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Biodiesel/Aquatic Species Project Report: FY 1992

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PREFACE

This report summarizes the progress and research accomplishments of the Biodiesel/Aquatic Species Project, field managed by the National Renewable Energy Laboratory (NREL), through October 1992. This report includes an overview of the entire project and a summary of individual research projects. The project receives its funding through the Biofuels Systems Division of the U.S. Department of Energy.

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I. PROJECT OVERVIEW

INTRODUCTION

Microalgae are microscopic aquatic plants with the potential to produce large quantities of lipids (plant oils). The primary goal of the Biodiesel/Aquatic Species Project is to develop the technology for growing microalgae as a renewable biomass feedstock for the production of a diesel fuel substitute (biodiesel), thereby reducing the need for imported petroleum. Microalgae are of interest because of their high growth rates and tolerance to varying environmental conditions, and because the oils (lipids) they produce can be extracted and converted to substitute petroleum fuels, such as biodiesel.

Biodiesel could play a significant role in meeting future domestic transportation fuel needs. It is expected that gasoline production in the United States will be reduced as petroleum feedstock availability becomes more limited and alcohol-based fuel production increases. Because diesel fuel is a coproduct of the gasoline refining process, the future reduction in gasoline production requires the development of a substitute, renewable source of diesel fuel. Biodiesel is an extremely attractive candidate to fulfill this need for a diesel fuel substitute. Biodiesel is a cleaner fuel than petroleum diesel. It is virtually free of sulfur, thereby eliminating the production of sulfur oxides during combustion. Emissions of hydrocarbons, carbon monoxide, and particulates during combustion are also significantly reduced in comparison to emissions from petroleum diesel. These properties make biodiesel useful in facilitating compliance with the Clean Air Act Amendments of 1990. An additional benefit of biodiesel is that it is thought to be a biodegradable fuel that can be utilized in standard, unmodified diesel engines. Biodiesel provides essentially the same energy content and power output as petroleum-based diesel fuel while reducing emissions. Biodiesel's low pollutant emissions can be extremely useful in Environmental Protection Agency (EPA) non-attainment areas, which are typically central cities with acute local air pollution problems. Buses and other fleet vehicles

running on biodiesel have the potential to make a major impact in these markets. Biodiesel is ordinarily considered to be derived from oilseeds, but a chemically identical biodiesel can be made from microalgae.

Microalgae can be grown in arid and semi-arid regions with poor soil quality where woody or herbaceous crops cannot be grown. Saline water from aquifers or the ocean can be used for growing microalgae. Such water has few competing uses and cannot be used for agriculture, forestry, or as potable water. This technology is complementary to ongoing efforts to grow lignocellulosic biomass in areas with good soil and water resources because microalgae are projected to be grown in those areas where lignocellulosics or oilseed crops will not grow well (desert southwestern United States and other areas with poor soils). Also, the yield of biomass per acre from microalgae is three- to five-fold greater than the yield from typical crop plant acreage.

Another area where microalgae are complementary to other energy crops is in quad potential and market readiness. The potential contribution of oilseeds to the energy supply is probably much less than 1 quad Btu ($1x10^9$GJ)/yr. However, oilseed biodiesel can be made available quickly, thus helping to solve local air pollution problems over the short term and establishing an early niche market for biodiesel. Microalgal biodiesel would come on line later to fulfill multi-quad market demand from the overall diesel market. Microalgal biodiesel enhances the long-term potential of biodiesel as renewable energy.

Projected global climate change provides another important rationale for the biodiesel project. Climate change, which has been linked to the accumulation of excess carbon dioxide in the atmosphere, has the potential of producing economic and geopolitical changes with profound impacts on our economy and the energy industry. The burning of fossil fuels, primarily in power plants, is the primary contributor to the excess carbon dioxide. Inasmuch as the primary nutrient for microalgal growth is carbon dioxide, operation of microalgal biomass farms has emerged as a promising candidate in the search for alternative approaches to ameliorate global climate change. The microalgae essentially recycle the carbon dioxide from the power

plant's stack gases into a secondary energy product (biodiesel). Although this carbon dioxide is eventually released when the fuel is burned, the process effectively doubles the amount of energy generated for a given quantity of carbon dioxide. Studies have shown that land and saline water are available in areas such as New Mexico and Arizona to support extensive microalgae facilities. The carbon dioxide emissions from all the power plants in these two states could be trapped by microalgae farms covering about 0.25% of the total land area. If this technology is expanded to other states, or projected future capacity is brought on line in Arizona and New Mexico, the farms could supply at least 2 quads (2.1×10^9GJ) of energy (equivalent to 15% of the gasoline used in the United States) in the form of liquid fuels.

