



EXPERIMENTAL EVOLUTION OF HETEROTROPHY IN A GREEN ALGA

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Laboratory populations of a green alga cultured in the dark with an organic substrate evolved into efficient heterotrophs with faster growth, higher fitness, and increased responsiveness to substrate concentration. Their phenotypes were almost entirely attributable to selection, rather than to history or ancestry. The fitness of evolved lines in the light was uniformly depressed, presumably through the accumulation of conditionally deleterious genes governing photosynthesis. Some evolved lines were no longer able to grow in the light and are thereby permanently isolated from their ancestors. Specialized autotrophs and heterotrophs may often evolve in algae through long-term shifts in the conditions of growth.

KEY WORDS: Acetate flagellate, adaptation, autotrophy, *Chlamydomonas*, experimental evolution, fitness, heterotrophy, mixotrophy, responsiveness.

New ways of life evolve from time to time. This is normally the province of paleontology or comparative biology, which record the acquisition of novel characters in ancient or modern lineages. Experimental evolution has been successful in delineating the course and illuminating the mechanism of adaptation, but has usually been confined to enhancing a general attribute, such as glucose utilization (Cooper and Lenski 2000), or resisting a specific stress, such as antibiotic application (MacLean et al. 2010). The evolution of qualitatively different ways of life, on the other hand, might seem to lie permanently beyond the ambition of laboratory studies.

This argument is based on the view that adaptation to different ways of life requires concerted changes in many genes that can occur only gradually over long periods of time. This is obviously true for the current state of attributes such as photosynthesis in plants, flight in birds, or parasitism in cestodes. These highly evolved states are modified versions of much simpler precursors, such as photon capture, down feathers, or commensalism. In their early stages, these new features may evolve through correspondingly simple genetic changes. Most teleosts are specialized for marine or freshwater existence, for example, although some species are broadly tolerant. Nevertheless, the evolution of

freshwater populations from marine ancestors has been studied in detail in the stickleback *Gasterosteus* (McKinnon and Rundle 2002), where a few major genes are primarily responsible for adaptation (Colosimo et al. 2005). Cultivated plants are often very different from their wild ancestors in morphology, phenology, and biochemistry, but in many cases they have evolved rapidly, with or without deliberate human manipulation, through the modification of a few genes of large effect (Doebly 2004). Even the fundamental shift from unicellular to multicellular body plans can be witnessed in experimental populations following selection by predators (Boraas et al. 1998) or simple centrifugation (Ratcliff et al. 2012). In all these cases, evolutionary change in the short term has both ecological causes and ecological consequences.

The major shift in life style that I shall describe is the evolution of heterotrophic populations from primarily photoautotrophic ancestors. This is a physiological shift that has profound ecological consequences. Heterotrophic and autotrophic growth use different metabolic pathways, often located in different cellular compartments, and a shift from one to the other involves a radical realignment of the entire economy of the cell. Nevertheless, there are many mixotrophic organisms that can use both pathways (Sanders 1991). The combination is found in many

unicellular eukaryotes, such as chryomonad flagellates and dinoflagellates. Some primarily autotrophic organisms such as sun-dews and parasitic red algae have evolved facultative or obligate heterotrophy, whereas primarily heterotrophic organisms such as ciliates, hydrozoans, and acoels have evolved a degree of autotrophy through harboring endosymbiotic green algae. Ciliates may retain the chloroplasts of their algal prey. There are many obligate heterotrophs incapable of photosynthesis because they lack plastids, either because their lineage has never acquired plastids or because (as in colorless euglenids) their plastids have been secondarily lost. There are also many obligate photoautotrophs that are incapable of utilizing organic compounds present in the environment. These are more difficult to interpret: photosynthesis builds sugars and other organic molecules from carbon dioxide, so it is not clear why such carbon substrates cannot be used when they are supplied directly (Chen and Chen 2006).

The evolution of any new attribute must proceed through the modification or improvement of an existing attribute, so the evolution of heterotrophy in autotrophs is based on preexisting metabolic systems (such as the Krebs cycle) common to all cells. *Chlamydomonas* is a unicellular chlorophyte that can use both autotrophic and heterotrophic metabolic pathways. It grows rapidly in the light by fixing CO₂ and more slowly in the dark by metabolizing acetate. I have previously described the experimental evolution of specialist and generalist types in light and dark conditions (Bell and Reboud 1997; Reboud and Bell 1997). In this report, I shall describe the evolution of efficient heterotrophic populations from primarily photoautotrophic ancestors within a few hundred generations and the associated impairment of autotrophic growth. To avoid misunderstanding, I emphasize that the ancestral populations in this study are not wholly incapable of heterotrophic growth; rather, through the modification of a limited initial capacity, they evolve to become much more efficient and in some cases obligate heterotrophs.

