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Culturing the marine cyanobacterium *Prochlorococcus*

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Abstract

Prochlorococcus is the numerically dominant primary producer in open ocean ecosystems. Analysis of *Prochlorococcus* genome sequences from cultured isolates and ocean samples has broadened interest in studying this tiny cell, and efforts are underway to develop it into a model system for studying marine microbial ecology. A critical component of these efforts has been the development of systematic culturing methods that will facilitate the distribution of *Prochlorococcus* to diverse labs that may be interested in studying it. This paper provides detailed methods for maintaining cultures of *Prochlorococcus*, including a comparison of growth rates of cells on two artificial seawater media and on a standard medium that uses a natural seawater base. Procedures for agar plating, cryopreservation, obtaining new isolates, and issues associated with culture volume and carbon limitation also are described.

The smallest, yet most abundant, unicellular marine cyanobacterium, *Prochlorococcus*, was first detected in the 1980s (Johnson and Sieburth 1979; Gieskes and Kraay 1983) and described soon thereafter (Chisholm et al. 1988; Li and Wood 1988; Chisholm et al. 1992). Extensive studies since then have established *Prochlorococcus* as a marine oxygenic phototroph of ecological significance, contributing significantly to total photosynthesis in oligotrophic regions of the world's oceans (Partensky et al. 1999). Isolates of *Prochlorococcus* have been obtained from diverse regions of the oceans and have been the source of many studies into the cell structure, physiology, genetics and molecular biology of this genus. More recently, the complete genomes of several strains of *Prochlorococcus* have been sequenced (Dufresne et al. 2003; Rocap et al. 2003; Coleman et al. 2006) or are in progress (see efforts in the United States at the Department of Energy's Joint Genome Institute, http://genome.jgi-psf.org/mic_home.html, and the Gordon and Betty Moore Foundation's Microbial Genome Sequencing Project, http://www.moore.org/microgenome/, and in the European Union through the Marine Picocyanobacteria Genome Project, http://www.sb-roscoff.fr/Phyto/Genome_Cyanos/).

Cultures of *Prochlorococcus* have been grown routinely in liquid rather than solid media because of the difficulty of growing *Prochlorococcus* cells on the latter. Since the first *Prochlorococcus* culture was isolated (by B. Palenik) and described (Chisholm et al. 1992), cultures have been isolated and maintained in natural seawater-based media, the compositions of which have evolved over the years (Table 1). They differ in a number of ways, primarily with respect to the type and concentration of the macronutrients and metal chelators. Anecdotal evidence suggested that increases in the macronutrients (K/10[-Cu] medium) and decreases in the EDTA concentration 10-fold (PRO2 medium) improved the frequency of successful isolations (Chisholm et al. 1992; Moore et al. 1998). Subsequently, macronutrient concentrations were increased and organic nutrient sources eliminated (PRO99 medium) to increase cell yields and reduce the substrates for heterotrophic contaminants. The two media recipes currently used in the two major *Prochlorococcus* culture collections are PRO99 (Moore et al. 2002; Andersen 2005) in the MIT Cyanobacterial Culture collection (Chisholm Laboratory, MIT) and PCR-S11 (Rippka et al. 2000) in the Roscoff Culture Collection (Station Biologique), which differ substantially in their trace metal composition (Table 1).

An artificial seawater (ASW)-based medium for the growth of *Prochlorococcus*, PCR-Tu_2, was first developed by Rippka et al. (2000), and has been used successfully in other studies (Laloui

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The data collected for this manuscript is a combination of work done by various researchers, including undergraduates from the Chisholm Lab at MIT and the Moore Lab at USM, since 1988 when *Prochlorococcus* was first brought into culture by Brian Palenik. Although we cannot name each person who contributed, we would like to acknowledge a few who carried out some of the culture experiments: Stina Drakare and Andrew Tolonen from MIT, and Tammy Swett, Daniela Robinson, and Kristen Krumhardt from USM. This work has been supported in part by NSF (to SWC, LRM, KK, DR, MAS, TS, and EZ), DOE, the Seaver Foundation, and the Gordon and Betty Moore Foundation (to SWC). C-MORE contribution number 39.

Table 1. Nutrient additions used in different *Prochlorococcus* growth media. AMP1 and PCR-Tu_2 use artificial seawater as the base, as indicated (Turk's Island Salt Mix). All others use filtered natural seawater as the base. Final concentrations in each medium in units of μM, unless otherwise noted.

	Final Concentrations						
Components	**CPTC-based[a]**	**K/10 (–Cu)[b]**	**PRO2[c]**	**PRO99**	**AMP1**	**PCR–Tu_2[d]**	**PCR–S11[d]**
Macronutrients:							
NH_4Cl		50	50	800			
$(NH_4)_2SO_4$					400	400	400
$(NH_2)_2CO$ (urea)	20	(100[e])	100 (50[f])				
$NaH_2PO_4 \cdot H_2O$		10	10	50	50[g]	50[g]	50
β-glycerophosphate	10						
Trace Metals:							
$Na_2EDTA \cdot 2H_2O$		11.7	1.17	1.17	0.117		
CPTC	100						
$Na_2EDTA/FeCl_3$						8	8
$FeCl_3 \cdot 6H_2O$		1.17	1.17	1.17	0.117		
FeSO4	0.10						
$MnCl_2 \cdot 4H_2O$	0.010	0.090	0.090	0.090	0.0090	0.460	
$MnSO_4 \cdot H_2O$							60
$ZnSO_4 \cdot 7H_2O$		0.008	0.008	0.008	0.0008	0.039	6.0
$CoCl_2 \cdot 6H_2O$		0.005 (0.010[e])	0.005	0.005	0.0005		
$Co(NO_3)_2 \cdot 6H_2O$						0.0085	3.0
$Na_2MoO_4 \cdot 2H_2O$	0.010	0.003	0.003	0.003	0.0003	0.0080	
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$							0.4
Na_2SeO_3		0.010	0.010	0.010	0.0010		
$NiCl_2.6H_2O$		(0.010[e])	0.010	0.010	0.0010		3.0
$CuSO_4 \cdot 5H_2O$						0.016	3.0
H_3BO_3						2.3	300
$Na_2WO_4 \cdot 2H_2O$							0.6
KBr							6.0
KI							3.0
$Cd(NO_3) \cdot 4H_2O$							3.0
$Cr(NO_3)_3 \cdot 9H_2O$							0.6
$VSO_5 \cdot H_2O$							0.6
$KAl(SO_4)_2 \cdot 12H_2O$							6.0
Buffers:							
HEPES, pH 7.5				[h]	1 mM	1 mM	1 mM
$NaHCO_3$				[h]	6 mM	2 mM	
Turk's Island Salt Mix:							
NaCl					481 mM	480 mM	
$MgSO_4 \cdot 7H_2O$					28 mM	28 mM	
$MgCl_2 \cdot 6H_2O$					27 mM	27 mM	
$CaCl_2 \cdot 2H_2O$					10 mM	10 mM	
KCl					9 mM	9 mM	
Vitamins:							
Vitamin B_{12}						10 μg l^{-1}	10 μg l^{-1}