Studies have shown that economic fuel production will require the microalgae to be grown in intensive culture in large outdoor ponds. The system design consists of 6-in(0.15m)-deep, raceway-shaped ponds with a paddlewheel for circulating the water. Carbon dioxide (perhaps in the form of power plant stack gases) and other nutrients are injected into the culture to optimize algal growth and oil production. Lipid accumulation is generally triggered by environmental stress, such as depletion of a key nutrient.

Project researchers have collected and studied more than 3000 strains of microalgae from desert and saline environments. From these, a number of promising lipid-producing species have been identified. These organisms grow over a wide range of salinities, produce significant quantities of lipid oils, and achieve growth rates of nearly three doublings per day. Some of these species tolerate temperatures of 100°F or higher.

Research is now focused on applying genetic techniques to enhance the lipid production of microalgae. This effort builds on extensive strain characterization research, as well as biochemical studies of the metabolic pathways for lipid synthesis. By manipulating culture conditions, scientists can already increase the lipid content of the cell from the 5% to 20% found in nature to more than 60% in the laboratory and more than 40% in outdoor culture. The goal is to develop genetically improved strains of microalgae that produce even higher lipid levels and for which the process of lipid accumulation could be better controlled.

OVERVIEW OF RESEARCH

Researchers in the Biodiesel / Aquatics Species Project focus on the use of microalgae as a feedstock for producing renewable, high-energy liquid fuels. In 1979, the U.S. Department of Energy (DOE) and the National Renewable Energy Laboratory (NREL) initiated the Aquatic Species Project as part of the overall effort in biofuels. The project began to focus exclusively on fuels from microalgae in 1982. Estimates show that the technology being developed by the project could provide as much as 7% of the total current U.S. energy demand.

The program's basic premise is that microalgae, which have been called the most productive biochemical factories in the world, can produce up to 30 times more oil per unit of growth area than land plants. It is estimated that 150 to 400 barrels of oil per acre per year could be produced with microalgal oil technology. Initial commercialization of this technology is envisioned for the desert Southwest because this area provides high solar radiation and offers flat land that has few competing uses (hence low land costs). Similarly, there are large saline aquifers with few competing uses in the region. This water source could provide a suitable, low-cost medium for the growth of many microalgae.

The project has supported research at NREL in Golden, Colorado, as well as in industry, other government laboratories, and universities.

Project Goal

The goal of the Biodiesel/Aquatic Species Project is to develop the technology base for large-scale production of oil-rich microalgae. The project is also developing methods to convert the microalgal lipids into liquid fuels needed for industry and transportation.

Project Objectives

Specific long-term objectives of the project are to

- Genetically engineer microalgae for high lipid production at high growth rates
- Identify "trigger" points in biochemical pathways of algae that turn lipid production on and off
- Develop inexpensive, large-scale, outdoor mass culture technologies to grow microalgae
- Evaluate resource requirements for large-scale production of oil from microalgae as well as the environmental impact of such activities in the U.S. desert Southwest
- Develop technologies for converting microalgal lipids into high-value liquid transportation fuels, particularly biodiesel
- Transfer the technologies to the private sector for continued development and rapid commercialization by involving industry in the research process as early as possible.

Description of the Biodiesel/Aquatic Species Project Elements

Production

The NREL culture collection contains microalgal strains that produce large amounts of lipids and grow rapidly, but not necessarily in the same growth phase or in a controllable manner. Genetic improvement will be necessary to develop strains with the characteristics necessary to meet the overall project technical and economic goals, including predictable growth performance

and controllable lipid yield. The genetic engineering process requires methods for getting genes (DNA) out of cells (cloning), modifying the genes, and reintroducing them into the microalgae. The modified genes would then confer one or more desired characteristics such as increased lipid content to the algae.

Extraction and Conversion

Methods need to be developed for economical extraction of lipids from microalgae and conversion of lipids to gasoline and diesel substitutes. Untreated lipids have oxygen contents and viscosities that are too high to be used in standard engines. The primary goal of the conversion element is to develop methods to economically convert a high proportion of the microalgal lipids to biodiesel fuels and to improve the overall economics by converting the balance of the biomass to biogas or other high-energy products.

Engineering Design

The technology to produce economic liquid fuels from microalgae will require the growth of microalgae on a large scale. Systems to maintain optimal levels of nutrients, carbon dioxide, salinity, and temperature must be developed and tested. The goal of the engineering design element is to develop large-scale outdoor facilities that allow the production goals to be met and to reduce the costs of such a system to those targeted by the project's economic analysis. It is hoped that future work in this area will allow the evaluation of various mass-culture and harvesting systems and technologies in an effort to increase outdoor algal productivities and decrease the cost of operating such a facility.