Materials and Methods

BASE POPULATIONS

There are three base populations, founded in 1997 from a complex cross between wild types (Bell 2005) and cultured since on Bold's basic medium (Bold 1949) solidified with agar. Each cycle of 14 days comprises vegetative growth with spontaneous mating, exposure to chloroform vapor to kill unmated cells, zygote germination, and respreading (see Harris 2008 for standard procedures). These outcrossed sexual populations have been maintained on minimal medium with strictly photoautotrophic growth for about 300 sexual and 2500 vegetative generations. These three populations are termed Anc 1, Anc 2, and Anc 3. They differ in the frequency of cells able to form macroscopic colonies when cultured on solid medium supplemented with acetate in complete

darkness: about 30% in Anc 2, 5% in Anc 3, and <1% in Anc 1. All three grow only very slowly in the dark.

SELECTION EXPERIMENT

The selection environment was a well of 200 μL volume on a microtiter plate containing Bolds medium made into slurry with 0.9 g L^{-1} low melting point agarose and supplemented with 1.2 g L^{-1} sodium acetate, placed in a dark chamber. Nine hundred and sixty replicate samples of 25 μL from each ancestral population (2880 selection lines in total) were used to inoculate the wells. Lines were at first transferred approximately every month. Following the initial wave of extinctions, the inoculum was reduced to 10 μL and the transfer interval to two weeks in the 240 surviving lines. They are currently transferred every week. Optical density and visual appearance were scored at every transfer. Experimental raw data are provided as Supporting information.

COMPETITIVE ASSAY IN THE DARK

Colony phenotypes of ancestral and derived lines are readily distinguished after incubation in the dark. Two attempts to estimate selection coefficients in mixtures of ancestral and evolved lines were unsuccessful, however, because the ancestral lines were almost completely eliminated within a single cycle of growth. To provide an estimate of selection coefficients, a surrogate tester with a stable yellow-in-the-dark phenotype was isolated from one of the dark lines. This phenotype is often caused by loss-of-function mutations in the later stages of chlorophyll biosynthesis. The yellow tester was mixed with each dark line and the mixture cultivated for six cycles, with the frequency of the tester estimated by plating. The selection coefficient was estimated as the rate of change of the logarithm of the ratio of tester to dark line cells over time (Dykhuizen and Hartl 1983). The selection coefficient of the tester relative to each ancestor was estimated in a similar way, adding one to the cell count to avoid zero values. Combining the two estimates yields the selection coefficient for each line relative to its ancestor. For Anc 1, most cell counts were zero and its selection coefficient relative to the tester is underestimated.

COMPETITIVE ASSAY IN THE LIGHT

Ancestral and derived lines were competed directly in the light with minimal medium, where colonies are clearly distinguishable, and selection coefficients calculated as in the dark assay, without requiring a standard tester. Only 60 lines (20 per ancestor) were assayed in the light.

GROWTH PARAMETERS IN THE LIGHT

The derived lines were also cultured by themselves in the light, with acetate supplementation (mixotrophic growth) or without (autotrophic growth) and their optical density was measured every day. The growth curves were used to estimate demographic

parameters such as the intrinsic growth rate r and the yield K by standard procedures.

GRADIENT ASSAY

The general adaptation of the lines to heterotrophic growth, beyond the specific concentration of acetate used in the selection experiment, was evaluated by measuring growth over a gradient of acetate concentrations. Each dark line and each ancestor was cultured in acetate concentrations ranging from 0.1 to 10 g L⁻¹ and their optical density scored at intervals of a few days.

EXOTIC SUBSTRATE ASSAY

To investigate the specificity of acetate as a substrate for heterotrophic growth, each line was tested for growth on media containing an alternative substrate as sole source of carbon, using the same protocols and molar concentrations as for growth on acetate. These exotic substrates were fructose, galactose, glucose, glycerol, lactose, maltose, mannose, sorbitol, and sucrose.