[a]Used for original isolation of SARG, MED, NATL1 and NATL2; published in Chisholm et al. (1992).
[b]Used for *Prochlorococcus* isolations between 1991 and 1992 (MIT 9107, MIT 9116, MIT 9123, MIT 9201, MIT 9202, MIT 9211, MIT 9215); published in Chisholm et al. (1992).
[c]Used extensively for culturing between 1994-1999; published in Moore and Chisholm (1999).
[d]Compiled from Rippka et al. (2000) and Rippka (1988).
[e]Referred to as PRO-TM; used for trace metal studies (Mann et al. 2002; Saito et al. 2002) and for obtaining axenic MED4 and MIT 9312 through plating with the addition of vitamins and spent media from Co-limited cultures (see text; Saito 2001).
[f]Used for *Prochlorococcus* isolations (MIT 9302, MIT 9303, MIT 9312, MIT 9313) published in Moore et al. (1998).
[g]pH adjusted to 7.5 with 1.0 M NaOH.
[h]If growing in volumes ≥1 L, can bubble with air or supplement the medium with 10 mM HEPES and 12 mM $NaHCO_3$ for increased final cell yield (see text).

et al. 2002). Because this medium contains a different trace metal mix from PRO99, including copper (Cu), to which we have learned that *Prochlorococcus* is particularly sensitive (Mann et al. 2002), we sought an alternative ASW-based medium that would be suitable for a wider range of *Prochlorococcus* isolates. Here, we present a comparative study of these two ASW-based media with that of a natural seawater-based medium, PRO99, to expand the media possibilities for culturing *Prochlorococcus*. In addition to media comparisons, we also include an analysis of the best microtiter plates to use for small volume culturing, the effect of added CO_2 to the cultures, approaches to culturing on agar plates, and cryopreservation methods for long-term storage of *Prochlorococcus* isolates. We begin with a detailed description of general culturing protocols and procedures to improve the probability of success of culturing this microbe, followed by specific experiments.

General culturing: Methods and procedures

Light and temperature conditions—Detailed descriptions of light and temperature optima for different strains of *Prochlorococcus* have been published (e.g., Johnson et al. 2006; Zinser et al. 2007; Moore et al. 1995; Moore and Chisholm 1999). For standard maintenance of stock *Prochlorococcus* cultures relatively low light levels (8–25 μmol Q m^{-2} s^{-1}, under either light : dark (LD) cycle or continuous light) are used to keep the transfer frequency down to about every 3–4 weeks. The light levels are obtained with cool white fluorescent lamps through a combination of neutral density screening (Roscolux 97, 98, 397 or 398, Rosco Laboratories; or Lee Filters) or window screening, and measured with a QSL-100 quantum scalar irradiance meter (Biospherical Instruments). For growth at higher irradiances, we recommend increasing the irradiance levels incrementally (not more than double) and allowing the culture to acclimate to each light level for two consecutive transfers before increasing again. Growth rates under continuous light conditions are equivalent to those observed under LD cycle if the cultures receive the same integrated photon flux over 24 h (K. Krumhardt and L. R. Moore, unpubl. data). Switching cells from LD cycle to continuous light works best if cultures are placed at the same net irradiance per 24 h.

The temperature range over which *Prochlorococcus* isolates have been grown is 13–31°C, with the minimum and maximum varying among strains (Zinser et al. 2007; Johnson et al. 2006; Moore 1997, Moore et al. 1995). Routinely, *Prochlorococcus* cultures in the MIT Cyanobacterial Culture Collection, the Roscoff Culture Collection in France, and the Provasoli-Guillard National Center for Culture of Marine Phytoplankton in USA (D. Andersen, pers. comm.), are grown between 20 and 24°C, which accommodates all strains. In all experiments reported here, growth temperatures were 23 ± 1°C.

