Trophic Conversion of an Obligate Photoautotrophic Organism Through Metabolic Engineering

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Most microalgae are obligate photoautotrophs and their growth is strictly dependent on the generation of photosynthetically derived energy. We show that the microalga *Phaeodactylum tricornutum* can be genetically engineered to thrive on exogenous glucose in the absence of light through the introduction of a gene encoding a glucose transporter (*glut1* or *hup1*). This demonstrates that a fundamental change in the metabolism of an organism can be accomplished through the introduction of a single gene. This also represents progress toward the use of fermentation technology for large-scale commercial exploitation of algae by reducing limitations associated with light-dependent growth.

Photosynthetic algae are dominant producers in aquatic environments, accounting for a substantial proportion of worldwide O_2 production and CO_2 fixation (*1*, *2*). They are also a component of feed for aquaculture and produce numerous valuable compounds including pigments (e.g., β-carotene, phycobiliproteins), oils (e.g., docosahexaenoic acid), and stable isotope-labeled biochemicals (e.g., [^{13}C]glucose); they also have potential in the discovery of new pharmaceuticals (*3*).

Commercial-scale cultivation of photosynthetic microalgae is typically performed in large, open outdoor ponds. This mode of growth, although it exploits natural sunlight for the production of energy (*3*, *4*), is associated with numerous disadvantages. Contaminants invade pond cultures, and seasonal and diurnal variations in temperature and light conditions make it difficult to predict both growth rates and final culture densities. Self-shading restricts light availability, severely limiting biomass production, and low cell densities prevent efficient harvesting of the cells. Together, these factors have restricted large-scale cultivation of microalgae to a small subset of genera that includes *Spirulina* and *Dunaliella*.

A strategy that can improve the efficiency and reduce the cost of microalgal biomass production involves heterotrophic growth of algae in conventional microbial fermenters (in the absence of light) (*3*–*5*). Fermenters provide controlled, sterile growth conditions that maximize productivity. Glucose or other forms of organic carbon, rather than light, supply energy and reducing equivalents. However, the use of fermentation technology is limited, because most microalgae are obligate photoautrophs and are unable to grow on fixed carbon compounds (6).

These considerations suggest advantages to genetically engineering microalgal metabolism for high rates of heterotrophic growth. One microalga that can be genetically modified by transformation (*7*–*9*), but is unable to grow heterotrophically (*6*, *10*, *11*), is the diatom *Phaeodactylum tricornutum* (UTCC646). We attempted a trophic conversion of this alga by transforming it with genes encoding glucose transporters. The transporter genes used included *Glut1* from human erythrocytes (*12*); *Hup1* from the microalga *Chlorella kessleri* (*13*); and *Hxt1*, *Hxt2*, and *Hxt4* from *Saccharomyces cerevisiae* (*14*). The coding regions of these genes were inserted into the *P. tricornutum* transformation vector pPha-T1 (*8*). A construct (Glut1-GFP) was also generated in which the green fluorescent protein gene, *GFP*, was fused to the 3′ end of the *Glut1* gene. Plasmids were introduced into *P. tricornutum* by using biolistic procedures, and transformants were selected for zeocin resistance in the light (*8*). The transformants were then transferred to solid or liquid medium containing 0.1 or 1.0% glucose, placed in complete darkness, and monitored for growth (*15*).

The *P. tricornutum* cell lines transformed with the *Glut1* gene (19 of 28) exhibited rates of glucose transport (*16*) between 0.2 and 13 nmol glucose/min for 10^8 cells (19 of 28 primary transformants). Cell lines with uptake rates of ≥1.6 nmol glucose/min for 10^8 cells (11 of 28) grew on glucose in the dark. For *Hup1*-containing transformants, 14 of 25 exhibited glucose uptake rates between 0.06 and 1.9 nmol glucose/min for 10^8 cells. Cell lines with uptake rates of ≥0.29 nmol/min for 10^8 cells (11 of 25) grew in the dark. None of the cell lines transformed with the control vector or the yeast transporter genes exhibited detectable glucose uptake (*17*). The inability of the transformants to express functional Hxt protein may reflect differences in codon usage between yeast and *P. tricornutum* (*8*).