Results

FEW LINES ADAPTED TO THE DARK

Most lines became extinct in the first eight growth cycles, despite the large inoculum volume and long transfer interval. Dark growth is a very severe stress that only a small minority of lines can survive: only 241 of 2880 (8.4%) of lines evolved a growth rate enabling them to persist indefinitely.

LINES EVOLVED SIMILAR FITNESS

Surviving lines had similar rates of growth (measured as change in optical density) after 50 cycles. In principle, their rates of growth should be constant, identical, and equal to the rate of dilution. In practice, there was a slight tendency for mean growth to increase, at a rate of about 1% per cycle. The among-line variance component (estimated by using successive values for cycles 46–55 as replicates) was positive and the coefficient of variation among lines calculated from it was about 0.2. The standard deviation among lines increased over time between cycles 25 and 55 at about the same rate as the mean. These trends indicate that some lines continued to evolve more efficient acetate metabolism after the initial phase during which lines incapable of sustained growth were eliminated.

The ancestral populations had very different selection coefficients, relative to a standard tester: < -0.67 per generation for Anc 1, -0.32 for Anc 3, and -0.13 for Anc 2. These values reflect the frequency of spores able to form macroscopic colonies in the dark, which is least in Anc 1 and greatest in Anc 2. After 50 cycles in the dark, these differences had been erased and the lines had evolved similar competitive ability regardless of ances-

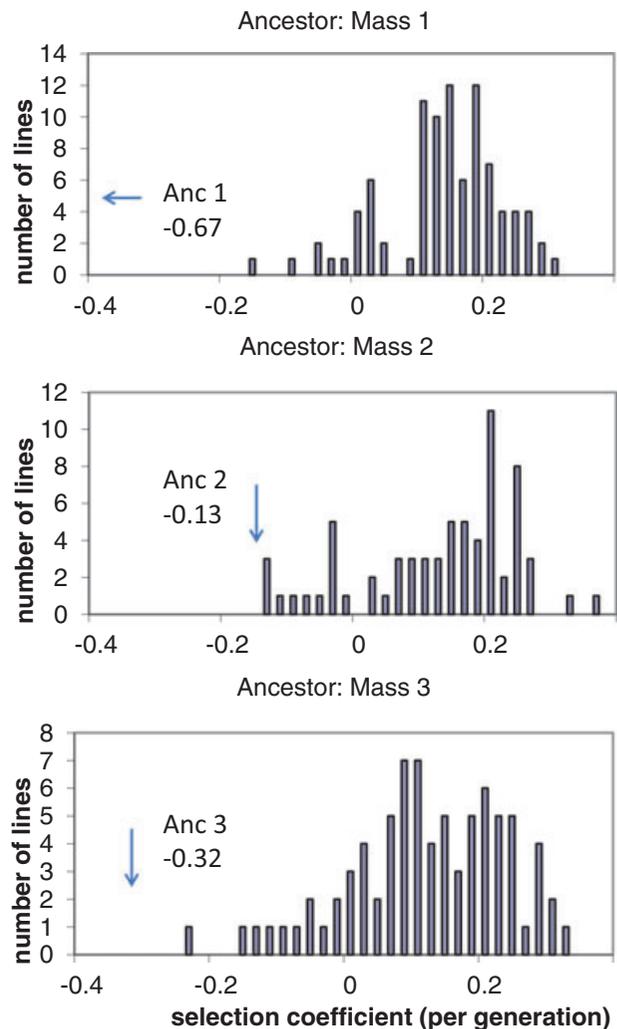


Figure 1. Distribution of fitness among the evolved dark lines. Values are estimates of fitness against the common tester. The fitness of the ancestor is also indicated.

try (single-classification analysis of variance [ANOVA], $F < 1$; Fig. 1). The average selection coefficient, relative to the arbitrarily chosen tester, was about $+0.11$. Anc 1 was transformed under very strong selection ($s > 0.78$ per generation) from a population incapable of substantial growth in the dark into a range of efficient heterotrophic lines. At the other extreme, the improvement of Anc 2 was more modest, although fitness differences ($s = 0.24$ per generation) were still intense by most standards.