Culture ware—Disposable, sterile polystyrene culture ware can be used to culture *Prochlorococcus*, and is used routinely for growth of *Prochlorococcus* cultures in the Roscoff Culture Collection (Vaulot et al. 2004). Moreover it has been used to isolate cultures from the wild. If glass or other reusable culture ware (i.e., polycarbonate or Teflon) is used, as is the case with most of the experiments presented here, we employ an acid washing protocol modified from the original trace-metal cleaning protocol introduced by J. Martin and colleagues (Fitzwater et al. 1982). It consists of an initial overnight soak with a detergent solution (~2% Micro-90; International Products Corporation), rinsing with tap water six times, then distilled water six times, followed by an overnight soak in 1 M hydrochloric acid (HCl, American Chemical Society grade) made with Type I ultrapure water (i.e., 18 MΩ; both Millipore and Barnstead/Thermolyne systems have been used with success) to remove trace amounts of metals. After soaking in acid (the 1 M HCl can be reused for up to 6 months), the culture ware is rinsed six times with Type I ultrapure water, filled with the same, soaked overnight, and autoclaved with the water in it. Prior to use, the water is poured out aseptically. All reusable culture ware and containers for nutrients and media are dedicated to culturing, and brushes are not used for washing, as these can be a source of metal contamination. Note that a systematic comparison of growth in the different reusable culture ware has not been carried out, but no major differences have been observed.

Natural seawater-based media —All media for growth of *Prochlorococcus* consist of inorganic nutrients added to a seawater base, which consists of either filtered natural seawater, or artificial seawater, e.g., Turks Island Salt Mix (Table 1). Natural seawater from the surface mixed layer of oligotrophic oceans, such as Sargasso Sea water (SSW) collected with Niskin or acid-cleaned GoFlo bottles, and even that collected with acid-cleaned buckets off the side of a slowly moving ship, seems to yield the most consistent growth results. When oligotrophic seawater is not available, we have used coastal seawater from the Environmental Systems Laboratory at the Woods Hole Oceanographic Institution, collected by filtration through serial 100 μm, 30 μm, and 1 μm string-wound cartridge filters, with success. Although a systematic study has not been done, we have observed up to a 25% reduction in growth rates of some strains on some batches of coastal seawater relative to SSW (T. Swett and L. R. Moore, unpubl. data). All seawater is stored in acid-cleaned polypropylene or polyethylene carboys at room temperature for several years.

For media preparation, the seawater is filtered into an acid-washed filter funnel through a 0.2 μm Supor 200 filter (Pall Gelman), or through stacked Whatman GF/F, then 0.2 μm polycarbonate filters. It is then placed into an acid-washed Teflon or polycarbonate bottle and autoclaved. Sterile nutrient stocks are added aseptically to the filtered, autoclaved seawater after cooling. Concentrated stocks of the macronutrients and trace metals are made with the highest grade chemicals (e.g., SigmaUltra) to avoid trace metal contamination. These chemicals are dissolved with Type I ultrapure water and filter sterilized through 0.2 μm, nylon or acetate syringe filters directly into acid-washed, sterile Teflon or polycarbonate bot-

tles for storage at 4°C. The trace metals with Na_2EDTA chelator are made as a single stock solution (10^3 X for PRO99 medium) by first dissolving the Na_2EDTA, then sequentially adding each additional trace metal (Andersen 2005).

For studies involving very controlled trace metal concentrations, a media variation, referred to as PRO-TM (Table 1e) has been used (Saito et al. 2002). In these media, seawater is not autoclaved due to potential metal contamination from autoclaves, but is sterilized by microwaving following the method of Keller et al. (1988). Furthermore, nutrient solutions are run through a prepared Chelex-100 resin (Bio-Rad Laboratories), and all nutrients and trace metals are filter sterilized and added aseptically using pipette tips that have been rinsed with 1 M HCl, then sterile, Type 1 ultra pure H_2O.

Comparison of growth on artificial and natural seawater-based media: Materials and procedures

PRO99 medium, which uses a natural seawater base, has been used in many published works on *Prochlorococcus* (e.g., Moore and Chisholm 1999; Moore et al. 2005; Steglich et al. 2006; Van Mooy et al. 2006) and is used to maintain *Prochlorococcus* isolates in the MIT Cyanobacterial Culture collection. We sought to compare growth rates on this medium with those on two artificial seawater-based media, PCR-Tu_2 (Rippka et al. 2000) and a modification called AMP1 (artificial medium for *Prochlorococcus*), the latter of which uses the major nutrients and salts found in PCR-Tu_2 but a different and lower concentration ($1/10^{th}$) of trace metal mix than that used in PRO99, and, therefore, doesn't contain added Cu (Table 1). For the experiment, the cultures were transferred from PRO99 into PCR-Tu_2 and AMP1 and PRO99, and followed for at least three transfers in their respective media. *Prochlorococcus* cultures used in this experiment (MED4ax, MIT9312ax, NATL2a, SS120, MIT9211, MIT9313ax) were chosen as representatives of the different phylogenetic clades (Rocap et al. 2002).

The Turk's Island Salt Mix (Table 1) was made following the method of Rippka et al. (2000) by dissolving each salt prior to adding the next and then sterilized by autoclaving. To this, syringe filter-sterilized macronutrients, HEPES and $NaHCO_3$ buffers, and trace metal working stocks were added aseptically, and the media stored in the dark at room temperature for up to 1 month. For both PCR-Tu_2 and AMP1 media, the NaH_2PO_4 and HEPES stocks were adjusted to pH 7.5 with 1 M sodium hydroxide (NaOH, Mallinckrodt 7708) made in Type 1 ultrapure water using a pH meter probe that is rinsed with 1 M HCl and then purified water prior to use (the probe is dedicated solely for this purpose to avoid contamination by other chemicals). For the PCR-Tu_2 medium, a $Na_2EDTA/FeCl_3$ stock was made separate from the other trace metal working stock (A5 + Co from Rippka [1988] according to Rippka et al. [2000]).

Monitoring growth, cell density & axenicity—Growth of *Prochlorococcus* cultures was monitored by fluorometric detection of bulk chlorophyll autofluorescence over the course of the growth cycles using Turner Design fluorometer TD-700 or 10-AU, and growth rates calculated by linear regression within the exponential growth phase. Bulk chlorophyll autofluorescence is directly proportional to cell concentration in exponentially growing (steady state) cultures acclimated to growth conditions, as evidenced by comparisons with flow cytometrically determined cell concentrations (data not shown). Comparison of final cell yield was quantified flow cytometrically with a FACS-Scan or FACS-Calibur (BD Biosciences) following standard methods (Marie et al. 2000).

