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Manipulation of Microalgal Lipid Production Using Genetic Engineering

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ABSTRACT

Genetic transformation of two species of diatoms has been accomplished by introducing chimeric plasmid vectors containing a bacterial antibiotic resistance gene driven by regulatory sequences from the acetyl-CoA carboxylase (ACCase) gene from the diatom *Cyclotella cryptica*. The recombinant DNA integrated into one or more random sites within the algal genome and the foreign protein was produced by the algal transformants. This is the first report of genetic transformation of any chlorophyll c-containing microalgal strain. We are using this system to introduce additional copies of the ACCase gene into diatoms in an attempt to manipulate lipid accumulation in transformed strains.

Index Entries: Biodiesel; diatoms; genetic transformation; lipid synthesis; microalgae.

INTRODUCTION

Microalgae are single-celled or colonial photosynthetic organisms that are attracting significant interest for industrial applications, including the production of specialty chemicals and nutritional supplements. In many microalgae, excess photosynthate accumulates as triacylglycerol storage lipids, and many species can be induced to accumulate increased amounts of lipids under conditions of nutrient deficiency *(1)*. The lipid, which is chemically similar to common vegetable oils, can be extracted and converted to fatty acid methyl esters for use as a diesel fuel substitute *(2)*. This type of fuel, which can be derived from animal, plant, or algal lipids, is known as "biodiesel." Biodiesel has advantages over fossil-derived diesel in that it is renewable, biodegradable, and produces fewer particulates and less SO_x when burned. Biodiesel from vegetable oil sources, such as soybean and canola, is currently used in a number of European markets, and is being tested in the US. Our goal is to develop microalgae as an economically viable source of biodiesel fuel.

One approach we are taking toward this goal is to use genetic engineering to optimize the lipid production capabilities of microalgae that show potential for biodiesel fuel production. A key step in this process is the development of genetic transformation systems for oleaginous microalgae. Prior to this article, the only

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reproducible genetic transformation systems that had been developed for any microalgal species were for the fresh-water green alga *Chlamydomonas reinhardtii* (reviewed in ref. *3*) and a related colonial species, *Volvox carteri (4)*. Efficient nuclear transformation in these strains was accomplished only by using homologous genes as selectable markers; thus, the techniques were not directly transferable to other microalgal species.

Diatoms are a group of microalgae that has shown significant potential for biodiesel production. These organisms are major primary producers in the global ecosystem, producing approx 25% of the world's oxygen. Diatoms are characterized by the presence of chlorophyll c and fucoxanthin in their chloroplasts, and by their intricately patterned, siliceous cell walls *(5)*. We report here on the successful genetic transformation of two species of diatoms. Chimeric plasmid vectors were constructed that utilize regulatory sequences from a diatom gene to drive the expression of a bacterial antibiotic resistance gene. These vectors were introduced into diatoms via microprojectile bombardment, and the transformants were selected based on their acquired resistance to the antibiotic. Transformants were analyzed to demonstrate the presence of the foreign DNA and production of the foreign protein. Progress toward developing recombinant microalgal strains with altered lipid production capabilities will also be discussed.

METHODS

Algal Strains

The centric diatom *Cyclotella cryptica* Reimann, Lewin, and Guillard strain T13L *(6)* was obtained from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (West Boothbay Harbor, ME). *C. cryptica* strain CYCLO1 and the pennate diatom *Navicula saprophila* NAVIC1 are from the NREL Microalgal Culture Collection *(7)*. Cells were grown axenically in artificial sea-water medium (ASW) *(8)* supplemented with 1.07 m*M* sodium silicate. In liquid culture, *C. cryptica* T13L was grown in 50% ASW, and *N. saprophila* and *C. cryptica* CYCLO1 were grown in 10% ASW. Cultures were grown at 26°C in Erlenmeyer flasks without agitation under a 16:8 h light/dark cycle, with a light level of 50 $\mu E/m^2/s$. For solid media, 10% ASW supplemented with 20 m*M* glucose (ASWG) and 1% agar was used for all strains.