LINES BECOME MORE RESPONSIVE

The ancestral populations show little response to acetate concentration over the range 0.1–10 g L⁻¹, whereas the selection lines have evolved a steep increase in growth over this range (Fig. 2). Their responsiveness can be expressed as the regression of growth (in units of optical density) on log acetate concentration in the linear portion (range 0.2–5 g L⁻¹) at the normal transfer interval of

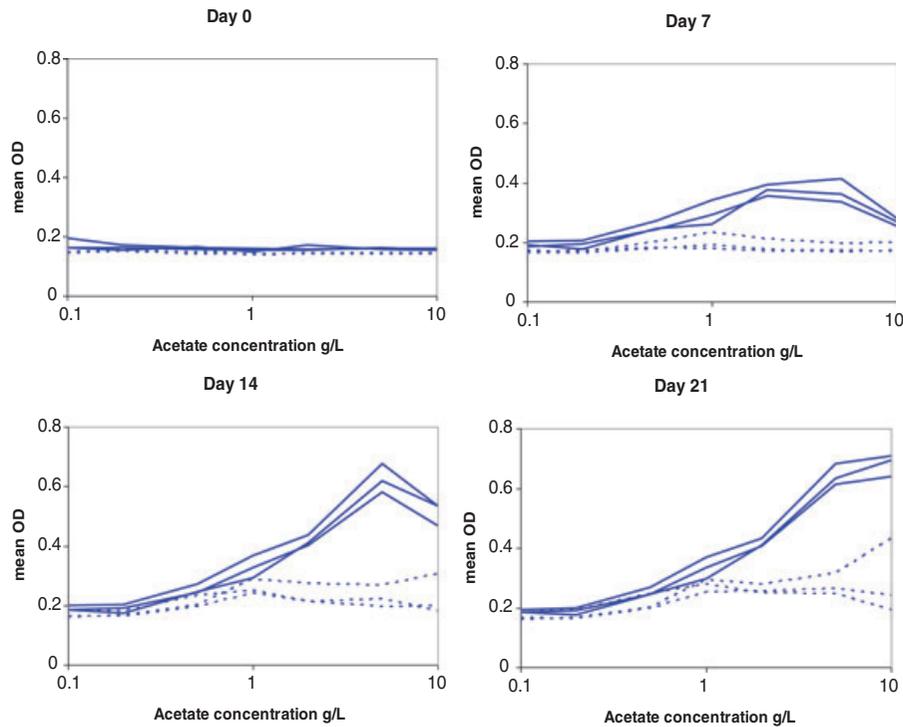


Figure 2. Responsiveness of the dark lines in relation to their ancestors. Response curves are given for growth after a specified number of days in the assay. The solid lines are the evolved lines, the broken lines the ancestors.

14 days. Like growth itself, responsiveness differed among ancestors (single-classification ANOVA $F = 45.4$; $df = 2, 57$; $P < 0.001$), reflecting their initial propensity for dark growth, but evolved to a similar level in their descendants ($F = 2.1$; $df = 2, 237$; $P > 0.05$). The regression coefficient for Anc 1 is $b = 0.066$, which increases to an overall average of $b = 0.35$ among the selection lines; for Anc 3, the corresponding values are $b = 0.15$ and $b = 0.37$; and for Anc 2, $b = 0.20$ for the ancestral population and $b = 0.33$ for the selection lines.

MOST PHENOTYPIC VARIATION WAS ATTRIBUTABLE TO NATURAL SELECTION

Each selection line had diverged from the average state of the ancestral populations after 50 cycles of dark growth. The extent of divergence with respect to a given character has three components: natural selection as the source of directional change, drift (or the idiosyncratic course of adaptation) as the source of nondirectional change, and ancestry as the determinant of initial state. The overall sum of squares can be partitioned among these three components. If the initial grand mean score is I and the final grand mean is F , then the sum of squared deviations among the m lines descending from each of n ancestors attributable to systematic change caused by natural selection is $mn(F - I)^2$; the quantity attributable to ancestry is $n \sum (A - F)^2$, where A is the mean score of all lines derived from a given ancestor; and the quantity attributable to drift is $\sum \sum (L - A)^2$, where L is the score of each line. Natural