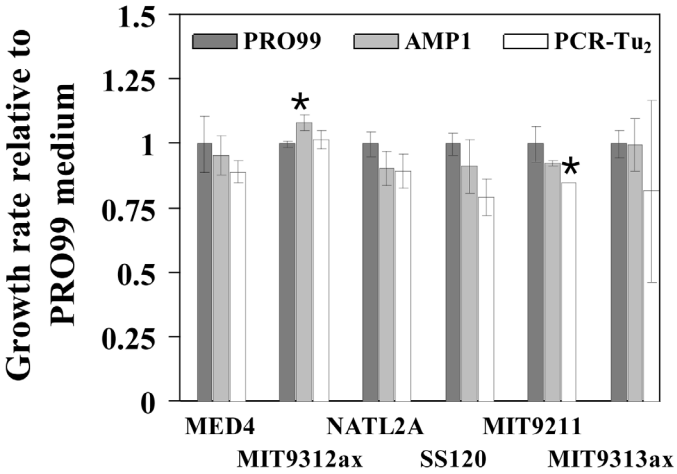

Fig. 1. Growth of *Prochlorococcus* strains on two different artificial seawater-based media (PCR-Tu_2 and AMP1) compared to growth on natural seawater-based media (PRO99). Light levels differed for each strain from ~7 to 37 μmol Q m^{-2} s^{-1} in 14 : 10 LD photoperiod, thus growth relative to PRO99 is presented for inter-strain comparison. Error bars represent standard deviation of relative growth for 2–5 replicate cultures. Asterisks indicate that only significant differences were found for MIT 9312ax grown on AMP1 versus PRO99 and for MIT 9211 grown on PCR-Tu_2 versus PRO99 ($P < 0.05$).

Throughout the experiments and regular maintenance of the two axenic *Prochlorococcus* cultures, axenic status was routinely checked using three methods. Growth of heterotrophic contaminants was checked by diluting a small amount of the *Prochlorococcus* culture in a marine purity test broth (Waterbury et al. 1986). Periodically, the potential presence of contaminants also was examined by epifluorescence microscopy (Leica Microsystems model DMLB) with 4′, 6-diamidino-2-phenylindole (DAPI) following the standard method of Hobbie et al. (1977) or by flow cytometry with SybrGreenI (Molecular Probes, Inc) following the procedure of Marie et al. (1997).

Assessment

Growth on the artificial seawater-based AMP1 medium was equivalent to growth on PRO99 for all six strains of *Prochlorococcus* tested, except MIT 9312ax, in which the growth was ~8% greater on AMP1 versus PRO99 ($P < 0.05$, two-tailed t test for independent samples; Fig. 1). Growth rate of *Prochlorococcus* in the PCR-Tu_2 media was slightly lower on average than

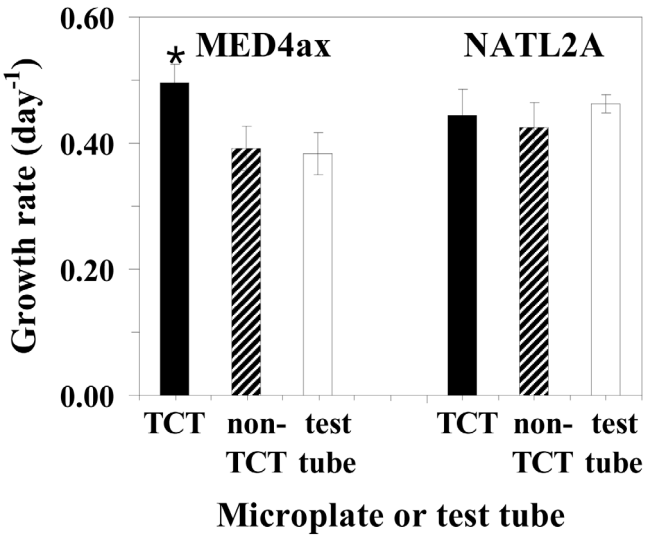

Fig. 2. Growth rate comparison for two strains of *Prochlorococcus*, MED4ax and NATL2A, grown in 96-well, tissue-culture treated (TCT) microplates (solid bars), non tissue-culture treated (non-TCT) microplates (diagonal bars), and glass test tubes (open bars). Cultures were grown in PRO99 media made with SSW in continuous light of 29 μmol Q m^{-2} s^{-1} (MED4ax) and 19 μmol Q m^{-2} s^{-1} (NATL2A) incident from below. Microplate data are average and standard deviation of 96 cultures from four different plates; test tube data are average and standard deviation of two cultures. The asterisk indicates significant differences between growth rates for MED4ax grown in TCT microplates versus non-TCT microplates and glass test tubes ($P < 0.001$).

in PRO99 but only significantly so for MIT 9211 ($P < 0.05$). One possible reason is the copper sensitivity of this low-light adapted strain (Mann et al. 2002), however, further tests are necessary. Our results indicate that either of these two artificial media can be used for growing *Prochlorococcus* cultures when natural seawater is not available or desired.

Effects of culture volume and carbon limitation on growth rate: Materials and procedures

Growth in microplates—A comparison of growth in 96-well polystyrene microwell plates (BD Falcon Clear 96-well Microtest Plates) that were treated for tissue culture (TCT; 353072) and those not treated (non –TCT; 351172) was carried out with *Prochlorococcus* MED4ax and NATL2A relative to controls grown in glass test tubes. To minimize evaporation and condensation, water was put into wells in the outer rows and columns, plate lids were sealed with parafilm, and fans were placed on the incubator shelves to maintain air circulation. Fluorescence from microplates was measured with a Bio-Tek Instruments Synergy HT Microplate reader using a bottom-reading fluorescence protocol without shaking.

