Brian Binder, 12:16 PM 2/18/98 , Grant ER61694 FINAL REPORT

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Date: Wed, 18 Feb 1998 12:16:49 -0500
To: John Houghton <john.houghton@oer.doe.gov>
From: Brian Binder <bbinder@uga.cc.uga.edu>
Subject: Grant ER61694 FINAL REPORT
Cc: "Sallie W. Chisholm" <chisholm@MIT.EDU>,
bbinder@uga.cc.uga.edu (Brian Binder)

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Dear Dr. Houghton:

Per our 2/11/98 phone conversation, here is the Final Report for the above-referenced DOE grant.
Please do not hesitate to contact me if you need any further information.

Sincerely,
Brian Binder

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FINAL REPORT, DOE Grant ER61694

"Measurement of Synechococcus In Situ Growth Rates Using Flow Cytometry and rRNA-Targeted Probes."

S.W. Chisholm and B.J. Binder

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INTRODUCTION

The overall goal of this project was the development of methods for the estimation of in situ Synechococcus growth rates using flow cytometrically-measured cellular properties. As an important picoplanktonic primary producer, Synechococcus can be expected to significantly influence the cycling of carbon in coastal marine environments. Traditional methods for estimating growth rates in natural populations of these and other phytoplankton have relied upon bottle incubations, which take a long time to perform and are subject to a well known suite of artifactual "bottle effects." The analytical approach we are developing would obviate the need for such incubations, thus avoiding bottle effects and allowing much higher sample through-put. Application of this approach to field populations of

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Synechococcus would contribute significantly to our understanding of the population dynamics of these organisms, and ultimately to our understanding of carbon cycling in coastal marine environments.

The first step toward developing the proposed method for in situ growth rate determination is to establish the relationships between growth rate and cellular properties (e.g. cellular rRNA and DNA content) in Synechococcus strains growing under a variety of conditions. Establishing these relationships has been the focus of this project.

RESULTS

I. DNA and Growth in Marine Synechococcus. The cell cycle behavior of 4 marine strains of Synechococcus has been analyzed by examining the DNA frequency distributions of exponentially growing and dark-blocked populations, and by considering the patterns of change in these distributions during growth under a diel light:dark cycle. The two modes of cell cycle regulation previously identified in a freshwater and coastal marine Synechococcus isolate, respectively, were represented among the three oceanic strains we examined. The first of these modes of regulation is consistent with the slow growth case of the widely accepted prokaryotic cell cycle model. The second appears to involve asynchronous initiation of chromosome replication, the presence of multiple chromosome copies at low growth rates, and variability in chromosome copy number among cells in the population. These characteristics suggest the involvement of a large probabilistic component in cell cycle regulation, and could make the application of cell cycle-based estimators of in situ growth rate to Synechococcus populations problematic. Details of this part of the project can be found in Binder and Chisholm 1995.

II. Ribosomal RNA (rRNA) Protocol Development: Whole-Cell Hybridization in Synechococcus. We tested the effect of buffer composition, probe concentration, and target cell concentration on probe-conferred fluorescence. Optimum probe concentration was determined for cells growing at 3 different growth rates. Whereas fluorescence was relatively insensitive to probe concentration in rapidly growing cells, it dropped off sharply below 1 ug /ml in cells growing at moderate and slow rates. Above this probe concentration, mean fluorescence increased slowly with increasing probe concentration in all cells. Owing to a parallel increase in non-specific binding (as reflected by NON338 fluorescence - see below), however, the difference between specific and non-specific fluorescence remained approximately constant at these higher probe concentrations. We therefore routinely used a probe concentration of 1 ug / ml. Under these conditions, probe-conferred fluorescence was insensitive to cell density in the hybridization mix over at least a factor of 10. In order to confirm that probe-conferred fluorescence was in fact originating from rRNA, cells were treated with RNase; EUB338 fluorescence in such cells was indistinguishable from the NON338 negative control. Inclusion of SDS in the hybridization buffer (as has been used in other studies) resulted in increased non-specific binding as well as increased concentrations of small

particulates which interfered with flow cytometric analysis. SDS was therefore excluded from the hybridization mix in these experiments.

III. RNA and Growth in Marine *Synechococcus*. The relationship between 16S rRNA content and light-limited growth rate in marine *Synechococcus* strain WH8101 has been established. WH8101 was grown in semi-continuous batch culture under continuous light, resulting in constant growth rates that could be maintained indefinitely. Specific growth rates ranging from 0.15 to 1.83 /day were achieved by exposure to light intensities ranging from 0.25 to $15 \times 10^{15} \text{ Q/cm-sqr/sec}$. The relationship between growth rate and light intensity was well-described by a hyperbolic tangent P vs. I relationship; there was no evidence of photoinhibition in the range of light intensities employed.