A detailed characterization was performed on a number of the *Glut1* transformants, including *Glut1-17* and *Glut1GFP-40*. Monospecific antibodies against the Glut1 polypeptide and GFP were used to demonstrate accumulation of Glut1 or the Glut1-GFP fusion protein in transformed cell lines (*18*). Membranes of the *Glut1-17* transformant contained two prominent polypeptides that reacted with Glut1-specific antibodies (Fig. 1); no cross-reacting material was present in the soluble phase of the cell (*17*). These polypeptides had molecular masses of 44 and 39 kD, which is less than the native protein (~55 kD) synthesized in human erythrocytes (Fig. 1, compare lanes B and G1-17), but which is close to the size of unglycosylated Glut1 (38 kD) (*19*). This implies that Glut1 synthesized in *P. tricornutum* is glycosylated differently from that in human erythrocytes. The Glut1-GFP fusion protein present in the *Glut1GFP-40* transformant had a molecular mass of ~75 kD, which is slightly smaller than the expected mass of Glut1-GFP (82 kD), presumably resulting from glycosylation differences.

Both the *Glut1-17* and *Glut1GFP-40* transformants showed high rates of glucose uptake (*16*) (Fig. 2). The *Glut1-17* transformant had a K_m for glucose of 1.2 mM and a V_{max} of 7.6 nmol glucose/min for 10^8 cells; *Glut1GFP-40* had a K_m of 1.0 mM and a V_{max} of 13 nmol glucose/min for 10^8 cells. The K_m values for glucose in the transformants are similar to those (1 to 2 mM) measured for human erythrocytes (*12*). Differences in the V_{max} between transformants probably reflect different levels of expression of the *Glut1* gene, which could depend on the site of integration into the diatom genome. In the presence of 5×10^{-4} units of cytochalasin B per milliliter, a specific inhibitor of Glut1-dependent transport (*20*), glucose uptake was reduced to undetectable levels (*17*). These results demonstrate that Glut1 facilitates glucose transport into *P. tricornutum* cells and that the affinity of the transporter for glucose is essentially the same as in human erythrocytes.

To determine the subcellular location of the Glut1 protein in transformed lines, the *Glut1GFP-40* strain was examined for GFP fluorescence by confocal microscopy (*21*). Untransformed cells showed strong chlorophyll fluorescence, but low fluorescence in the green channel (Fig. 3, A, B, and C).

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Transformed cells containing the *GFP* gene exhibited GFP fluorescence consistent with localization in the cytosol and the lumen of the cell nucleus (Fig. 3D). A similar distribution of soluble GFP in plant cells has been observed (*22*, *23*). In contrast, *Glut1GFP-40* showed fluorescence associated with the extreme periphery of the cells (Fig. 3E). These results demonstrate that the Glut1 protein targets GFP to the cytoplasmic membrane, a pattern consistent with exclusive localization of the chimeric protein to the membrane fraction and the function of Glut1 in membrane-associated transport.

Commercial exploitation of metabolically engineered diatoms requires high rates of glucose-dependent growth and the achievement of high cell densities. Growth of the *Glut1-17* transformant was measured in the light and dark in liquid medium supplemented with glucose (*15*). As shown in Fig. 4, both untransformed cells and the *Glut1-17* transformant grown in the light without glucose reached the same cell densities (3×10^7 cells/ml). The addition of glucose did not change the growth characteristics of the untransformed strain. In contrast, the transformed strain attained a cell density about five times as high. Untransformed cells are unable to grow in the dark in the presence of glucose, but *Glut1-17* grew at the same rate and to the same cell density in the presence of glucose in the light or dark. As the cultures became more dense, and as light absorption was attenuated by self-shading, the rate of growth of *Glut-17* in the presence of glucose exceeded that of untransformed cells. If heterotrophic growth is conducted in a microbial fermenter with continuous addition of glucose and other nutrients, the density attained by Glut-17 exceeded that of untransformed cells 15 times (*17*), reaching densities of 5×10^8 cells/ml.