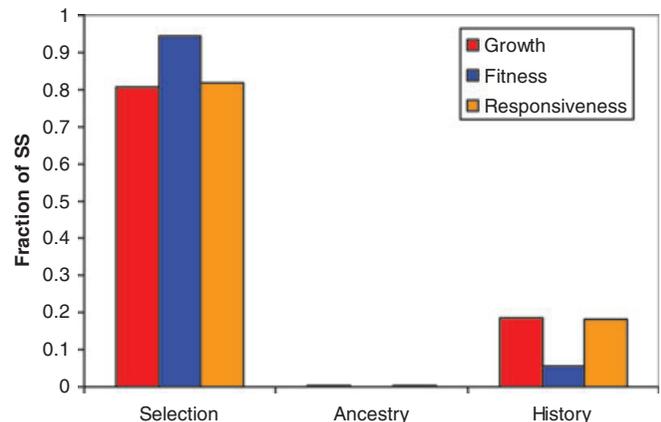


Figure 3. The contributions of selection, ancestry, and history to the divergence of the evolved dark lines. "Growth" refers to growth in monoculture and "Fitness" to competition in mixtures.

selection is by far the largest component for fitness, growth, and responsiveness; drift and other forms of sampling error make a much smaller contribution; and the effect of ancestry is scarcely perceptible (Fig. 3).

ADAPTATION WAS QUALITATIVELY SPECIFIC

The average combined growth (difference between initial and final values of OD) on fructose, galactose, glucose, glycerol, lactose, maltose, mannose, sorbitol, and sucrose, in units of optical density,

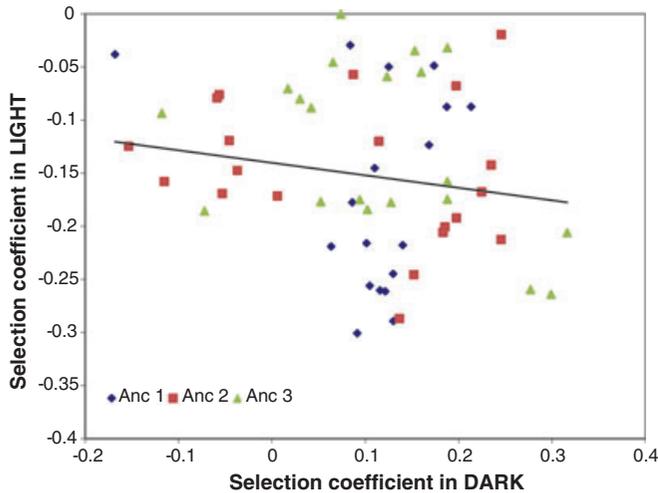


Figure 4. The fitness of the dark lines cultured in the light relative to their fitness in the dark. Fitness is measured relative to a common tester in the dark and against the ancestor in the light. The regression equation over all lines is $y = -0.12x - 0.14$ ($r^2 = 0.05$).

was 0.0085 for blank (uninoculated) wells, 0.00815 for ancestral populations, and 0.00675 for dark selection lines. There is no evidence for general adaptation to carbon substrates other than acetate. No line showed consistently enhanced growth on any individual substrate.

ADAPTATION IS ANTAGONISTIC

The average selection coefficient acting against the dark lines when cultured with their ancestral population in the light was -0.15 per generation (SD 0.088, SE 0.01), with all estimates negative. There was thus a very general and, on average, substantial reduction of fitness in the ancestral environment associated with adaptation to heterotrophic growth.

ADAPTATION AND ANTAGONISM ARE ONLY WEAKLY RELATED

There is only a weak negative correlation between selection coefficients in light and dark ($r = -0.22$; $0.01 < P < 0.05$ for a one-tailed test; Fig. 4).

SOME DARK LINES ARE UNABLE TO GROW IN THE LIGHT

Growth parameters for autotrophic and mixotrophic growth are all positively correlated among selection lines, with mixotrophic conditions associated with lower average growth rate and productivity (Fig. 5). The corners of the four quadrants of this graph illustrate four different possible outcomes of selection.

- (1) The upper right quadrant contains lines able to grow well in both autotrophic and mixotrophic conditions. Because all lines grow well in heterotrophic conditions, these are true