Analysis

Economic and resource analyses provide input to project management in order for research directions and priorities to be set. The goal of the analysis element is to support the technology development by determining cost goals, economic sensitivities, resource assessments,

and environmental impacts as new data are developed. To accomplish this, researchers will conduct ongoing economic analyses. Resource and environmental assessments will be conducted to identify potential constraints, identify and address data gaps, and provide project guidance.

Project Highlights

FY 1992 Accomplishments

The primary area of research during FY 1992 was the effort to genetically improve microalgae in order to control the timing and magnitude of lipid accumulation. Increased lipid content will have a direct effect on fuel price, and the control of lipid content is a major project goal. Previously, the enzyme acetyl-CoA carboxylase (ACC) was purified and biochemically characterized from *Cyclotella cryptica*; ACC appears to play an important role in controlling the levels of lipids accumulated in microalgal cells. A major accomplishment in FY 1992 was the further characterization of the gene encoding ACC from this diatom. Work also began on the manipulation of the gene into a form that can be utilized more readily in other organisms. A manuscript is being prepared detailing the gene isolation and characterization work (Roessler and Ohlrogge 1993).

Attempts have been made to isolate two other algal genes as well: nitrate reductase (NR) and orotidine-5'-phosphate decarboxylase (OPD). NR is an enzyme that is important in the partitioning of photosynthetic carbon products among lipids and other cellular components - understanding the genetics and biochemistry of this enzyme may lead to another method of controlling lipid production. OPD is involved in pyrimidine biosynthesis. Both the NR and OPD genes will be useful as selectable markers for microalgal transformation. Algal cells mutant in one or the other of these two gene activities have been characterized.

A major step in the cloning of these and other genes was achieved during FY 1992 by generating a representative genomic "library." This library contains sequences of DNA from the green alga *Monoraphidium minutum* inserted into a vector that replicates in the bacterium

Escherichia coli. The library appears to be of good quality and of sufficient size such that every gene in the organism should be represented in multiple copies. The library should allow specific genes to be isolated by hybridization to probe sequences.

Another important accomplishment in the development of algal transformation systems was the testing of a method of introducing DNA into algal cells. A published method for plant transformation that involves agitating cells in the presence of silicon carbide fibers and DNA was applied to a test alga, *Chlamydomonas reinhardtii*. Relatively efficient DNA introduction was achieved with this method (Dunahay 1993).

Work on the analysis of some of the key microalgal strains for their DNA composition was published during FY 1992 (Jarvis et al. 1992).

FY 1993 Plans

Genetic engineering will continue to be a major area of effort in the project. This area has the potential to have a positive effect on product yield. In FY 1993, research will focus on manipulation of the ACC gene from *C. cryptica* and on the cloning of two other genes, OPD and NR, for use as selectable markers in transformation studies. Efforts will concentrate on the application of gene probes to the newly generated genomic libraries. Work will also continue on refining the methods for introducing genes into microalgal cells.

II. CULTURE COLLECTION

The microalgal culture collection was transferred to fresh culture medium quarterly during FY 1992, and the comprehensive culture collection data base was updated. There is a total of 432 strains of algae in the collection: 34 in the cold water collection, 80 in the clone collection, and 318 in the main collection. During the past year only two strains failed successful transfer, which represents an excellent record for a collection of this size. Experiments were performed to decrease the amount of filter-sterilized media required for transfer of the collection. Such efforts have allowed the complete elimination of some media types, thus streamlining the process of culture collection transfer. The level of automation of culture media handling has also been increased.

III. LIPID GENE CHARACTERIZATION

INTRODUCTION

A gene is a specific molecule of DNA that gives an organism the ability to perform a specific function. For many genes, the DNA is "transcribed" into messenger RNA, which is then "translated" into a protein. In many cases, the protein product is an enzyme that catalyzes a particular chemical reaction. In order to genetically engineer an organism, such as a microalga, to better carry out a particular function (e.g., lipid accumulation), it is necessary to alter the DNA composition of the organism by manipulating the genes that encode proteins of regulatory significance.

One of the key enzymes controlling the rate of lipid accumulation is acetyl-CoA carboxylase (ACC). ACC is a biotin-containing enzyme that catalyzes the carboxylation of acetyl-CoA to form malonyl-CoA. This reaction has been shown to be highly regulated and is probably the rate-limiting step in fatty acid biosynthesis (Kim et al. 1989; Lane et al. 1974). In addition, recent evidence suggests that differences in the rates of fatty acid synthesis in plants may be attributable to changes in ACC activity (Post-Beittenmiller et al. 1991, 1992). Increased ACC activity also appears to play a role in environmentally induced triacylglycerol accumulation in the diatom *C. cryptica* (Roessler 1988). However, little is known about the regulation of ACC gene expression in photosynthetic organisms.