Most diatoms do not have the capacity to grow in the absence of light on exogenous glucose (*6*). This report demonstrates that trophic conversion of the obligate photoautotrophic diatom, *P. tricornutum*, can be achieved by transforming the alga with a single gene encoding a glucose transporter. Functionality of the algal and human glucose transporters in *P. tricornutum* suggests that the heterologous proteins are correctly targeted and inserted into diatom membranes. The inability of the diatom to glycosylate Glut1 normally suggests that the carbohydrate moiety may not play a critical role in targeting Glut1 to the cytoplasmic membrane or in transport function. *Hup1* has also been expressed in the microalgae *Volvox* (*24*) and *Cylindrotheca* (*25*), but neither were able to grow heterotrophically.

Trophic conversion by introduction of a glucose transporter requires that the complete glycolytic pathway is present within the cells and that it can support a high flux of metabolites. Photosynthetic organisms transfer fixed carbon out of plastids into the cytoplasm where it can be metabolized into hexose sugars; these sugars can then be used to support growth or stored as polysaccharides. Diatoms synthesize chrysolaminarin, a β-1,3-glucan polymer, as the primary carbohydrate storage compound (*26*). The catabolism of this glucose polymer would require glycolysis. Hexokinase activity, required for conversion of glucose to glucose-6-phosphate, the first metabolite of the glycolytic pathway, has also been reported in diatoms (*11*). Thus all of the necessary activities for glucose metabolism already exist in diatom cells. As a result, exogenous glucose entering the cell can be metabolized at a high flux, allowing the cells to thrive in the absence of light.

The trophic conversion of microalgae such as diatoms is a critical first step in engineering algae for successful large-scale cultivation using microbial fermentation technology. In addition to providing a means for maintaining culture conditions, glucose and other nutrients can be continuously provided to maximize productivity. The use of fermentation technology elim-

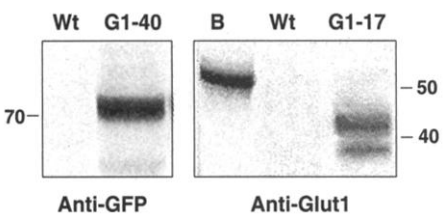

Fig. 1. Reactivity of antibodies specific for Glut1 or GFP to membrane proteins extracted from untransformed and transformed cell lines. The proteins were resolved by SDS–polyacrylamide gel electrophoresis after solubilization of total membranes from wild-type (Wt), untransformed cells; G1-40, the *Glut1GFP-40* transformant; B, human erythrocytes; G1-17, the *Glut1-17* transformant. The antibodies used were specific for GFP (left panel) or Glut1 (right panel).

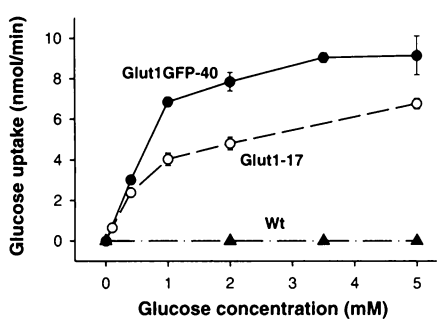

Fig. 2. Uptake of glucose by 10^8 cells of transformants *Glut1GFP-40* and *Glut1-17*, compared with wild-type untransformed cells (Wt). Glucose uptake was assayed as described (*16*).