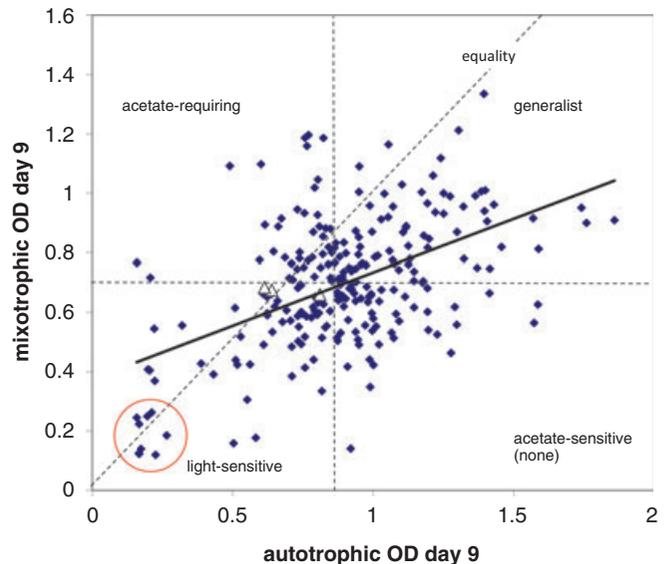


Figure 5. The growth of the dark lines in autotrophic and mixotrophic condition. The plot refers to yield at nine days; plots for estimates of the intrinsic rate of increase and the limiting density give similar results. The white triangles are mean values for the three ancestors; solid circles are the evolved lines. The plot is divided into four quadrants by the means of heterotrophic and mixotrophic growth; the diagonal broken line represents equality. The horizontal and vertical broken lines show the overall means on the axes. The regression equation is $y = 0.359x + 0.359$ ($r^2 = 0.25$). The circle identifies light-sensitive lines.

generalists. They are not rare; despite the overall depression of fitness in the light, most of the dark lines retain the ability to grow well in the light.

- (2) The lower right quadrant represents the suppression of growth by acetate in the light; not surprisingly, there are no lines with this phenotype.
- (3) The upper left quadrant contains (along its left-hand edge) acetate-requiring lines that grow well in the light only when supplied with acetate. Two lines have this phenotype.
- (4) The lower left quadrant contains light-sensitive lines that grow poorly in the light whether acetate is present or not. There are at least eight lines whose growth is strongly repressed by light. The most noteworthy lines are those for which the ancestral environment has become lethal, and that cannot be rescued by acetate. This phenotype was confirmed by pinning these lines on solid agar together with ancestors and selection lines able to grow in the light.

Discussion

RAPID EVOLUTION OF EFFICIENT HETEROTROPHY

Cells able to use acetate efficiently in the dark were rare in the base populations after several thousand generations of exclusively

autotrophic growth. Consequently, more than 90% of derived lines soon become extinct when these populations were abruptly transferred to the dark. The surviving lines nevertheless recovered within about 20 generations and evolved greatly enhanced heterotrophic growth within a few hundred generations, regardless of ancestry. A similar result for maltose utilization by experimental populations of *Escherichia coli* selected on glucose was reported by Travisano et al. (1995). The evolved lines have higher rates of growth, greater competitive fitness, and enhanced responsiveness to acetate. This is most likely to have been attributable to the expansion of lineages still capable of some level of heterotrophic growth in the base populations. It is possible that beneficial mutations subsequently improved the efficiency of heterotrophic growth, but unlikely that they were responsible for the difference between survival and extinction.

MIXOTROPHY

There are fundamental constraints on metabolism that follow from the incompatibility of alternative metabolic pathways. Glucose can be degraded either by fermentation or by respiration, for example, but not by both simultaneously. Cells may therefore achieve high growth rate through fermentation, at the expense of low yield, or high yield through respiration, and the expense of low growth rate (MacLean et al. 2010). There is no such incompatibility between heterotrophic and autotrophic pathways in *Chlamydomonas* and other acetate flagellates. Acetate is brought across the membrane by a monocarboxylic transporter protein in eukaryotic microbes, although this does not seem to have been decisively established for *Chlamydomonas*. Once in the cytosol, it combines with coenzyme A to form acetyl CoA, which is then oxidized by one of two routes. The first is through the tricarboxylic acid (Krebs) cycle in the mitochondria. The second is through the glyoxalate cycle, an abbreviated version of the Krebs cycle that normally takes place in the glyoxysome, a specialized kind of plastid, although its location in *Chlamydomonas* is at present uncertain. The glyoxalate cycle is necessary for acetate to be used to synthesize carbon skeletons, and its key enzymes are induced by dark incubation (Neilson and Lewin 1974). Inorganic carbon, by contrast, enters the cell by diffusion or through a carbon concentrating mechanism involving a chain of carbonic anhydrases, and is processed through the Calvin cycle in the chloroplast. The reactions, the cellular compartments in which they take place and the enzymes involved for heterotrophic and autotrophic growth are largely distinct and the two pathways are therefore compatible.