During FY 1991, the ACC gene was cloned from the diatom *C. cryptica*, as reported previously. ACC enzyme had been purified from several higher plants and algae, but prior to this accomplishment there were no reports describing the isolation of an ACC-encoding gene from a photosynthetic organism. This represents a major advance in both microalgal biotechnology and the understanding of lipid accumulation in photosynthetic organisms (plants) in general. During FY 1992, the *C. cryptica* ACC gene was further characterized, and the gene cloning results were prepared for publication.

DNA SEQUENCE ANALYSIS

The complete sequence of DNA bases in the ACC gene from *C. cryptica* has now been determined. This effort, in conjunction with RNA-based sequencing, has pointed out the presence and location of two intervening sequences, or "introns," which are spliced out of the RNA transcript. In addition, computer analysis of the sequence has identified a number of important features of the gene.

Identification of Introns

The lack of an "open reading frame" (stretch of contiguous amino-acid coding sequence) long enough to encode a full-size polypeptide within the DNA sequence suggested the presence of an intron. Introns, or intervening sequences, are stretches of nucleotides that are spliced out of the RNA transcript of a gene. The presence of introns has implications for predicting the correct protein sequence and affects future gene manipulation strategies. The possibility that introns exist in the ACC gene of *C. cryptica* was tested using the polymerase chain reaction (PCR; Saiki et al. 1988; Lee and Caskey 1990) procedure to amplify sequences generated from *C. cryptica* total RNA. A PCR product was obtained in this manner that was slightly smaller than the product obtained when using genomic DNA as the PCR template. Sequence analysis confirmed that a 73-base-pair (bp) intron is located approximately 125 bp upstream from the region of the gene that encodes the biotin binding site (see below).

Because a logical translation initiation codon for the gene was not apparent in the first analysis, it seemed likely that an intron might also exist near the 5' end of the coding sequence. The 5'-RACE procedure (Rapid Amplification of cDNA Ends; Frohman et al. 1988) was used to examine this possibility. The longest RACE product obtained indicated the presence of a 447-bp intron. The product did not extend in the 5' direction far enough to include a potential initiation codon, although analysis of the genomic sequence indicated that an in-frame ATG (initiation) codon was present less than 50 bp upstream from the 5' end of the RACE clone.

Therefore, it is likely that this ATG represents the true translation initiation codon; experiments are in progress to confirm this supposition. Removal of the 73 bp and 447 bp introns yields an open reading frame of nearly 6.3 kb. Additional RNA-PCR experiments have not indicated the presence of other introns in the gene.

Characteristics of the ACC Protein Sequence

Based on the above analyses, the ACC polypeptide from *C. cryptica* is predicted to be composed of 2089 amino acids and have a molecular weight of 229,836 daltons. Past research, using size comparisons in gel electrophoresis, has indicated that the *C. cryptica* ACC enzyme has a molecular weight of 185 to 200 kD (Roessler 1990). This discrepancy is most likely attributable to inaccurate size estimation in the gel procedures, but could also be accounted for in part by post-translational cleavage. Unfortunately, we have not been able to determine the N-terminal amino acid sequence of the actual mature polypeptide. It is interesting to note, however, that the N-terminal sequence of the predicted protein has characteristics of a "signal sequence" (von Heijne 1990). In eukaryotes, signal sequences direct polypeptides into the endoplasmic reticulum (ER). Signal sequences have also been shown to be necessary for transport of nuclear-encoded proteins into the chloroplasts of diatoms (Bhaya and Grossman 1991), which is consistent with the fact that diatom chloroplasts are completely enclosed by closely appressed ER membranes (Gibbs 1979). Because fatty acid biosynthesis occurs primarily in the chloroplasts of higher plants (Harwood 1988), it is assumed that ACC is located in the chloroplasts of diatoms, and therefore a signal sequence would be necessary for chloroplast targeting. Alternatively, it is possible that the ACC gene that we have cloned codes for an ER-localized ACC that is responsible for producing the malonyl-CoA utilized for elongation of fatty acids from C16 or C18 to C20 and C22. The location of the cleavage site in the putative ACC signal sequence is not clear.