Large volumes and carbon limitation—As culture volume increases, the probability of carbon (C) limitation in unstirred cultures increases. We examined this phenomenon by studying the effects of buffer additions and bubbling in large volume cultures (>1 L). To test the hypothesis that C availability limits culture yield in large volume cultures, a 4 L culture of *Prochlorococcus* MED4ax was grown in PRO99 to stationary phase, and then split into two 2 L aliquots and two 20 mL test tubes. $NaHCO_3$ (6 mM from freshly made, 0.6 M syringe-filter sterilized stock) was added to one of each size culture and gently shaken to mix, and growth was monitored.

To test whether the amount of $NaHCO_3$ added would influence final yield, as well as pH, we compared final yields of MED4ax grown with increasing concentrations of $NaHCO_3$ (0, 2, 6, 10 mM) as well as increasing concentrations of the buffer, HEPES (pH 8; 1 and 6 mM), in PRO99 medium. When the control culture reached stationary phase, both 6 mM $NaHCO_3$ and 5 mM HEPES (pH 7.5) were added to further document the effect. Growth was followed fluorometrically, and pH was examined throughout.

We also tested the extent to which bubbling with air can alleviate C limitation and result in higher final cell yields in large volume cultures of *Prochlorococcus* MED4ax. Replicate 1 L cultures of MED4ax were grown in PRO99 medium. One bottle was bubbled using an acid-washed Teflon transfer/venting closure (Savillex, 600-038-30) with air intake through a 0.22 μM Acro 37 TF filter (Pall Gelman, 4464) attached with silicone tubing to an aquarium pump. The other bottle was not bubbled, but the cap slightly loose to allow air flow.

Assessment

Growth in microplates—Both MED4ax and NATL2A grew as well or better in either type of microplate as in glass test tubes (Fig. 2). MED4ax grew at a significantly higher rate (25%) in TCT microplates ($P < 0.001$, two-tailed t test for independent samples), although the reason for this is not understood.

Large volumes and carbon limitation—The addition of $NaHCO_3$ to 2 L and 20 mL cultures in stationary phase causes them to resume growth immediately (Fig. 3A), indicating that stationary phase is caused by C limitation in these cultures. The 2 L bottle without added $NaHCO_3$ did not resume growth, whereas the 20 mL tube without added $NaHCO_3$ did, supporting the idea that surface-to-volume ratios of cultures are important in determining when the supply of C through diffusion will become limiting.

Addition of increasing concentrations of $NaHCO_3$ to 2 L batch cultures grown in PRO99 medium resulted in dramatically increased final yields relative to the PRO99 control (Fig. 3B). Higher concentrations of $NaHCO_3$ resulted in lower pH and less dramatic change in pH as cultures grew (Fig. 3C). Addition of HEPES buffer alone also resulted in increased final yields (Fig. 3B,C) possibly because the buffer helps maintain a relatively constant pH during exponential phase of 8.22 ± 0.04, which is close to the pH at which HCO_3^- constitutes > 95% of the carbonate species (Morel and Hering 1993). The combined addition of $NaHCO_3$ and HEPES to an unamended PRO99 culture in stationary phase resulted in an immediate drop in pH in the culture from 9 to 7.9 (Fig. 3C) and resumption of

