and contributed to the long-term response we observed. If so, this reservoir of neutral and nearly neutral variation may play an important part in shaping the response of populations.

In this study, we investigated the effect of selective history on adaptive outcomes and found that, on average, selection lines were able to adapt to low CO<sub>2</sub> and reached similar end points regardless of their history of selection. The particular founder used had an effect on the end point reached after

adaptation, seen as growth rate attained after selection at low CO<sub>2</sub>, although it did not have a significant effect on the population sizes attained or on the direct response to selection. In addition, the particular ancestor (the particular High or Ambient population used for selection at low CO<sub>2</sub>) used for low selection had an effect on the response to selection in terms of growth rate. This suggests that ancestry plays an important role in adaptive outcomes, even between very similar or closely related taxa. Three of the four Low selection lines measured evolved higher-affinity net CO<sub>2</sub> uptake relative to their ancestor at low CO<sub>2</sub>, which may partly explain the direct response to selection. The finding that both Low(High) and Low(Amb) lines showed a direct response to selection at low CO<sub>2</sub>, and that a subset showed an improvement in a system thought to be under selection, demonstrates that long-term decreases in CO<sub>2</sub> alone can produce evolutionary change in microalgae with CCMs. This suggests that microalgae in the recent past were different than contemporary algae. In addition, the Amb(High) lines had the same fitness as the Amb(Amb) lines, demonstrating that the High selection phenotypes were evolutionarily reversible in terms of fitness. Taken together, this demonstrates that over the range of CO<sub>2</sub> and time investigated, selective history did not appear to constrain the ability of *Chlamydomonas* to adapt to lower CO<sub>2</sub> environments, measured as growth in pure culture. It also demonstrates that fitness can be recovered quickly (about 175 generations) by natural selection in large populations. This is consistent with the findings of microevolutionary experiments, in which fitness between replicate lines tends to converge when they are selected in the same environment (Travisano and Lenski 1996; Travisano 1997; Riley et al. 2001; Cuevas et al. 2002), and loss of fitness is usually reversible (Estes and Lynch 2003).

In this system, adaptation to a given level of CO<sub>2</sub> is specific to that level. Trade-offs are a general feature of adaptation to specific levels of CO<sub>2</sub>, which we have previously reported in High adapted lines (Collins and Bell 2004). In this case, some of the Low selection lines failed to grow reliably at levels of CO<sub>2</sub> higher than the one at which they were selected. An apparently less severe trade-off is that a minority of Low selection lines did not show a detectable increase in growth rates with increasing CO<sub>2</sub>. These results are in agreement with the evolution of specialist and generalist types in microevolutionary experiments (Reboud and Bell 1997, where constant environments lead to the evolution of specialists, whereas environments that fluctuate in time permit the evolution of generalists. The rapid evolution of specialists for different levels of CO<sub>2</sub> is consistent with several levels of induction of a CCM over a range of CO<sub>2</sub> levels, rather than all or nothing CCM induction (reviewed in Matsuda et al. 1998). If the CCM was simply turned on at ambient levels of CO<sub>2</sub> and continued to be turned on at low CO<sub>2</sub>, one would expect all Low selection lines to be able to grow at ambient CO<sub>2</sub> and vice versa. This points toward the existence of a subset of CCM genes that are specific to ambient and/or low CO<sub>2</sub>, which is supported by some CCM knockout phenotypes. For example, the *pmp-1* knockout grows at very low, but not at ambient, levels of CO<sub>2</sub> (Spalding et al. 2002). Trade-offs associated with adaptation to a specific level of CO<sub>2</sub> suggest that a highly regulated algal CCM may have evolved as a

result of fluctuations of CO<sub>2</sub>, which would be likely to occur in blooming phytoplankton, rather than in response to the mean concentration of CO<sub>2</sub> present in the environment. Studies on *Peridinium gatunense* showed an increase in CCM activity during an annual bloom in natural populations, although CO<sub>2</sub> limitation leading to oxidative stress eventually led to a population crash (Berman-Frank et al. 1995).