There are two potential advantages for maintaining both autotrophic and heterotrophic metabolism. The first is the immediate advantage of utilizing both organic and inorganic carbon by mixotrophic growth when both are supplied simultaneously. The second is the long-term advantage when each is supplied alternately.

It may seem obvious that access to two sources of carbon skeletons and energy must be advantageous, but this is not necessarily so. The yield from autotrophic growth per mole carbon is about twice as great as that from heterotrophic growth, because about half of the acetate supply must be diverted to provide energy (Boyle and Morgan 2009). Because heterotrophic and autotrophic pathways are largely separated, they can be operated simultaneously; the maximum theoretical yield of mixotrophic growth is intermediate between autotrophic and heterotrophic growth. The results reported here are consistent with the predictions of flux-balance analysis: our long-term laboratory populations of *Chlamydomonas reinhardtii*, maintained for a decade as autotrophs, have much lower growth rates when cultured on acetate in the dark than they do in the light. Acetate weakly inhibited growth and yield in the light, both in the ancestral and evolved populations. In most reports in the literature, however, growth is usually faster in heterotrophic conditions (Perez-Garcia et al. 2011), at least for *Chlorella* supplied with glucose, which is much more energy-rich than acetate (e.g., Endo et al. 1974). This suggests that there may be a trade-off between faster growth (by heterotrophy) and higher yield (by autotrophy). Laliberté and de la Noüe (1993) found that heterotrophic growth and yield greatly exceeded autotrophy in *C. humicola* cultured on acetate, whereas mixotrophy had much higher growth rate and yield than autotrophy and heterotrophy combined. The autotrophic growth rates they reported (about 0.2 per day) were extremely low, however. In any particular case, the growth that is actually achieved will depend on the physiological potential of the stocks used and on the conditions of growth, such as the nature and concentration of the substrate and physical factors such as light and aeration. Mixotrophy will be beneficial in some combinations of factors but not in all. The mixotrophic (phagotrophic) flagellate *Ochromonas* coexisted with either obligately heterotrophic or obligately photoautotrophic competitors in laboratory culture when both light and bacterial prey were available, but was excluded by specialist heterotrophs in the dark (Rothhaupt 1996).

ALTERNOTROPHY

The other possibility is that lineages often experience an alternation of light and dark. Most wild types of *C. reinhardtii* have been isolated from soil, where they can grow by photosynthesis only if exposed on the surface but can use acetate when blocked from light deeper in the soil. Lineages able to use both kinds of site are likely to flourish because they can maintain high long-term rates of growth. Chlorophytes do not secrete extracellular enzymes, and can only feed on substrates leaked by bacteria and fungi, such as the acetate released by the anaerobic fermentation of many kinds of substrate. They act as scavengers that are able to use only a limited range of substrates produced by other organisms, but subsidized by their ability to grow without organic carbon during

periods when light is available. Specialized autotrophs will grow only slowly, if at all, during dark periods, whereas specialized heterotrophs are penalized during periods when there is little or no organic carbon. Generalists will tend to evolve when the environment alternates between states requiring different ways of life because their long-term geometric mean fitness will exceed that of either specialist (Gillespie 1977). Reboud and Bell (1997) showed that experimental populations of *Chlamydomonas* subjected to an environment fluctuating between light and dark conditions every few generations tended to evolve into generalists capable of growing well in both. Jones et al. (2009) have speculated that mixotrophic protists could survive the “catastrophic darkening” associated with volcanic eruptions and asteroid impacts and be responsible for the rapid resumption of primary production after the event.

SPECIALISTS

Despite the advantages of having two different modes of growth, most algae can grow only as autotrophs or (much less often) as heterotrophs. Obligate photoautotrophs may lack mechanisms to transport relatively large molecules across the cell membrane. One species of *Nostoc* lacks a glucose transport system and is an obligate autotroph, whereas another has a functional system and can grow heterotrophically (Beauclark and Smith 1978). *Phaeodactylum tricorutum* is an obligate photoautotroph that can be transformed into a facultative heterotroph by the insertion of a glucose transporter gene (Zaslavskaja et al. 2001). Alternatively, substrates that are taken up by the cell may not be processed successfully because of lesions in metabolic pathways. Some obligately autotrophic algae, for example, lack α -ketoglutarate dehydrogenase and are thus unable to use the Krebs (tricarboxylic acid) cycle to generate ATP (Benedict 1978; Wood et al. 2004). The inability of the evolved dark lines to use any of the carbon substrates tested suggests that lack of functional transport systems is the basis of acetate specialization in *C. reinhardtii*.