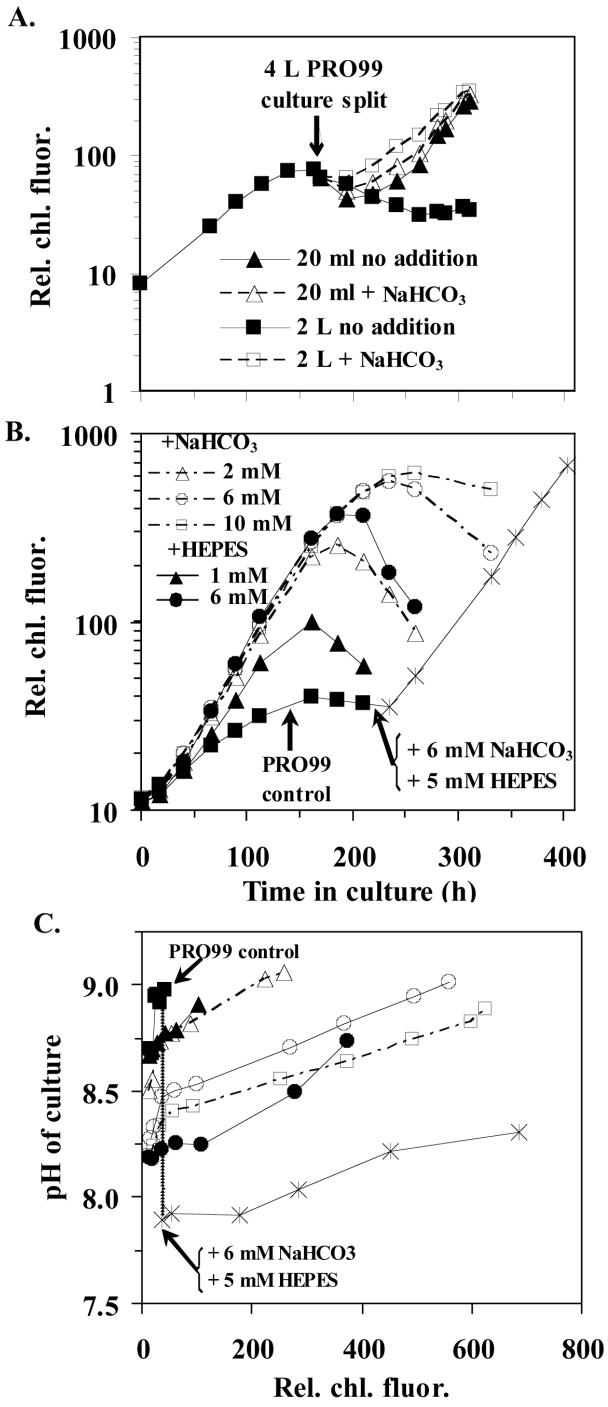

Fig. 3. Growth response of *Prochlorococcus* MED4ax to various additions of $NaHCO_3$ and/or HEPES. (A) Comparison of growth in small and large batch cultures with and without addition of 6 mM $NaHCO_3$. (B) Comparison of growth as concentration of $NaHCO_3$ and HEPES buffer increase. The "no addition control" culture (solid squares) was rescued after 210 h of growth with the addition of both 6 mM $NaHCO_3$ and 5 mM HEPES (asterisks). (C) pH as a function of relative chlorophyll fluorescence during exponential phase and approaching stationary phase of growth with increasing additions of $NaHCO_3$ or HEPES or both. All cultures were grown in a 14 : 10 LD photoperiod at 70 μmol Q m^{-2} s^{-1}. Symbols and relative chlorophyll fluorescence data correspond to that presented in (B).

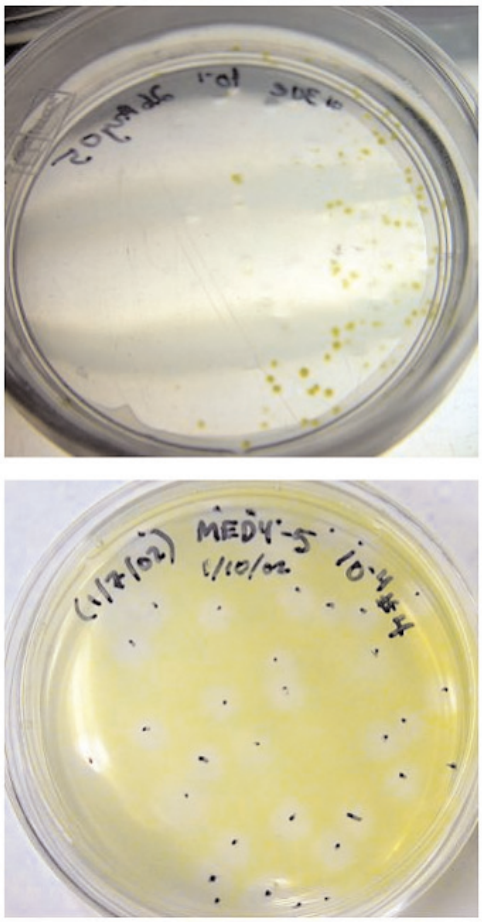

Fig. 4. Growth of isolated colonies (A) and lawn (B) of *Prochlorococcus* MED4ax using the pour plate method with LMP agarose made with PRO99 medium. Viral plaques are visible in (B), scored on the lawn of MED4ax cells.

growth (Fig. 3B). The subsequent pH change due to growth was minimized even more than with the addition of either $NaHCO_3$ or HEPES alone (Fig. 3C).

As was observed with the addition of buffers to PRO99, bubbling with humidified air resulted in seven-fold greater final yields relative to a non-bubbled culture (from 2.3×10^8 to 1.4×10^9 cells mL^{-1}). These results clearly indicate that C limitation is an issue in large volume cultures of *Prochlorococcus* and can be alleviated through addition of buffers or bubbling with air.

Growth on solid medium: Materials and procedures

Lawns and colonies of *Prochlorococcus* cells can be obtained (fig. 4) using a modification of the pour-plate method used for *Synechococcus* (Brahamsha 1996). To determine what type of agar would allow for a lawn of cells to grow up the fastest and last the longest, a variety of types were tested: Invitrogen ultra-pure low melting point (LMP) agarose, Novagen Bac-Plaque agarose, JW Superclean agar (Difco Bacto-agar purified according to Waterbury et al. [1986]), Baculovirus agarose (Invitrogen – discontinued) and Merck's Gelrite. *Prochlorococcus* cells

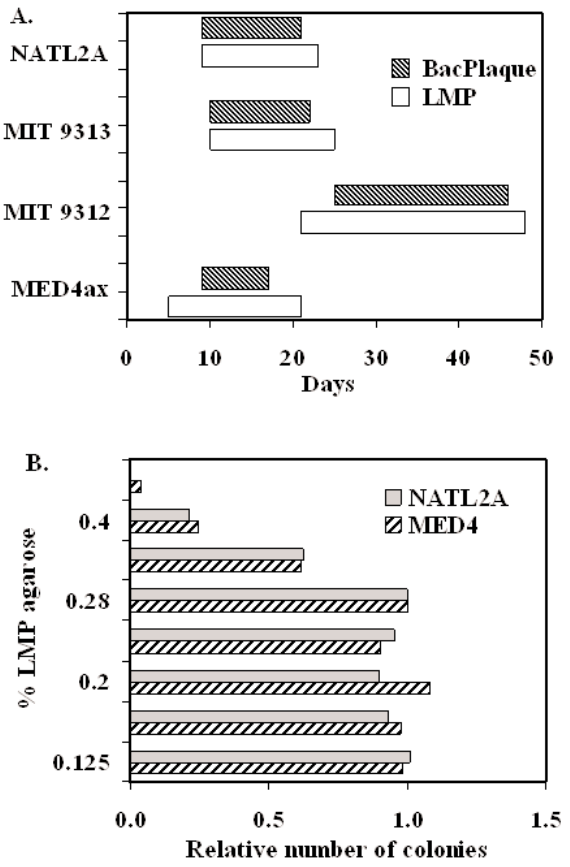

Fig. 5. Comparison of agarose types/brands and percentage for growing *Prochlorococcus* on solid medium using pour plates. (A) Initial detection and duration in days after plating for four strains of *Prochlorococcus* plated on LMP and BacPlaque agarose. (B) Number of colonies produced (normalized to the number obtained using 0.28% agarose) as a function of percentage of LMP agarose for two strains of *Prochlorococcus*. All plates were grown in 15 μmol Q m^{-2} s^{-1} continuous light and 20°C.