In addition to trade-offs in growth at different CO<sub>2</sub> concentrations, we show that CCM regulation is affected by selection at low CO<sub>2</sub>. Of the lines investigated, all Low selection lines (regardless of ancestor) and one Amb(High) line gained CCM regulation. Higher-affinity CO<sub>2</sub> uptake was induced at ambient CO<sub>2</sub>, but was not further improved at low CO<sub>2</sub>; this is qualitatively similar to wild-type regulation we measured in *Chlamydomonas* (Collins et al. 2006). However, in the Low selection lines, this was done by decreasing the CO<sub>2</sub> uptake affinity at high CO<sub>2</sub> by at least 6.6-fold relative to their ancestor. This suggests that fitness recovery happened by compensatory mutations rather than by back mutation. Back mutation would have led to a wild-type phenotype with ancestral values of CO<sub>2</sub> uptake affinity at high CO<sub>2</sub> instead of loss of function in the ancestral environment. Compensatory mutations have been shown to be responsible for fitness recovery in experiments with viruses and microbes (Elena et al. 1998; Burch and Chao 1999; Moore et al. 2000; Rokytka et al. 2002) as well as metazoans (Estes and Lynch 2003). Alternatively, these phenotypes could have occurred by the fixation of a neutral or nearly neutral type already present in the ancestral (High or Ambient selected) population, although this type would have had to be present at an extremely low frequency, since the High selected populations that were used as ancestors for this experiment did not show this degree of CCM regulation (Collins et al. 2006). The presence of both CO<sub>2</sub>-sensitive and CO<sub>2</sub>-insensitive Low selection lines demonstrates that how *Chlamydomonas* responds to changes in CO<sub>2</sub>, although predictable at physiological timescales, is fundamentally changeable over evolutionary ones.

In this experiment, both back selection and selection at low CO<sub>2</sub> resulted in evolved lines with a range in growth rates that were either sensitive or insensitive to CO<sub>2</sub> concentration. Along with differences in growth, selection at low CO<sub>2</sub> led to at least two different types of carbon uptake kinetics (CO<sub>2</sub> sensitive and CO<sub>2</sub> insensitive). Thus, the variability seen in the affinity and regulation of CCMs in microalgae could be partially attributable to neutral variation produced by chance during repeated changes in CO<sub>2</sub> and bicarbonate levels. Examples of both CO<sub>2</sub>-sensitive and CO<sub>2</sub>-insensitive growth have been reported in natural populations of phytoplankton (Hein and Sand-Jensen 1998; Tortell and Morel 2002), as have different CCM regulation strategies, even among phytoplankton with similar biology (Raven et al. 2002; Raven 2003; Rost et al. 2003). It is clear that historical factors constrain adaptive outcomes over very long timescales in phytoplankton, as can be seen by differences in Rubisco affinities between taxa, in which affinity correlates with atmospheric CO<sub>2</sub> at the epoch when each taxon emerged (Tortell 2000). However, our data suggest that the diversity of CO<sub>2</sub> growth and uptake strategies may not be attributable to selective history or adaptation over shorter timescales, but

are instead mainly attributable to ancestry and chance events, even in closely related populations.

Our results show that selective history does not affect adaptive outcomes measured as fitness over microevolutionary timescales in this system. However, over a timescale of hundreds of generations, Amb(High) lines did not regain the CO<sub>2</sub> uptake characteristics usually seen in standard laboratory populations of *Chlamydomonas*. As in back-selection experiments in *Drosophila* (Teotónio et al. 2002; Estes and Lynch 2003), this suggests that complex phenotypes are unlikely to be faithfully restored by back-selection. We have demonstrated that chance events can drastically change the affinity and regulation of carbon uptake, as well as the CO<sub>2</sub> dependence of growth rates, after only about 1200 generations of selection. This diversity of phenotypes occurs without systematically affecting fitness, and corresponds qualitatively to growth and CO<sub>2</sub> uptake strategies seen between contemporary taxa of phytoplankton. More experimental work over a range of environments is needed to understand the role of reverse selection and chance in explaining the biology and diversity of contemporary populations.

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