REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY USE ONLY *(Leave blank)*	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	12-92 to 12-95	Final Report

4. TITLE AND SUBTITLE

Genetic transformation systems for characterization of gene promoters in marine algae

5. FUNDING NUMBERS

N00014-93-10182

R&T 4101-144

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Arlington, VA 22217-5660

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT

Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

This report summarizes the results of ONR funded research focused on the development of a general method for directed genetic manipulation of marine algae based on DNA transfection. The research established the use of electroporation for DNA transfection and macromolecular loading of walled diatom cells through the development of an osmotically compatible electroporation buffer, Seapore Buffer. This technique was extended to several other diverse groups of marine phytoplankton. Expression of transfected genes was demonstrated and stability of transfection assessed. Kanamycin and formaldehyde were identified as two useful selective agents for enrichment of transformed cell lines and enhancement of expression from plasmids bearing the resistance markers. A variety of protein encoding genes where characterized from the diatom *Skeletonema costatum* and used to identify potential requirements for efficient translation of heterospecific genes in diatoms. Flanking regulatory sequences of highly expressed genes were targeted for future development of diatom specific transformation vectors. The protocols developed through this research provide a foundation for biotechnolgical utilization of marine chromophyte algae.

14. SUBJECT TERMS

diatoms, electroporation, transfection, transformation, antibiotic selection, gene expression, gene structure, phytoplankton

15. NUMBER OF PAGES

4

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
U	U	U	UL

FINAL REPORT

GRANT #: N00014-93-10182

PRINCIPAL INVESTIGATOR: Dr. G. Jason Smith

INSTITUTION: University of California, Los Angeles

GRANT TITLE: Genetic transformation systems for characterization of gene promoters in marine algae

AWARD PERIOD: 15 December 1992 - 31 December 1995

OBJECTIVE: To develop a general method for directed genetic manipulation of marine microalgae based on DNA transfection. Exploit DNA transfection technology to establish the use of foreign gene cassettes as sensitive reporters of heterologous and homologous promoter function in these cells. Characterize the microalgal sensitive to a variety of antibiotic and chemical agents for use as dominant selection markers and utilize genes conferring resistance to these agents in the development of microalgal specific transformation vectors for isolation of stably transformed cell lines.

APPROACH: Use electroporation for DNA transfection of marine microalgae. Electroporation conditions are optimized for each algal species to balance uptake of exogenous DNA (and other macromolecules) with cell viability. Activity assays for the products of reporter genes are used to assess the function of linked gene promoter and termination sequences. Antibiotic and chemical sensitivity is used to determine the availability of dominant selection markers for mutagenesis and stable transformation studies.

ACCOMPLISHMENTS: Electroporation was identified as a technique which met three criteria essential for development of a general transformation system for marine algae, these include: i) the capacity to induce DNA uptake through a variety of organic and inorganic cell walls, ii)high efficiency of transfection without the requirement for extensive manipulation of the target cells, iii) ability to induce the uptake of a broad size range of plasmids or chromosomal fragments for transient or stable expression assays. Our efforts have established the technical requirements for implementation of these criteria for electroporation-dependent transfection and transient gene expression in marine algae.

Implementation of electroporation required the development of an osmotically substituted medium which sustained comparable levels of cell viability, yet substantially lower conductivity than seawater and supports electrical fields in excess of 10 kV/cm. Experiments with the diatom *Skeletonema costatum* (CCMP1332) revealed that significant membrane electroporation, as evidenced by dextran uptake, occurs at field strengths above 1.0 kV/cm while cell viability drops sharply above 2.0 kV/cm. Experiments with a non-calcifying strain of the coccolithophore *Pluerochrysis sp.* (CCMP299) revealed membrane poration at lower field strength of ca 0.5 kV consistent with the lack of a siliceous frustule. The demonstration of electroporative loading of molecules 70 kD in mass (ca. 5 nm particles) in representative chlorophyte, prymnesiophyte, pyrrophyte algae indicates that *in vivo* genetic and physiological manipulations utilizing nucleic acids, peptides and antibodies are now feasible with marine microalgae.

A variety of plasmid constructs (size range 3-13 kb) have been transfected into the diatom *S. costatum;* plasmid uptake exhibited field-

strength responses similar to those observed for dextran uptake (>50% of surviving cells transfected). These results suggest that electrically induced membrane pores are larger than the nominal size indicated by dextran loading and that electroporation can be reliably used for dsDNA transfection of intact diatom cells. DNA hybridization analysis revealed that while log phase cultures yielded highest transfection efficiencies, maintenance of plasmids was negatively correlated with post-transfection growth rate indicating that plasmids containing the ColE1 origin of replication will only be suitable for transient expression assays. In contrast, transfections with plasmids containing the origin of replication derived from the yeast 2μm-circle plasmid resulted in their maintenance in the supercoiled conformation for at least 14 days in the absence of direct selection, and abundance could be amplified through sublethal selection for gene products encoded by passenger DNA. These results indicate that the yeast 2 μm origin functions as an autonomous replicating sequence (ARS) in *S. costatum* and provides a vector system for hyperresistance cloning of endogenous genes in this species.