Several other features of the predicted ACC primary structure warrant discussion. In particular, it is of interest to attempt to determine the regions or domains of the mature protein that are responsible for the three enzymatic functions of ACC: biotin carboxylase,

carboxyltransferase, and biotin carboxyl carrier protein. Computer alignment programs were used to search for regions of the ACC amino acid sequences from rat, yeast, and *C. cryptica* that were similar. In the portion of the *C. cryptica* ACC polypeptide that includes the biotin carboxylase domain (residues 1 to 620), there is 52% and 50% identity with the rat and yeast ACC sequences, respectively. Furthermore, in the portion of *C. cryptica* ACC that includes the carboxyltransferase domain (residues 1120 to 2089), there is 42% identity with both the rat and yeast sequences. There is less sequence conservation in the middle region of the protein between any of these ACC enzymes (30% to 32% identity, with the bulk of this similarity occurring in the vicinity of the biotin binding site). This middle region, which includes portions of the biotin carboxyl carrier protein domain, may be little more than a spacer region that facilitates the physical movement of the carboxylated biotin from the biotin carboxylase active site to the carboxyltransferase active site. In this case, a high degree of sequence conservation would not be expected.

The presumed biotin binding site is a lysine residue (No. 770) that is flanked by two methionines. This tripeptide occurs in every biotin-containing enzyme for which the amino acid sequence is known. Another characteristic of this region is the presence of one or more proline residues approximately 25 to 30 positions ahead of the biotin binding site that are believed to serve as a hinge region for carboxybiotin movement (Samols et al. 1988). Proline residues are also found at this location in *C. cryptica* ACC, although they are displaced five to six residues toward the N-terminus in *C. cryptica* ACC relative to yeast and animal ACC.

Regions of the carboxyltransferase subunit from *E. coli* that are proposed to be involved in acetyl-CoA binding and carboxybiotin binding have been identified (Li and Cronan 1992a). Related sequences can be found in the corresponding sequences of ACC from *C. cryptica*, rat, and yeast. Another highly conserved region is the putative ATP-binding site of the biotin carboxylase domain/subunit (Li and Cronan 1992b; Al-Feel et al. 1992).

GENE CONSTRUCTS

Work is currently under way to create a version of the *C. cryptica* gene that should be more readily expressible for use in the genetic engineering of microalgae for enhanced lipid production. This primarily involves splicing together fragments generated by RNA-PCR in order to make an intron-free open reading frame; the sequence information described above is critical to the success of this effort. In addition, these intron-free constructs will be placed downstream of plant promoters to allow for enhanced transcription in plant tissues. In the long term, future modification work on this gene may lead to significant commercial opportunities in both microalgae and oilseed crops.

SUMMARY

The gene that encodes the lipid biosynthetic enzyme acetyl-CoA carboxylase in the eukaryotic alga *C. cryptica* has been isolated and cloned, representing the first time that a full-length gene for this enzyme has been cloned from a photosynthetic organism. The gene contains a 447-bp intron that is located near the putative translation initiation codon and a 73-bp intron that is located slightly upstream from the region of the gene that encodes the biotin binding site of the enzyme. The gene encodes a polypeptide that is predicted to be composed of 2089 amino acids and have a molecular weight of approximately 230 kD. The deduced amino acid sequence is similar to the sequences of animal and yeast ACCs in the biotin carboxylase and carboxyltransferase domains. However, there is less sequence similarity in the biotin carboxyl carrier protein domain, although the highly conserved met-lys-met of the biotin binding site is present. The amino terminus of the predicted protein has characteristics of a signal sequence, suggesting that the enzyme may be imported into chloroplasts via the endoplasmic reticulum.

IV. MICROALGAL TRANSFORMATION

INTRODUCTION

Genetic transformation is the process of introducing DNA into a cell and having a gene contained on that DNA be "expressed" (read by the cell's biochemical machinery) to make a protein. In order to verify transformation, a method must be devised to detect the protein biochemically or to apply a genetic selection such that only cells producing that protein are able to reproduce. Thus, transformation requires not only the physical introduction of DNA into the cell, but also requires a means of monitoring gene entry and expression. In addition, the creation of stably transformed cell lines requires that the DNA become fixed into the cell's genome.

Research in microalgal transformation has focused primarily on methods to assess the successful introduction of foreign DNA. A significant effort has been devoted to the development of appropriate selectable markers for microalgae that will provide a powerful selection for rare, stable transformation events. Testing of DNA introduction methods has also been a major focus.

HOMOLOGOUS SELECTABLE MARKERS

We are currently working to develop two genes as potential selectable markers for algal transformation. Our DNA analysis data, and the precedent set in *C. reinhardtii*, suggest that heterologous selectable markers (e.g., antibiotic resistance) may not be effective in the algae. By using homologous genes, problems in promoter specificity and codon bias will not inhibit gene expression, allowing successful selection for transformed cells.