Cellular rRNA content was assayed by whole-cell hybridization of methanol-fixed samples, as described above. rRNA-targeted oligonucleotide probes were labeled at the 5' end with a single fluorescent molecule. We used two probes, EUB338 and NON338, both labeled with the fluorochrome BODIPY-FL-X (Molecular Probes, Inc.). EUB338 is a well-established probe for all Bacteria (but not Archaea); NON338 is complimentary to EUB338, and is used as a negative control.

Hybridized cells were analyzed by flow cytometry. Flow cytometry is not only fast and accurate, but also provides the means to restrict analysis to specific picoplankton groups, defined according to their autofluorescence and light scattering properties. Although this ability is not particularly valuable for the culture studies reported here, it is extremely important for the analysis of *Synechococcus* cells in the context of natural mixed microbial assemblages.

The relationship between cellular rRNA content, as reflected in EUB338-derived fluorescence, and growth rate in *Synechococcus* WH8101 appears to comprise 3 different phases: at the lowest growth rates ($\mu < \sim 0.7$ per day), rRNA per cell remained approximately constant; at intermediate growth rates ($\sim 0.7 < \mu < \sim 1.5$ per day), rRNA per cell increased proportionally with growth rate, and at the highest growth rates ($\mu > \sim 1.5$ per day), rRNA per cell appeared to shift down sharply. The first two phases of this relationship are similar to the pattern observed in *E. coli* and in some studies of the freshwater *Synechococcus* strain PCC6301. The down-shift in cellular rRNA at the highest growth rates to our knowledge has not been observed previously; we hypothesize that it is in some way related to the shift from light-limited to light-saturated growth in *Synechococcus*.

The BODIPY fluorescence of cells hybridized with the negative probe, NON338, was approximately an order of magnitude less than the fluorescence of EUB338-hybridized cells at all growth rates tested. This observation indicates 1) that non-specific binding by the oligonucleotide probes was insignificant under the conditions employed here, and 2) that fluorescence "spill-over" from photosynthetic pigments was likewise insignificant (since

such spill-over would be present in NON- as well as EUB-hybridized cells).

In order to further confirm that EUB338-conferred fluorescence does in fact reflect cellular rRNA content, we developed an assay for cellular RNA based on the nucleic acid stain SYBR Green II (Molecular Probes, Inc.). If rRNA constitutes the majority of total cellular RNA (a reasonable expectation, based on what is known in other prokaryotes), then a good correspondence between the stain-based total RNA assay and the probe-based rRNA assay would provide further evidence that the latter approach is reliable.

Because SYBR Green II is a general nucleic acid stain that binds to both RNA and DNA, we analyzed aliquots of cells that were treated with RNase or were untreated. Cellular DNA content is reflected by the SYBR Green fluorescence of RNase-treated cells, and cellular RNA content is reflected by the difference between untreated and RNase-treated cells. (DNase could in theory be used for a more direct estimate of cellular RNA, but we were unable to completely digest cellular DNA by this approach).

SYBR Green-based estimates of RNA per cell were very well correlated with probe-based estimates of rRNA ($r=0.96$, $p<0.01$), providing strong evidence that our probe-based estimates were indeed valid. Furthermore, the relationship between SYBR Green-based RNA and growth rate corresponded very well to the relationship described above between probe-based rRNA and growth rate. SYBR Green-based estimates of DNA were also very well correlated with estimates from the DNA-specific stain Hoechst 33342.

We conclude that although the relationship between rRNA and growth rate in *Synechococcus* WH8101 is not simple, the observed systematic variation of the former with the latter suggests that cellular rRNA content may be used as an indicator of in situ growth rate in natural *Synechococcus* populations. The utility of this approach will ultimately depend upon the sensitivity of the rRNA - Growth Rate relationship to changes in growth-limiting environmental factors, and the extent to which this relationship holds among different *Synechococcus* strains. These issues are currently under investigation.

PAPERS AND PRODUCTS DELIVERED

Papers:

Binder, B.J., and S.W. Chisholm. "Cell cycle regulation in marine *Synechococcus* sp. strains." *Appl. Environ. Microbiol.* 61:708-717 (1995).