(10 mL of late exponentially growing cultures of MED4ax, MIT 9312, MIT 9313, and NATL2A; ~10^7 cells mL^{-1}) were added to 30 mL PRO99 medium supplemented with 0.5% agar, resulting in final agar concentration of 0.4%. Note that the LMP, BacPlaque, and Gelrite solidifying agents were dissolved by autoclaving or microwaving and cooled to 29°C, whereas the JW Superclean agar and Baculovirus agarose were cooled to 39°C, before cells were added. In order to determine the best % agar for obtaining lawns of *Prochlorococcus* for viral plaque assays, LMP agarose concentrations ranging from 0.15–0.70% were tested. Late exponential phase cultures of MED4ax, MIT 9312, MIT 9313 and NATL2A were diluted 1000-fold and 10 mL added to 40 mL of PRO99 medium supplemented with various percentages of agar cooled to 29°C.

Assessment

The growth of lawns started sooner and lasted longer before losing their green color when LMP agarose was used (Fig. 5A). The optimal % LMP agarose for obtaining the most discrete colonies in pour plates was found to be 0.125–0.28 % (Fig. 5B). However, LMP agarose of 0.28% produced a more solid medium that allowed the cells to be evenly distributed without mixing together within the matrix and worked best for relatively fast and long-lasting growth of lawns of *Prochlorococcus*.

Growing colonies of *Prochlorococcus* that are sufficiently isolated from heterotrophic contaminants in order to develop axenic cultures has been difficult. Utilizing pour plate method for purifying cultures has not yielded axenic cultures; however, some success has been achieved instead through streak plating techniques (MED4ax and MIT 9312ax; Saito 2001). These streak plates as well as the medium used to resuspend picked colonies, contained "spent media," i.e., media from which stationary phase *Prochlorococcus* cells have been removed. In these experiments, agar plates were prepared by dissolving Baculovirus agar (0.35% final concentration; Invitrogen – discontinued) in a PRO-TM medium (Table 1e) with added vitamins (1 μg L^{-1} each of biotin, cobalamin, and thiamin) made in 65% seawater, 25% Type I ultrapure water, and 10% spent media. Spent media was prepared from cobalt stressed cultures by growing *Prochlorococcus* cells in the K/10-Cu media (Table 1) with no added cobalt, filtering the culture through 0.2 μm sterile syringe filter into acid-clean polycarbonate container, and storing in darkness at 4°C until use.

Note that the seawater was not autoclaved for these plating experiments due to potential metal contamination from autoclaves but rather was sterilized by microwaving following the method of Keller et al. (1988). Cells used for streaking were grown to late stationary phase. Green lawns of cells typically appeared within 2–4 weeks, with well-separated colonies appearing in areas with the most dilute inoculum after that. Picked colonies were placed in small volumes of media (~5 mL) and monitored visually for growth and heterotrophic contamination for several months. Anecdotally, agar made without spent media or with 100% seawater-based medium also can be used. One possible explanation for the consistent production of *Prochlorococcus* colonies in agar enriched with spent media from cobalt stressed cultures may be due to increased production of dissolved organic carbon, and related cobalt ligands or metallophores therein, in the cobalt stressed cultures (Saito 2001; Saito et al. 2002).

In addition to the strains reported here for testing the type and percentage of agarose, *Prochlorococcus* strains SS120, MIT 9215, MIT 9515, and NATL1A also have been plated successfully by both the pour and streak plating methods. Although the above methods for pour and streak plating *Prochlorococcus* do yield colonies, the plating efficiencies obtained are quite low, at 1% to 10% efficiency. Thus, there is much room for improvement.

Cryopreservation: Materials and procedures

Methods for long-term cryopreservation and recovery of *Prochlorococcus* strains were developed from those established

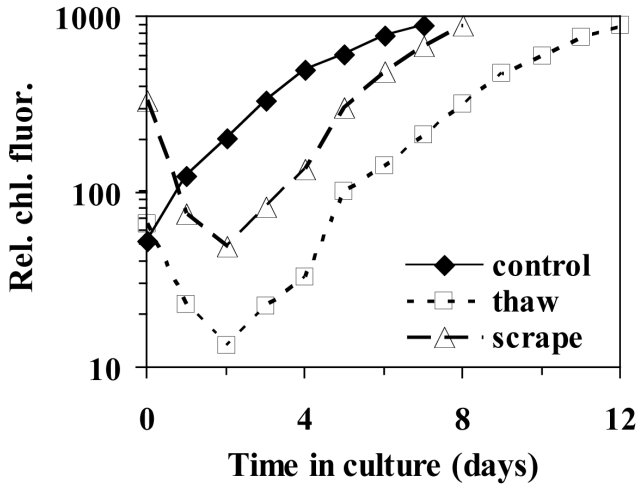

Fig. 6. Growth recovery of cryopreserved *Prochlorococcus* MED4ax using the scrape method (open triangles) and the thaw method (open squares) relative to a non-cryopreserved control (solid diamonds) culture, which is a descendent from the culture used for the cryopreservation. The culture was grown under low light levels (10 μmol Q m^{-2} s^{-1} continuous light) at 21°C in PRO99 medium bubbled with air.

for marine *Synechococcus* (Waterbury et al. 1986), except that we concentrated the *Prochlorococcus* cells by centrifugation prior to preservation, and we used a final concentration of 7.5% dimethyl sulfoxide (DMSO; EMD Chemicals) rather than 10%. Here we describe in detail cryopreservation and two recovery methods (scrape method and thaw method) we have used with success, and report results from both recovery methods.