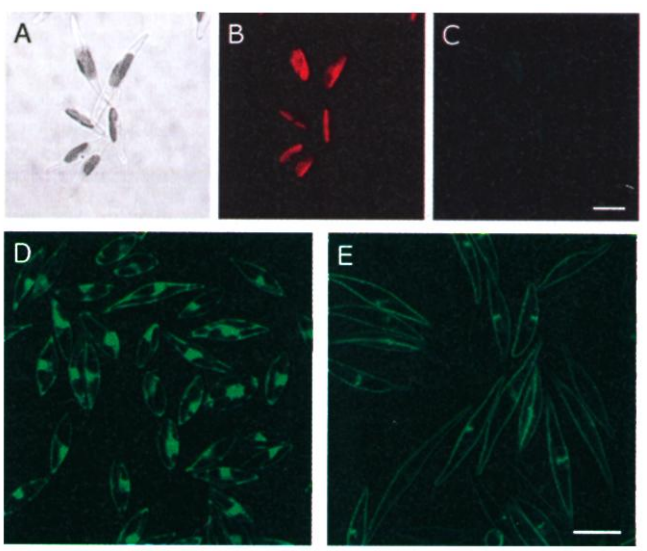

Fig. 3. Localization of Glut1 in *P. tricornutum* transformed with the chimeric gene encoding the Glut1-GFP fusion protein. The top panel shows a transmitted light image of untransformed *P. tricornutum* cells (**A**), fluorescence from the cells in the red channel (**B**), representing chlorophyll; and fluorescence in the green channel (**C**). The lower panel shows fluorescence in the green channel of cells that were transformed with the *GFP* gene (**D**), and of cells transformed with the chimeric *Glut1-GFP* gene [(**E**), strain designated Glut1-40]. Scale bars, 10 μm.

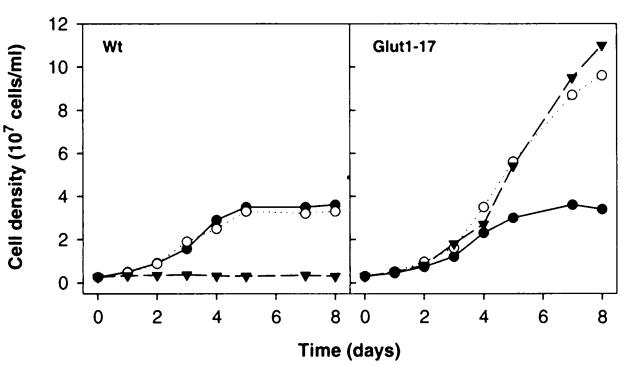

Fig. 4. Growth of untransformed cells and *Glut1-17* under different conditions. The left panel shows the growth of untransformed, wild-type cells (Wt), and the right panel shows the growth of *Glut1-17*. Growth was in the light (filled circles), in the light plus glucose (open circles), or in the dark plus glucose (filled triangles).

inates contamination by microbes, which is an important criterion for maintaining food industry standards. Fermentative growth of naturally heterotrophic microalgae has resulted in dry biomass accumulation to 100 g/liter (*3*, *27*), which is 10 to 50 times the yields obtained by using light-dependent culture systems. Fermentation-based systems can reduce production costs of microalgae by an order of magnitude relative to that incurred by photosynthesis-based production; cost reduction analyses factor in expenses for both fixed-carbon supplementation and equipment operation (*28*). Commercial benefits of fermentation-based systems result from increased biomass, productivity, harvesting efficiency, and reduced losses from contamination. The ability to grow microalgae heterotrophically increases the feasibility for developing a large range of new algal products.

Marine ecosystems also depend on diatoms, which contribute substantially to the reduction of inorganic carbon in marine habitats. Such a contribution may increase substantially as the ecology of oceanic environments is altered (*29*–*32*). The exploitation of diatoms that can be genetically manipulated and that can grow heterotrophically will facilitate the use of mutants to augment our understanding of both photosynthesis and other metabolic pathways that are essential for competing in marine ecosystems.