Nitrate reductase

The NR gene has been used successfully as a transformation marker in a number of organisms, including fungi (Daboussi et al. 1989) and *C. reinhardtii* (Kindle et al. 1989). Algal cell mutants lacking functional NR can be selected based on their resistance to chlorate. Cells having a functional NR protein will take up chlorate along with nitrate and reduce it to chlorite, which is toxic to the cells. The NR-minus mutants do not reduce the chlorate and therefore do not experience the cytotoxic effects of chlorite. Consequently, cells can be subjected to a positive selection regime by growing target organisms in the presence of chlorate and picking out resistant colonies. Using this protocol, we previously isolated several putative NR-deficient mutants of *M. minutum* and of the diatom *C. cryptica*. These candidates grow in the presence of chlorate and are unable to utilize nitrate on plates or in liquid culture. Experiments this year have been aimed at the further characterization of these mutants and at the use of one of the *M. minutum* mutants in preliminary transformation tests. Unfortunately, the *C. cryptica* mutants have been problematical in that they appear to grow poorly in liquid media. This problem is being addressed currently.

Preliminary transformation experiments using physical means (see below) to introduce the *C. reinhardtii* NR gene into one of the *M. minutum* mutants have been performed. Colonies were obtained on selective medium at a low rate in these experiments, but further analysis using Southern blotting (Sambrook et al. 1989) to detect introduced sequences by gene hybridization suggested that these were probably not true transformants; i.e., foreign gene sequences were not present. Most likely the colonies that did arise were the result of genetic reversion of the NR mutation. It is unknown whether the failure in transformation was due to poor introduction of the *C. reinhardtii* gene or to an inability of the gene to be expressed or stabilized.

Although NR genes from other algae may be able to complement the mutations in our strains of interest, the likelihood of achieving transformation would be much greater if the homologous genes could be isolated. In other words, transforming a mutant of *M. minutum* with

a gene from *M. minutum* has a much better chance of success; this is expected because the gene from *M. minutum* carries all of the correct signals to be read by the algal cell. We are therefore working to isolate wild-type NR genes from both *M. minutum* and *C. cryptica*. Once the gene is cloned, complementation of a NR-minus mutant with a functional NR gene will result in cells that can use nitrate as the sole nitrogen source, providing a powerful selection for transformants.

Previous attempts to clone the *M. minutum* NR gene focused on the use of PCR with primers based on NR gene sequences from related organisms. This approach has not yet proven to be successful. A second approach is to isolate the gene by direct hybridization to NR sequences from another alga. Preliminary hybridization experiments reported previously indicated that, using DNA from the NR gene of the green alga *Chlorella vulgaris*, a single band was detected in genomic DNA from *M. minutum*. This result encourages the use of direct screening of a "genomic library" to identify the *M. minutum* NR gene. The first step in this approach, the generation of the library, was accomplished during FY 1992.

A genomic library contains the entire genome (DNA content) of an organism broken down into smaller pieces; these pieces are then incorporated into the genome of bacteria or viruses so that the incorporated foreign genes are naturally amplified (i.e., the genes are "cloned"). This library of cloned DNA pieces is then screened with a labeled probe consisting of a small piece of DNA that specifically hybridizes to the cloned gene of interest, thereby allowing the investigator to quickly identify and isolate the desired gene. A genomic library was created in a lambda phage vector called λGEM12 (Promega), using genomic DNA from *M. minutum* that was partially cleaved with the restriction enzyme *Sau*3A. The library contains approximately 300,000 separate clones. Because this particular phage will not be viable unless it has received an insert of at least 9,000 bp, preliminary estimates would suggest that the library contains multiple copies of each gene in the organism (assuming a reasonable DNA content of $<5 \times 10^7$ bases per cell). This library has been amplified and frozen for future screening with various genetic probes.

Orotidine-5'-phosphate decarboxylase

The OPD gene codes for an essential enzyme in the pyrimidine biosynthesis pathway. OPD mutants grow only if provided with an alternate source of pyrimidines, such as uracil. There is a positive selection for OPD mutants; OPD converts the drug 5-fluoroorotic acid (FOA) into a toxic compound, killing wild-type cells, whereas OPD mutants can grow on FOA-containing media. In addition, a spectrophotometric assay can be used to measure OPD activity in cell extracts (Donovan and Kushner 1983). This gene has been cloned and sequenced from a number of organisms (e.g., Rose et al. 1984; Newbury et al. 1986; Ohmstede et al. 1986; Turnbough et al. 1987), and has been used successfully in several transformation systems (e.g., Buxton and Radford 1983; Boy-Marcotte et al. 1984; van Hartingsveldt et al. 1987).