Plasmids

Two plasmids containing selectable markers that were used to transform the diatoms (pACCNPT10 and pACCNPT5.1) are shown in Fig. 1. Details of the construction of these plasmids will be described elsewhere *(9)*. Both plasmids contain the coding region of the neomycin phosphotransferase gene (*npt*II) from *E. coli* transposon Tn5; the neomycin phosphotransferase enzyme (NPTII) confers resistance to aminoglycoside antibiotics, such as G418 (geneticin). The *npt*II coding sequence is flanked by putative promoter and terminator sequences from the acetyl-CoA carboxylase (ACCase) gene (designated *acc1*) from *C. cryptica* T13L *(10)*. Plasmid pACCNPT10 contains 445 bp of upstream and 592 bp of downstream untranslated region (UTR) from *acc1*; this vector also contains 275 bp from the 3' end of the *acc1* coding region between the translational stop signal of the *npt*II gene and the *acc1* 3'-UTR. Plasmid pACCNPT5.1 was derived from pACCNPT10 by removing all

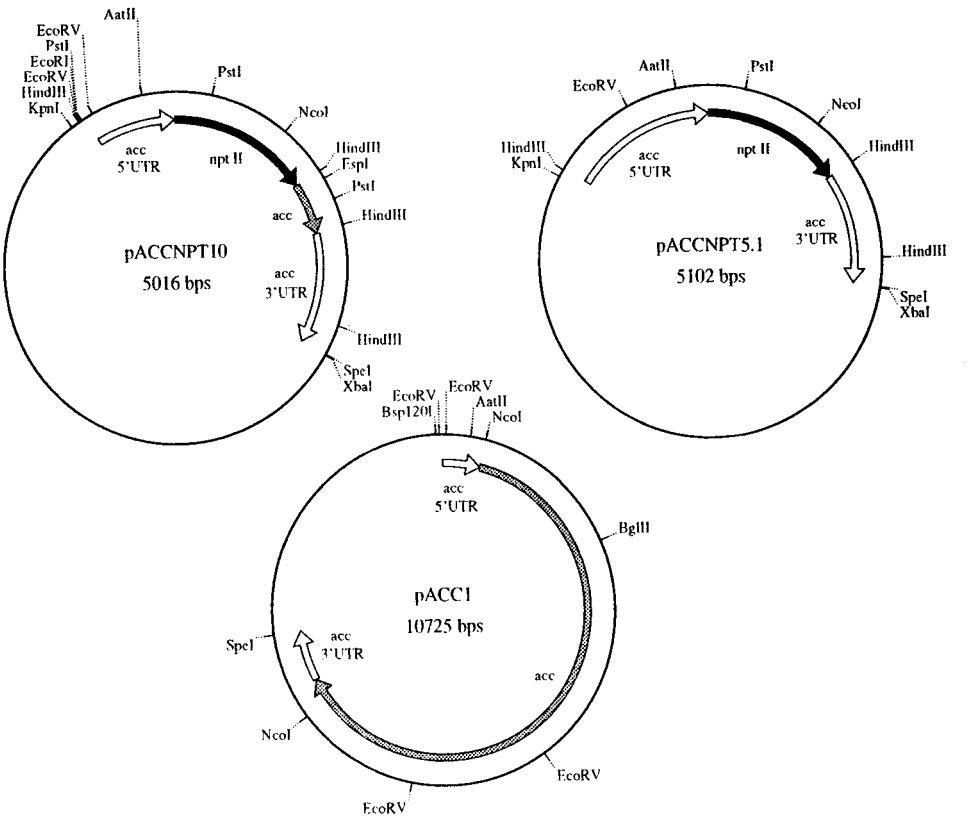

Fig. 1. Maps of the two plasmid vectors, PACCNPT10 and pACCNPT5.1, used as selectable markers for microalgal transformation. Also shown is pACC1, which contains the entire ACCase coding sequence from *C. cryptica* T13L cloned into pBluescript KS+. The *npt*II coding sequence is shown in solid black, *acc1* regulatory regions are in white, and *acc1* coding regions are shaded.