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15. After 4 weeks in the dark, the transformants that grew were restreaked and maintained on 1.0% glucose. Liquid cultures were grown with 1.0% glucose at 20°C on an orbital shaker. All characterized transformants were generated from independent particle bombardments. Cells were grown at 20°C with continuous illumination at 75 μmol photons m^{-2} s^{-1} in Provasoli's enriched seawater medium with 10× the nitrogen and phosphorus by using Instant Ocean artificial seawater, at 0.5× concentration. Glucose was maintained between 5 and 10 g/liter. Growth rates were determined in 250-ml flasks (50 ml of media) with silicon foam closures. Daily samples measured cell numbers and nutrients. Flasks were stirred at 100 rpm. Fermentations were done in a 2-liter Applikont vessel by using an agitation rate of 100 rpm, dissolved oxygen was maintained at >20% saturation.
16. Cells in logarithmic phase growth were harvested, washed two times, and resuspended in fresh medium. Assays were initiated by adding unlabeled glucose and [D-^{14}C]glucose to 0.05 μCi/ml; the cells were maintained in the light. Samples were removed at 0, 2, 5, 10, and 15 min after the addition of labeled glucose. The cells were collected by filtration, washed with medium containing 1.0% unlabeled glucose, and transferred to scintillation vials.
17. L. A. Zaslavskaia *et al.*, unpublished data.
18. The cells were broken by using a MinibeadBeater by two cycles at full speed on ice. Cell membranes were pelleted by centrifugation at 100,000*g* for 30 min, solubilized in 2.0% SDS, resolved on 7.5% polyacrylamide gels, and transferred to nitrocellulose membranes.
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21. Confocal microscopy was performed using a Nikon 60× N.a. = 1.2 water immersion objective on a Nikon TMD 200 inverted microscope outfitted with a BioRad MRC 1024 confocal head mounted in a Koehler configuration. EGFP was excited at 488 nm and visualized with a 522/25-nm bandpass filter. Plastid autofluorescence was excited at 456 nm and visualized with a 585-nm-long pass filter.
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Telomere Position Effect in Human Cells

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In yeast, telomere position effect (TPE) results in the reversible silencing of genes near telomeres. Here we demonstrate the presence of TPE in human cells. HeLa clones containing a luciferase reporter adjacent to a newly formed telomere express 10 times less luciferase than do control clones generated by random integration. Luciferase expression is restored by trichostatin A, a histone deacetylase inhibitor. Overexpression of a human telomerase reverse transcriptase complementary DNA results in telomere elongation and an additional 2- to 10-fold decrease in expression in telomeric clones but not control clones. The dependence of TPE on telomere length provides a mechanism for the modification of gene expression throughout the replicative life-span of human cells.

Most normal human cells lack the enzyme telomerase, which maintains telomeres, and as a consequence, telomeres shorten with each division until the cells reach replicative senescence (the Hayflick limit). This growth arrest is mediated by p53 and has been suggested to be the result of a DNA damage response to telomeres that have become too short (*1*–*3*). No mechanism has been demonstrated in vertebrates that can account for differences between young and old (but not yet senescent) cells. In *Saccharomyces cerevisiae*, telomere position effect (TPE) can result in the reversible silencing of a gene near a telomere by a mechanism that depends both on telomere length and on the distance to the gene (*4*–*6*). Because telomeres in most human cells shorten with age, TPE would provide a mechanism to incrementally alter phenotype with increasing cellular age (*7*). However, previous efforts to identify TPE in mammalian cells have not been successful (*8*–*10*). We demonstrate here the presence of TPE in human cells and that the strength of the silencing effect is dependent on telomere length.

We seeded de novo telomere formation in (telomerase-positive) HeLa cells by introducing a linear plasmid containing a luciferase reporter adjacent to 1.6 kb of telomere repeats (Web fig. 1) (*11*). Integration of a repeat-containing plasmid can result in breakage of the chromosome, followed by extension of the plasmid telomeric sequences by telomerase and loss of the distal chromosome fragment (*12*). Clones with a telomeric reporter were identified by Southern blotting of purified telomeres (Fig. 1A and Web fig. 2) (*11*) and confirmed by in situ hybridization (Fig. 1B). The mean length of the healed telomeres (after subtracting 3 kb of attached plasmid sequence) was estimated from Southern blots to be between 1.5 and 2 kb. Control clones were generated by transfection of an other-

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