Previous work using the FOA mutant selection method allowed us to obtain several putative OPD mutants from UV-mutagenized cells of the green alga *M. minutum*. These cells require uracil for growth, thus allowing a powerful selection for cells that have been transformed with the wild-type OPD gene. The next step in the development of this system is the isolation of the wild-type algal OPD gene. Two approaches were attempted during FY 1992; both were based on PCR methodologies. The first involved the use of standard PCR with degenerate primers based on two regions of sequence that appear to be fairly well conserved between species. Following extensive manipulation of PCR conditions, sequences could be amplified from algal DNA. These fragments were cloned into standard vectors and their nucleotide sequences were determined. Unfortunately, the fragments appeared to be unrelated to any other OPD gene, suggesting that random DNA sequences had been amplified during the PCR reactions. A second series of experiments involved the use of an "inverse" PCR technique in which the genomic DNA is circularized before amplification. This strategy made better use of the most highly conserved region of the gene and had the potential for cloning the entire gene without having to do subsequent library screening. This approach was also unsuccessful. An attempt to clone the gene by complementation of the *E. coli pyrF* marker using inverse-PCR amplified sequences failed as well.

Other approaches to cloning the *M. minutum* OPD gene are currently being explored. In particular, the genomic libraries described above provide a valuable tool for cloning this gene by homology to other known OPD sequences. The OPD gene from *Neurospora crassa* will be used in initial hybridization experiments because this organism has an elevated guanosine plus cytosine (GC) content as does *M. minutum.* A chemically synthesized oligonucleotide probe based on the most highly conserved region of the gene will also be used.

DNA INTRODUCTION METHODS

Once suitable selectable marker systems have been devised, the main barrier to transformation becomes the physical introduction of DNA into the algal cell. The presence of well-developed cell walls in most microalgae may make this a difficult problem to overcome. However, progress was made in FY 1992 in that DNA introduction was accomplished in a test alga using a method published in the literature that had not previously been applied to algal cells.

Chlamydomonas reinhardtii is currently the only eukaryotic unicellular alga for which efficient transformation systems exist. Initial transformation of *C. reinhardtii* was facilitated by the use of wall-less cells, either genetic mutants (*cw-15*) or cells whose walls were degraded using autolysin, a species-specific cell wall-degrading enzyme produced during mating by *C. reinhardtii* gametes (Kindle 1990). High-frequency, stable nuclear transformation can be achieved by agitating wall-deficient cells in the presence of plasmid DNA, glass beads, and polyethylene glycol (Kindle 1990). Stable nuclear transformation also requires the use of homologous genes (Debuchy et al. 1989; Kindle et al. 1989); *C. reinhardtii* nuclear DNA contains a high GC content and exhibits a codon bias towards C and G nucleotides, which apparently results in poor expression of foreign genes (Mayfield and Kindle 1990). Transformation of intact (walled) *C. reinhardtii* has been accomplished by using microprojectile bombardment to introduce plasmid DNA into the cells (Boynton et al. 1988; Kindle et al. 1989). Although both nuclear and chloroplast transformation have been achieved by this method, particle

bombardment is generally more efficient for chloroplast transformation than nuclear transformation, presumably because of the large relative size of the chloroplast compared to the nucleus in *C. reinhardtii* cells (Kindle et al. 1991). Low frequencies of nuclear transformation of walled cells were also reported by Kindle (1990) using the glass bead protocol and via electroporation (Brown et al. 1991).

A simple, generally applicable technique for high-frequency transformation of intact (walled) microalgal cells is desirable due to the difficulty of producing viable cell-wall-deficient cells from most species. The composition of microalgal walls is highly variable and attempts to determine the enzymatic conditions for wall degradation for each isolate can be tedious and frustrating. Several recent reports demonstrated the feasibility of using silicon carbide (SiC) whiskers to facilitate the entry of DNA into intact higher plant cells. Kaeppler and coworkers demonstrated both transient and stable expression of foreign genes in maize and tobacco following agitation of suspension culture cells with plasmid DNA and SiC whiskers (Kaeppler et al. 1990, 1992), and Asano and coworkers reported ß-glucuronidase (GUS) expression in cultured cells of *Agrostis alba* using a similar protocol (Asano et al. 1991). NREL researchers were able to use SiC whiskers for the production of stable nuclear transformants of intact cells of *C. reinhardtii*.

The protocol for transformation of *C. reinhardtii* cells using SiC whiskers is relatively straightforward. An NR-deficient strain of *C. reinhardtii* (*nit1-305 mt*$^-$) was used as the recipient strain. The plasmid used to complement the NR marker contains a 14.5-kb sequence of genomic DNA that encodes the full-length sequence for the NR gene from *C. reinhardtii*. Cells in late exponential or early stationary growth phase were harvested and placed into a small volume of fresh medium. Plasmid DNA, polyethylene glycol (PEG), and a slurry of SiC whiskers were added to each tube. The samples were then agitated vigorously using a laboratory mixer, after which the cells were spread onto petri plates containing selective medium (nitrate being the sole nitrogen source). Transformants appeared within 10 to 14 days.