Several reporter plasmids have been used to test for genetic expression of the transfecting DNA genes and provide novel enzymatic functions and gene sequences for detection in the target algae. Functional expression of these artificial gene constructs, has been observed following their transfection into *S. costatum*, verifying the utility of our approach to microalgal transformation. Electroporation of *S. costatum* with reporter genes under the control of the plant viral CaMV35S promoter (pBI221, pJD300, pBI121) resulted in a 2-4 fold enhancement of reporter gene activity by 24 h post-transfection. Reporter gene expression did exhibit a significant transfection dose-response, indicating that the induced activity is a direct result of expression of the foreign gene cassette. Luciferase is proving to be a sensitive reporter for gene expression in *S. costatum* and has been used to demonstrate that the CaMV35S promoter supports higher levels of expression than the SV40 promoter in this diatom.

Our examination of potential dominant selection markers for use in directed genetic manipulation of *S. costatum* was limited to those antagonists for which gene cassettes conferring resistance were available. Sensitivity was determined as the lowest inhibitory concentration (LIC) for growth in liquid or solid media. Growth bioassays of cells transfected with pBI121, containing a *nptII* expression cassette conferring kanamycin resistance linked to a *uidA* expression cassette enabled us to demonstrate that positive selection for antibiotic resistance is feasible in *S. costatum*, that it can be used as a means to stabilize expression from sequences linked to the resistance marker. While liquid culture bioassays permitted us to detect expression of antibiotic resistance genes, the incompatibility of many species of marine phytoplankton, including *S. costatum*, with growth on solid media has hampered our ability to isolate clonal lines of transformants. To overcome this limitation, a technique was developed where cells are resuspended in soft agarose and then plated onto nutritive media; this plating assay has been optimized for use with *S. costatum* permitting us to isolate individual colonies within 7 days of plating. This method has been successful with several other strictly planktonic species.

Based on evidence that *S. costatum* has the capacity to produce and assimilate formaldehyde (FA), the use of this compound as an endogenous selectable marker was explored with the aid of an ONR High School Fellow. *S. costatum* and other marine algae tested exhibited a 10-fold

lower LIC for FA than reported for yeast from which a FA resistance gene (SFA) had been isolated. In addition, homology probing of genomic DNA from *S. costatum* with the yeast SFA gene to revealed the presence of several restriction fragments hybridizing at ca. 70% level of identity, indicating that a SFA homolog is present in this diatom. Although transfection experiments with the yeast shuttle plasmid YFRp1, bearing the intact *sfa* gene, did not significantly enhance the LIC for FA in *S. costatum*, sublethal exposure to FA following transfection did induce the amplification of YFRp1. Therefore a low level of SFA expression from the yeast promoter was sufficient for positive selection for the *sfa* gene and demonstrated that hyperresistance cloning is feasible in this diatom.

An ongoing motivation of our research efforts is the characterization of regulatory elements in chromophyte genes which can be used to control expression of transgenes. Towards this end we have constructed both genomic and cDNA libraries for *S. costatum* and have characterized clones for the fucoxanthin chlorophyll *a/c* binding protein (FCP), glutamine synthetase (GS), nitrate reductase (NR), and several unidentified light-regulated open reading frames. These genes exhibit a range of expression patterns from constitutive with substrate modulation (FCP, GS), to substrate inducible and endproduct repressible (NR), and can also serve as endogenous targets for selection.

The analysis of these genes has yield insights as the the requirements for efficient translation in diatoms. The sequence bounding the translation initiation domain is highly divergent from the classic Kozak consensus with the exception of the absolute conservation of an A residue at position -3 relative to the AUG codon. Codon usage is biased towards the used of pyrimidines in the third position of degenerate codons and GpG dinucleotides are avoid in the second and third positions. Little sequence conservation is observed in either the 5' or 3' UTR of the genes characterized although the UAA stop codon is strongly prefered (>90%). No consensus polyadenylation sequence has been observed in the 3' UTR. Northern analysis of the *fcp* gene family in *S. costatum* indicates that these comprise highly expressed transcripts (ca. 10% total mRNA) and abundance is positively correlated with growth rate, making the 5' and 3' flanking domains for these genes ideal sources of regulatory sequences for *S. costatum* specific transformation vectors. These regions from the sfcpB gene were being used for the construction of *nptII* and *sfa* based selection vectors in work continuing after the present funding period.

CONCLUSIONS: Electroporation has proven to be a highly efficient method (>50% of cells) for transfection of eukaryotic microalgae with DNA and other macromolecules. Our development of Seapore Buffer has permitted extention of this technology to a variety of marine algae and offers advantages over particle based transfection systems of minimal culture manipulation and high post transfection viability. The bulk of electroporated DNA does however, seem to remain extrachromosomal and may contribute to the expression variability encountered. The high efficiency of electroporation mediated transfection offers the possibility to overcome these problems by application of restriction enzyme mediated integration utilizing microalga-specific selection vectors developed here, and may permit utilization of insertional mutagenesis strategies for the molecular dissection of the unique metabolic processes characterizing marine microalgae.

SIGNIFICANCE: Our studies have provided a basic and essential technology for directed genetic manipulation of marine diatoms and microalgae in

general. This work has provided a foundation for biotechnological exploitation of these cells and for controlling microalgal production in bioreactors, in biofilms, and in natural phytoplankton communities.

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