but 13 bp of the *acc1* coding region following the *npt*II gene, and by replacing the 445 bp of 5'-untranslated sequence with a corresponding 816-bp fragment to extend the putative promoter region. Plasmid pACC1 (also shown in Fig. 1) contains the entire coding region of *acc1*, including 445 bp of upstream and 592 bp of downstream sequence. The parental plasmid in all cases was pBluescript KS + (Stratagene, La Jolla, CA). Plasmid pCaMVNEO (not shown) was a gift from Michael Fromm.

Transformation

DNA was introduced into algal cells using the DuPont/Bio-Rad PDS1000-He microprojectile accelerator (Bio-Rad Laboratories, Hercules, CA). In this technique, plasmid DNA is precipitated onto tungsten or gold beads and propelled into the algal cells by a burst of helium pressure *(11)*. Before bombardment, concentrated algal cells were spread onto the center two-thirds of an ASWG agar plate that had been supplemented with 50 μg/mL ampicillin to reduce bacterial contamination. Approximately 3×10^7 cells were used for each bombardment for the *C. cryptica* strains, and 3×10^8 cells for *N. saprophila*. Plasmid DNA was coated onto tungsten

particles (0.5-μm m3N particles from Alfa Research Chemicals, Ward Hill, MA), and the cells were bombarded with the particles using a burst pressure of 1100 psi. Ten microliters of the DNA/particle suspension, containing 3 mg tungsten and 0.8–1.0 μg plasmid DNA, were used for each transformation. After bombardment, the plates were placed in the growth room for 2 d to allow the cells to recover and express the foreign gene. To select transformants, cells on each plate were resuspended in 2 mL of 10% ASW and replated onto eight ASWG plates containing G418 (Sigma, St. Louis, MO). The concentration of G418 required to inhibit the growth of the cells completely was determined empirically for each algal strain, and depended on the cell plating density and on the salt concentration of the media used to grow the cells. G418-resistant strains of *C. cryptica* T13L, *C. cryptica* CYCLO1, and *N. saprophila* were selected on 100, 50, and 25 μg/mL G418, respectively. G418-resistant colonies were typically seen within 7–10 d. The putative transformants were picked after 14 d, and tested for continued growth on G418 plates and for the presence of the *npt*II gene and NPTII protein.

For the cotransformation experiments, pACCNPT5.1 and pACC1, in a molar ratio of approx 1:1, were coprecipitated onto tungsten beads and used to transform *C. cryptica* T13L or *N. saprophila*. G418-resistant colonies were screened for the presence of pACC1 sequences using the polymerase chain reaction (PCR).

Analysis of Transformants

DNA was isolated for Southern analysis by the glass bead/vortexing protocol as described previously *(12)*. The DNA was digested with restriction endonucleases, separated on 0.8% agarose gels, and transferred to nylon membranes. The foreign DNA sequences within the algal DNA were detected using the Genius nonradioactive DNA detection system (Boehringer Mannheim Biochemicals, Indianapolis, IN) according to the manufacturer's instructions. The probes were labeled with digoxigenin using PCR *(13)*. To detect *npt*II sequences, the entire *npt*II coding region was used as a probe. *C. cryptica* T13L *acc1* sequences in cotransformants were detected using a probe that recognizes a 312-bp conserved sequence at the 5'-end of the *acc1* coding region.