Stable nuclear transformation of walled *C. reinhardtii* cells was achieved by this method. Although the absolute number of transformants varied between experiments and between replicates in individual experiments, 10 to 100 transformants per 10^7 cells were obtained consistently. This is comparable to the efficiency obtained with a published protocol using glass beads instead of SiC whiskers. However, the use of glass beads rapidly reduces cell viability, with less than 10% survival after 60 s of agitation. In contrast, mixing the cells with SiC had little effect on cell viability; greater than 80% cell survival was observed even after 10 min of agitating the cells in the presence of SiC.

Several factors were tested for their influence on the efficiency of SiC-mediated transformation of walled *C. reinhardtii* cells. The presence of PEG in the transformation mix was important, as transformation efficiencies were five- to ten-fold lower when PEG was omitted. In addition, the use of linearized plasmid DNA resulted in 2 to 6 times as many transformants as did supercoiled DNA, while the addition of 40 µg of salmon sperm DNA as carrier had little effect.

SiC whiskers have been reported to be cytotoxic and possibly mutagenic in cultured mammalian cells (Vaughan et al. 1991), suggesting that some or all of the putative transformants expressing the wild-type NR phenotype could have arisen from SiC-induced reversion at the *nit1* locus. However, several experiments suggest that this did not occur. No transformants appeared when plasmid DNA was omitted. In addition, the number of putative transformants obtained was dependent on the concentration of plasmid DNA in the transformation mix, with increasing numbers of colonies obtained up to 10 µg DNA. Finally, total DNA isolated from the putative transformants was analyzed for the presence of foreign DNA sequences by Southern blotting. Many of the putative transformants exhibiting the wild-type NR phenotype contained DNA that hybridized to the introduced plasmid.

In conclusion, agitation with SiC whiskers was found to be a successful method for introducing DNA into walled cells of a test alga, *C. reinhardtii*. Although the transformation efficiency is similar to that achieved by treatment with glass beads, SiC vortexing results in very

little cell death and therefore appears to be a more gentle procedure. Glass beads (200-300 μm) are much larger than algal cells; glass bead-mediated transformation apparently works by physically damaging the cell wall and membrane, often resulting in cell death. The SiC fibers may work by nicking or puncturing the wall and/or membrane, allowing DNA entry through a less severe wound. The only other available method for reproducibly introducing DNA into walled plant or algal cells is microprojectile bombardment. Although this method produces very high numbers of chloroplast transformants in *C. reinhardtii*, the efficiency of nuclear transformation by the biolistic method (Kindle et al. 1989; Zumbrunn et al. 1989) is comparable to that achieved here with SiC whiskers. In contrast to biolistics, SiC-mediated transformation has the obvious advantage of requiring no costly special equipment. Furthermore, particle bombardment may be difficult to optimize for very small algal cells (Smith et al. 1992). Experiments are currently in progress to adapt the SiC transformation protocol to other microalgal species that have greater potential for lipid production in outdoor mass culture.

V. ENVIRONMENT, SAFETY AND HEALTH AND QUALITY ASSURANCE

During FY 1992 the new standard of excellence in environment, safety, and health (ES&H) was maintained. Because of these efforts, routine laboratory inspections surfaced only very minor problems, and these were dealt with promptly.

The inventory of chemicals in the laboratories was updated on a monthly basis during FY 1992. The inventory, which can be accessed through the laboratory computer, includes information such as order and receipt dates and particular hazards associated with a chemical. Laboratory-Specific Chemical Hygiene Plans, Safe Operating Procedures, and similar documents were written or updated for each of the Biodiesel/Aquatic Species Project laboratories.

The calibration of equipment within the laboratories was maintained according to schedule.

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The primary goal of the Biodiesel/Aquatic Species Project is to develop the technology for growing microalgae as a renewable biomass feedstock for the production of a diesel fuel substitute (biodiesel), thereby reducing the need for imported petroleum. Microalgae are of interest as a feedstock because of their high growth rates and tolerance to varying environmental conditions, and because the oils (lipids) they produce can be extracted and converted to substitute petroleum fuels such as biodiesel. Microalgae can be grown in arid and semi-arid regions with poor soil quality, and saline water from aquifers or the ocean can be used for growing microalgae.

Biodiesel is an extremely attractive candidate to fulfill the need for a diesel fuel substitute. Biodiesel is a cleaner fuel than petroleum diesel; it is virtually free of sulfur; and emissions of hydrocarbons, carbon monoxide, and particulates during combustion are significantly reduced in comparison to emissions from petroleum diesel. Biodiesel provides essentially the same energy content and power output as petroleum-based diesel fuel.

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