Binder, B.J., and Y.C. Liu. Growth Rate-Regulation of Ribosomal RNA Content in a Marine *Synechococcus* (Cyanobacterium) Strain." Submitted to *Applied and Environmental Microbiology*.

Binder, B.J. "Cell Cycle Regulation During Light-Limited Growth in Marine *Synechococcus* Strains WH8101 and WH7803." In preparation for submission to

Journal of Phycology.

Binder, B.J., and Y.C. Liu. "Variation in rRNA Content as Function of Growth Rate in the Marine *Synechococcus* Strain WH8101." In preparation for submission to "Marine Cyanobacteria," L. Charpy and A. Larkum, eds.

Abstracts:

"Estimating *Synechococcus* in situ growth rates using flow cytometry: Correlations between growth rate and cellular rRNA, DNA, and protein content." Dept. of Energy Ocean Margins Program Meeting, 9/95.

"Relationship between growth rate and biochemical composition in light-limited *Synechococcus* cells." American Society of Limnology and Oceanography Ocean Sciences Meeting, 2/96.

"Cell Cycle Regulation in Two Strains of Marine *Synechococcus* Sp. (Cyanobacteria) Growing Under Light-Limitation." Phycological Society of America Annual Meeting, 7/96.

"Cell Cycle Regulation in Marine *Synechococcus* Sp. (Cyanobacteria) During Light- and Nitrogen-Limited Growth." American Society of Limnology and Oceanography Aquatic Sciences Meeting, 2/97.

"~~Variation in~~ rRNA Content as a Function of Growth rate in Marine *Synechococcus* Strain WH8101." International Symposium on Marine Cyanobacteria and Related Organisms, Paris, France, 11/97.

TRAINING:

Graduate Students Supported:

Alexandra Worden (Ph.D. candidate, Dept. Marine Sciences, University of Georgia)

Post-Docs Supported:

Brian Binder (Prior to transfer to Dept. Marine Sciences, University of Georgia)

FINAL TECHNICAL REPORT,
MIT subcontract (PO No. CB-S-522560) under DOE grant DE-FG02-93ER61694.
[UGA reference: 10-21-RR100-203, RF MIT BINDER]
Brian Binder, P.I.

SUMMARY OF RESULTS

I. DNA and Growth in Marine *Synechococcus*. The cell cycle behavior of 4 marine strains of the unicellular cyanobacterium *Synechococcus* has been analyzed by examining the DNA frequency distributions of exponentially growing and dark-blocked populations, and by considering the patterns of change in these distributions during growth under a diel light:dark cycle. The two modes of cell cycle regulation previously identified in a freshwater and coastal marine *Synechococcus* isolate, respectively, were represented among the three open ocean strains we examined. The first of these modes of regulation is consistent with the slow growth case of the widely accepted prokaryotic cell cycle paradigm. The second appears to involve asynchronous initiation of chromosome replication, the presence of multiple chromosome copies at low growth rates, and variability in chromosome copy number among cells in the population. These characteristics suggest the involvement of a large probabilistic component in cell cycle regulation, and could make the application of cell cycle-based estimators of in situ growth rate to *Synechococcus* populations problematic. Details of this part of the project can be found in Binder and Chisholm 1995.

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For each experimental culture growing under a specific light intensity, a constant growth rate was maintained for the equivalent of at least 10 generations prior to sampling. Once 10 generations had transpired, each culture was generally sampled twice, at the end of two different "transfers." These two samples were treated as replicates in all subsequent analyses. Cells were preserved in methanol and stored at -20C prior to analysis.

Cellular rRNA content was assayed by whole-cell hybridization of methanol-fixed samples with an rRNA-targeted oligonucleotide probe labeled at the 5' end with a single fluorescent molecule. We used two probes, EUB338 and NON338, both labeled with the fluorochrome BODIPY-FL-X (Molecular Probes, Inc.). EUB338 is a well-established probe for all Bacteria (but not Archaea); NON338 is complimentary to EUB338, and is used as a negative control.

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"Variation in rRNA Content as a Function of Growth rate in Marine *Synechococcus* Strain WH8101." International Symposium on Marine Cyanobacteria and Related Organisms, Paris, France, 11/97.

Manuscripts in Preparation:

"Growth Rate-Regulation of Ribosomal RNA Content in a Marine *Synechococcus* (Cyanobacterium) Strain." To be submitted to Applied and Environmental Microbiology.

"Cell Cycle Regulation During Light-Limited Growth in Marine *Synechococcus* Strains WH8101 and WH7803." To be submitted to Journal of Phycology.