A rapid assay for the presence of NPTII protein in putative transformants was performed by Western blotting. Cells were scraped from plates using a sterile inoculating loop (approx 10-μL packed cell volume) and placed in 50 μL of water in a microfuge tube. An equal volume of SDS 2X extraction buffer (125 m*M* Tris, 4% SDS, 20% glycerol, 10% β-mercaptoethanol) was added, and the sample was boiled for 5 min. Cell debris was removed by centrifugation, and 10–20 μL samples were separated on 8–16% SDS-polyacrylamide gels (Novex, San Diego, CA). The separated proteins were transferred to a nitrocellulose membrane, and NPTII protein was detected using anti-NPTII primary antibodies (5-Prime→ 3-Prime, Inc., Boulder, CO) and alkaline phosphatase-conjugated goat antirabbit IgG secondary antibodies.

RESULTS

Two chimeric plasmid vectors were constructed that contain the bacterial *npt*II gene flanked by regulatory sequences from *acc1* from the diatom *C. cryptica* T13L (Fig. 1); these vectors were introduced into microalgal cells via microprojectile bombardment. The results from several transformation experiments are summarized in Table 1. Both plasmids were used successfully to mediate the transforma-

Table 1
Transformation of *C. cryptica* and *N. saprophila*

Recipient Strain	Plasmid	Form of input DNA	# Plates treated	# G418-resistant colonies	Ave # transf. per treatment	Transformants per 10^8 cells[a]
T13L	pACCNPT10	Supercoiled	5	25	5.0	16.7
T13L	pACCNPT5.1	Supercoiled	10	19	1.9	6.3
T13L	pACCNPT5.1	Linear[b]	7	16	2.3	7.7
T13L	pACCNPT10	Linear	7	9	1.3	4.3
T13L	Control[c]	—	4	0	0	0
T13L	pCaMVNEO	Supercoiled	2	0	0	0
CYCLOI	pACCNPT10	Supercoiled	2	2	1.0	3.4
CYCLOI	pACCNPT5.1	Supercoiled	2	12	6.0	20.4
CYCLOI	Control	—	2	0	0	0
NAVIC1	pACCNPT5.1	Supercoiled	5	42	8.4	2.8
NAVIC1	pACCNPT10	Supercoiled	1	18	18.0	6.0
NAVICI	Control	—	1	0	0	0

[a]*C. cryptica* plates contained ten times as many cells as the *N. saprophila* plates.

[b]Linearized plasmids were produced by digestion with *KpnI*, which cuts in the polycloning site of pBluescript KS+ at the 5'-end of the *acc1* promoter region (Fig. 1).

[c]Controls consisted of cells not bombarded with plasmid or cells bombarded with supercoiled pBluescript KS+.

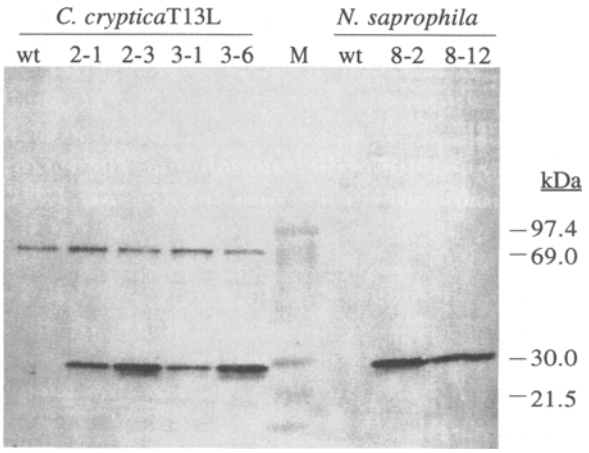

Fig. 2. Western blot showing the presence of the 30-kDa NPTII protein in transformants of *C. cryptica* T13L and *N. saprophila* NAVIC1. The sizes (in kDa) of the mol- wt markers in the 6th lane (M; Rainbow Markers, Amersham, Arlington Heights, IL) are indicated on the right.

tion of two strains of the centric diatom, *C. cryptica*, as well as the pennate diatom *N. saprophila*. The numbers of transformants obtained in each experiment were low, from about 1–18 from each plate treated. Similar numbers of transformants were obtained using either supercoiled or linearized plasmids. No G418-resistant colonies were seen on untreated controls or on plates containing cells bombarded with pBluescript KS+. We also did not obtain any transformants using pCaMVNEO as the transformation vector; this plasmid contains the *npt*II gene driven by regulatory sequences shown to work well in a number of higher plants systems *(14,15)*.