EVOLUTION OF SPECIALIZATION BY MUTATIONAL DEGRADATION

Mutations in genes that encode components of the photosystems will often be neutral or nearly so in dark conditions, and will therefore tend to accumulate because they are not efficiently eliminated by purifying selection. Few if any will become fixed, because the number of generations required for fixation is of the order of the population size, but many will occur at low frequency, so that as time goes on the number of lineages which bear none of these mutations will gradually diminish. A population that lives perpetually in the dark will eventually lose the ability to grow in the light, when the last lineage free of conditionally deleterious mutations become extinct. It may even lose protection against light-induced damage. Conversely, a population that lives perpetually in the

light will eventually accumulate mutations in genes responsible for active transport, and possibly substrate metabolism, and will evolve into an obligate photoautotroph. In this way, generalists will decay into obligate autotrophs or obligate heterotrophs when they are confined for long periods to a single kind of environment. Within the polyphyletic genus *Chlamydomonas*, there are roughly equal numbers of obligately autotrophic species and facultative heterotrophs, with no obvious phylogenetic clustering. There are also obligate heterotrophs among related forms, such as *Polytoma* and *Polytomella*, which have lost the chloroplast. These examples suggest that transitions between facultative heterotrophy and obligate autotrophy may occur quite readily and rapidly.

A consistent and rather uniform loss of autotrophic fitness evolved subsequently among lines cultured in the dark. The degree of enfeeblement is not strongly related to the advance in dark growth, and can be attributed largely to loss-of-function mutations among the many loci encoding the photosystems. Most lines could still grow quite well in the light, whether supplied with acetate, and would readily survive a reversal of conditions. A small fraction (about 5%) of lines showed severely impaired growth in the light. Two grew normally when acetate was supplied, and hence require both light and acetate. Eight grew poorly whether acetate was supplied or not; three of these appeared to be unable to grow in the light and represent obligate heterotrophs. Acetate-requiring mutants deficient in photosynthesis have been isolated by chemical mutagenesis and replica-plating and found to have lesions in photosystem or pigment biosynthesis genes, many located on the chloroplast genome (Spreitzer and Metz 1981). These mutants tend to be sensitive to light, perhaps because reductants generated by an intact photosystem electron transport chain would not be used up by an impaired Calvin cycle. Compensatory nuclear mutations have also been identified (Spreitzer and Ogren 1983). “White” strains lacking carotenoids have loss-of-function mutations in the nuclear phytoene synthase gene *PSY*, whose product is required for the initial stages of carotenoid synthesis (McCarthy et al. 2004). They can grow heterotrophically in the dark, but are unable to grow in the light, with or without acetate. They retain the plastid envelope but lack thylakoid membranes and the pyrenoid bodies that normally hold Rubisco (Inwood et al. 2008). It is a short step from this phenotype to colorless relatives of *Chlamydomonas*, such as *Polytoma*, that lack all but small nonfunctional remnants of the plastid. Our experimental protocol was not designed to favor light-sensitive types, as the lines were routinely exposed to light during transfer, but this minimal exposure did not prevent white and light-sensitive phenotypes from emerging in several populations.

The three lines unable to grow in the light, with or without acetate, all evolved from Anc 1, in which the great majority of cells are unable to grow in the dark. They illustrate a simple route to ecological speciation, resulting within a few hundred

generations in populations that would be almost completely isolated in nature through an inability to grow in a common environment. The obligately autotrophic and obligately heterotrophic members of the Chlamydomonadaceae presumably diverged through the same mechanisms that are involved in the experimental evolution of efficient heterotrophic growth.

This experiment shows that how evolutionary change can lead to ecological change within a short span of time. Mixotrophy in natural communities links the microbial and planktonic food webs and can have important ecological consequences (Ptacnik et al. 2004). Although the balance between autotrophic and heterotrophic metabolism is the most fundamental feature of the food web, it can shift rapidly as some members of the community evolve in response to altered conditions of growth. Microbes do not recognize any clear boundary between ecology and evolution.

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Supporting Information

The following supporting information is available for this article:

Table S1. Summary of attributes of AcAg Expt Dark selection lines. Assays in which all 240 surviving lines were scored

Table S2. Summary of attributes of AcAg Expt Dark selection lines. Assays of 60 lines.

Supporting Information may be found in the online version of this article.

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