From a late exponential growth phase MED4 culture, 50 mL was concentrated by centrifugation at 10,000 g for 15 minute at 20–22°C in sterile centrifuge tubes. The supernatant was decanted leaving ~1 mL for resuspending the pelleted cells. Filter-sterilized DMSO (75 μL) was added to the resuspended cells in a sterile, cryogenic vial (Nalgene 5000-0012), inverted to mix, immersed immediately in liquid nitrogen, and then stored at – 80°C. For the scrape method a small amount of frozen cells was scraped with a sterile toothpick (either autoclaved wooden toothpicks or ethanol-sterilized plastic toothpicks) and placed into 25 mL fresh, sterile PRO99 medium. The frozen concentrated cells can be returned to –80°C freezer as many as five times for later recovery. For the thaw method, the entire frozen sample was placed in 37°C water bath until the sample was almost completely thawed (the small ice chunks that remain thaw upon gentle mixing). Then it was split (500 μL each) into two tubes each containing 25 mL fresh medium. Cultures from both methods were incubated at 10 μmol Q m^{-2} s^{-1} continuous light, the same low light conditions used for growth before cryopreservation.

Assessment

Chlorophyll fluorescence of DMSO-cryopreserved *Prochlorococcus* cells decreased immediately after thawing and inoculation into the same media and growth conditions from which they were frozen (Fig. 6). However, growth began 3 d after cells were inoculated with little to no difference in growth rate between cells that were recovered by the scrape method (0.55 day^{-1}), the thaw method (0.53 day^{-1}), and the non-cryopreserved, control culture (0.55 day^{-1}). The timing of recovery can vary significantly, ranging from 3 d (Fig. 6) to 8 weeks post thawing, depending on the size of the inoculum. All strains in the MIT Cyanobacterial Culture collection have been cryopreserved and recovered using the above method, as evidenced by visual inspection of chlorophyll color appearing in the tubes after recovery. It has been observed that the bacterial contaminants recover from the cryopreservation method as well, although a study of any potential difference in response between contaminants and *Prochlorococcus* has not been carried out.

One of the most critical factors in successful recovery of DMSO-cryopreserved *Prochlorococcus* appears to be the requirement of low growth irradiance (~10 μmol Q m^{-2} s^{-1} continuous light) during the pre-freezing and recovery incubation processes, possibly due to sensitivity of *Prochlorococcus* cultures to light changes (small incremental changes are required for acclimating to higher irradiances; Moore et al. 1995). This has not been tested systematically, but recovery does not occur if cells are put into higher light intensities relative to their pre-freezing culture conditions. We have found that cells frozen in continuous light can be recovered in 14 : 10 LD photoperiod (and *visa versa*) as long as it is under low light conditions. Additionally, some DMSO-cryopreserved *Prochlorococcus* can be recovered in different media from which the original culture was grown; for instance we have successfully recovered MED4ax in AMP1 when originally grown and cryopreserved in PRO99 (data not shown).

Procedures for isolating Prochlorococcus from the wild

The procedures for isolating *Prochlorococcus* from natural populations have been described (Chisholm et al 1992) and have not changed significantly since the initial isolations. A variety of different media have been used for isolating *Prochlorococcus* strains now in the MIT Cyanobacterial Culture Collection, however, the most success has been with media containing lower concentrations of macronutrients, such as PRO2 (Table 1). One of the biggest challenges in obtaining *Prochlorococcus* isolates is the elimination of other phytoplankton, particularly *Synechococcus* cells, which often outcompete the *Prochlorococcus* cells in culture. In our experience, selection of growth conditions that favor *Prochlorococcus*, such as very low light, and frequent transferring over the course of months appear to provide the best means of obtaining unialgal isolates. Regular monitoring of cell density using flow cytometry is also helpful. Of course, marine bacteria are co-cultured with the isolates, as they cannot be removed by size exclusion, and need to be eliminated via other means, such as plating (Saito 2001), serial dilution (Rippka et al. 2000), and flow cytometric sorting (Moore et al. 2005). Another potential

problem associated with isolating *Prochlorococcus* is the possible artificial selection of strains due to isolation methods, such as the filter pore size (typically 0.6 μm polycarbonate), low-light conditions, and selective culture media. For example, no known NO_3-using *Prochlorococcus* strains have been isolated, possibly due to the fact that only ammonia (NH_4) has been used as a N source for the isolation of *Prochlorococcus* strains in MIT Cyanobacterial Culture collection (Moore et al. 2002). Other methods of isolation, such as the high-throughput methods introduced by Connon and Giovannoni (2002), should be explored to isolate potentially novel *Prochlorococcus*.

Discussion

In order to develop *Prochlorococcus* as a model system for studying marine microbial ecology, systematic culturing methods are essential. Since *Prochlorococcus* was brought into culture in the late 1980s, methods for culturing the isolates have evolved substantially as our understanding of their physiology has grown. Here we have tried to present the most comprehensive and current culturing methods for maintaining a wide variety of *Prochlorococcus* ecotypes. The methods for growth in microplates allow for rapid, large-scale comparative growth experiments, whereas the methods for alleviating carbon limitation in large cultures allow for researchers to obtain high biomass necessary for molecular analyses such as whole genome and protein expression under various experimental conditions. The difficulty in plating *Prochlorococcus* is still a challenge that needs to be met for improving yields necessary for better genetic screens, but the methods presented here provide a basis for development of a robust plating system for *Prochlorococcus*. For any type of microbial model system, the need to isolate and maintain strains representative of natural populations is paramount. The isolation issues and culturing methods, as well as the cryopreservation and recovery methods, presented herein should allow other researchers to do so.

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