DNA was isolated from wild-type and G418-resistant strains of the algae to test for the presence of the *npt*II gene by Southern blot analysis. DNA fragments that hybridized to the digoxigenin-labeled *npt*II probe were seen in all G418-resistant isolates tested; the probe did not hybridize to any fragments in the DNA from wild-type cells (data not shown). In the *C. cryptica* transformants, the hybridization patterns suggest that the plasmid DNA integrates into one or more random sites in the host cell genome, often in the form of tandem repeats. Similar analyses were also performed on DNA isolated from transformed strains of *N. saprophila* NAVIC1. All G418-resistant isolates of these transformants also contained DNA fragments that hybridized to the *npt*II probe. In contrast to the results with *C. cryptica*, the data suggest that *N. saprophila* may be less likely to integrate the foreign DNA in the form of tandem repeats, and more likely to integrate the plasmid at multiple sites or to insert rearranged copies of the plasmid DNA.

To ensure that the G418-resistant phenotype of the transformants was caused by the expression of a functional NPTII protein, and not by a spontaneous mutation, crude cell extracts were analyzed for the presence of the NPTII protein by Western blotting. A blot containing protein extracts from wild-type and transformed cells of *C. cryptica* T13L and *N. saprophila* is shown in Fig. 2. All G418-resistant isolates produced a 30-kDa protein that is recognized by the anti-NPTII antibody. This protein is not seen in wild-type cells. Of the G418-resistant isolates listed in Table 1, 58 were

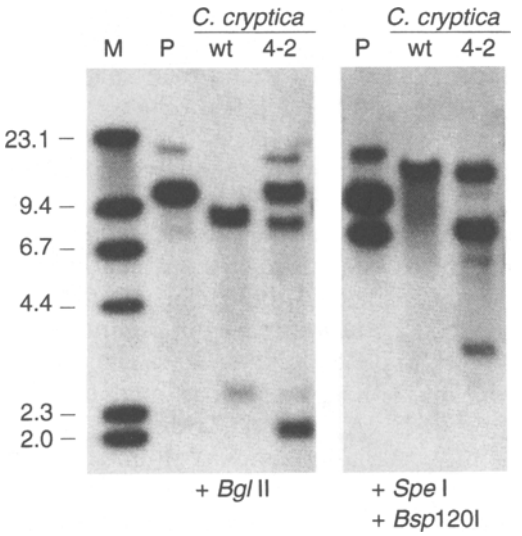

Fig. 3. Southern blot showing the presence of additional *acc1* sequences in an isolate of *C. cryptica* T13L cotransformed with pACCNPT5.1 and pACC1. The DNA was hybridized to a digoxigenin-labeled probe specific for a 312-bp region at the 5'-end of *acc1*. *Bgl*II cuts pACC1 once, within the ACCase coding sequence (*see* Fig. 1). The presence of a band of 10.7-kb in the cotransformant that comigrates with linearized pACC1suggests that the plasmid has integrated in the form of two or more tandem repeats, as was seen for the *npt*II containing plasmids. *Bsp*120I and *Spe*I recognize single sites in pACC1 that flank a 7.9-kb fragment that includes the entire *acc1* coding region. The presence of a band of this size demonstrates that at least one full-length copy of *acc1* from the plasmid is present in the recombinant strain. The first lane (M) contains digoxigenin-labeled λ DNA digested with *Hin*dIII as size markers (Boehringer Mannheim Biochemicals). Lanes marked "P" contain pACC1 plasmid, digested with the indicated enzymes.

chosen at random and tested for the presence of NPTII protein by Western blotting. All contained the NPTII protein.

As discussed above, one goal of this research is to use the genetic transformation technology to manipulate microalgal lipid biosynthetic pathways. One potential engineering target is the enzyme ACCase, which catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA, the primary building block of fatty acids. This enzyme is believed to be a key regulatory enzyme in lipid production for higher plants *(16)*, and results from our laboratory suggest a role for ACCase in the accumulation of storage lipids in *C. cryptica* in response to nutrient deficiency *(17)*. Therefore, we decided to test whether overexpressing the ACCase gene (*acc1*) in diatoms could affect lipid accumulation. We successfully introduced additional copies of *acc1* into *C. cryptica* T13L and *N. saprophila* by cotransforming these organisms with pACC1, which contains the genomic ACCase gene, and pACCNPT5.1. At least one isolate of each strain has been shown by Southern analysis to contain one or more full-length copies of *acc1* in addition to the endogenous sequences (Fig. 3). Analyses of these recombinant strains for enhanced ACCase protein production and activity, and for their effects on lipid accumulation, are in progress. Preliminary results suggest that ACCase activity may be increased two- to threefold as compared to wild-type cells in several recombinant strains.

DISCUSSION

There are two basic aspects to the development of a genetic transformation system: (1) a method to get DNA into the cell; and (2) a way to detect expression of the foreign gene.

For microalgae, the cell wall is a significant barrier to DNA entry. There has been little success with producing and regenerating microalgal protoplasts because of the diversity and complexity of microalgal cell walls. For this reason, we have been exploring methods to introduce DNA into microalgal cells directly through the cell walls. We recently reported the use of silicon carbide fibers to mediate the entry of DNA into walled cells of the green alga *C. reinhardtii (18)*, but preliminary studies suggested that this method would not work to penetrate the cell walls of diatoms, such as *C. cryptica*. During the past few years, microprojectile bombardment (also known as biolistics), has been widely used to introduce DNA into walled cells of higher plants and *C. reinhardtii*, as well as intact cells of some bacteria and fungi. We found that biolistics also works very well to mediate DNA entry into at least two species of diatoms through the siliceous cell wall. Based on results in *C. reinhardtii* and the results reported here, biolistics should work well to introduce DNA into most microalgal species.

To be able to detect the expression of the foreign gene, we chose a selectable marker gene, since this allowed us to screen for rare transformation events in a large population of cells. In *C. reinhardtii*, nuclear transformation required the use of homologous genes as selectable markers. The DNA of these organisms is unusually rich in the nucleotides guanosine and cytosine, which may relate to codon bias and poor expression of foreign genes *(3)*. Previous work from our laboratory indicated that DNA from diatoms does not have an elevated G + C content *(12)*, so expression of bacterial marker gene by the diatom should not be problematic. We believe that the use of diatom regulatory sequences was critical to obtaining high enough expression of the bacterial gene to allow growth of the transformed cells in the presence of G418. This is supported by our inability to recover *C. cryptica* T13L transformants when pCaMVNEO, which contains the *npt*II gene driven by a strong plant promoter (the 35S RNA promoter from the cauliflower mosaic virus, CaMV), was introduced into the algal cells (Table 1). However, when we cotransformed *C. cryptica* T13L with pACCNPT5.1 and plasmid pDO432 (which contains a bacterial luciferase gene driven by the same CaMV promoter *[19,20]*), a number of transformants selected based on their resistance to G418 also demonstrated significant luciferase activity (unpublished results). Thus, although the microalgae can recognize and utilize foreign promoter sequences, the level of expression may be insufficient to allow the use of the foreign promoters in selectable marker systems.

We have recently begun experiments designed to use genetic engineering to optimize lipid production in microalgae. The preliminary results are encouraging, because it appears that introducing additional copies of the ACCase gene into *C. cryptica* does result in enhanced activity of this enzyme. We are working to characterize these transformants and to identify other key genes in the various algal metabolic pathways that would be likely genetic engineering targets for modifying the lipid production capabilities of microalgae.

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