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Measurement of *Prochlorococcus* ecotypes using real-time polymerase chain reaction reveals different abundances of genotypes with similar light physiologies

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Summary

***Prochlorococcus* is a marine cyanobacterium which is found at high abundances in world's tropical and subtropical oligotrophic oceans. The genus *Prochlorococcus* can be divided into two major groups based on light physiology. Both of these groups can be further subdivided into genetically distinct lineages, or ecotypes. Real-time polymerase chain reaction (PCR) assays based on sequence differences in the 16S-23S rDNA internal transcribed spacer or the 23S rDNA were developed to examine the distribution of each ecotype in the field. The real-time PCR assays enabled linear quantification of concentrations ranging from 10 to 4×10^5 cells ml^{-1}. These assays were applied to a stratified water column in the Sargasso Sea. The majority of *Prochlorococcus* cells above 110 m belonged to the one of the low chlorophyll *b/a* ratio (high-light adapted) ecotypes, while two types of high chlorophyll *b/a* ratio (low-light adapted) cells dominated below 110 m. The other three types were found at significantly lower numbers or not detected at all. Differences in the abundance of ecotypes within the major light physiology groupings suggest that other factors, such as nutrient utilization and differential mortality, are driving their relative distributions. Real-time PCR assays will enable further exploration of these factors and temporal and geographic variability in ecotype abundance.**

Introduction

Prochlorococcus marinus is a small marine cyanobacterium (Chisholm *et al*., 1988; 1992) distributed ubiquitously across the oligotrophic portions of the world's tropical and subtropical oceans at high concentrations (up to 4×10^5 cells ml^{-1}) (Campbell *et al*., 1997; Partensky *et al*., 1999; Crosbie and Furnas, 2001; DuRand *et al*., 2001). They contribute significantly to the primary production in these regions (9–82% of total gross primary productivity) (Goericke and Welschmeyer, 1993; Li, 1995; Liu *et al*., 1997; Veldhuis *et al*., 1997). Experiments on cultured strains combined with molecular phylogeny have revealed that the genus *Prochlorococcus* is composed of multiple genetically and physiologically differentiated subgroups, or ecotypes (Moore and Chisholm, 1999; Moore *et al*., 2002; Rocap *et al*., 2002). Strains can be divided into two main groups, low and high B/A (also referred to as high- and low-light adapted respectively), based on their characteristic ratios of divinyl chlorophyll *b* and *a* molecules and their 16S rDNA sequences (Moore *et al*., 1995; 1998; Urbach and Chisholm, 1998; Moore and Chisholm, 1999). Low B/A strains have higher optimal light intensities for growth than high B/A strains, whose larger chlorophyll *b/a* ratio allows them to grow under low and blue-shifted light, typical of the lower portion of the oceanic euphotic zone (Moore *et al*., 1998; Moore and Chisholm, 1999).

Phylogenetic analysis of single loci (Palenik, 1994; Moore *et al*., 1998; Urbach and Chisholm, 1998; Rocap *et al*., 2002) and complete genome sequences of three *Prochlorococcus* strains (Dufresne *et al*., 2003; Rocap *et al*., 2003) have provided substantial evidence for the division of *Prochlorococcus* into ecotypes. Molecular phylogeny using the 16S-23S rDNA internal transcribed spacer (ITS) divides *Prochlorococcus* into six genotypes, two within the low B/A ecotype and four within the high B/A group (Rocap *et al*., 2002). The two low B/A types (I and II) exhibit differences in phosphorus acquisition (Moore *et al*., 2005) and their strikingly different geographic distributions in the Atlantic and Red Sea (West *et al*., 2001) suggest that they have ecologically significant physiological differences. The four high B/A genotypes exhibit differences in their ability to grow on nitrite as a sole N source (Moore *et al*., 2002). Strains belonging to both the most evolutionarily derived (I) and the most basal (IV) high B/A lineages can utilize nitrite, which is found in peak concentrations just below the mixed layer (Olson, 1981; Dore and Karl, 1996). In contrast, the two strains repre-

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senting the remaining two types, high B/A II and III are unable to grow on nitrite. Genome sequences of the high B/A IV strain MIT 9313 and the high B/A III strain SS120 have revealed large differences in gene content, including in genes for nitrogen utilization that account for the observed differences in physiology (Dufresne *et al*., 2003; Rocap *et al*., 2003).

Differences observed in light physiology of cultured isolates suggested that *Prochlorococcus* ecotypes vertically partition the euphotic zone (Moore *et al*., 1998; Urbach *et al*., 1998). This hypothesis was tested directly by West and colleagues (2001) using fluorescent *in situ* hybridization (FISH), providing the first quantitative confirmation of niche-partitioning of *Prochlorococcus* ecotypes. In both the Eastern North Atlantic and the Red Sea low B/A ecotypes dominated surface waters (3, 20 and 40 m). A single probe was used to detect all high B/A ecotypes together, and in both the Atlantic and Red Sea, this probe detected significantly more cells in the deep euphotic zone (at 80 and 100 m respectively) than at shallower depths (West *et al*., 2001). The four cultured high B/A ecotypes were not measured independently, mostly because the probes target the 16S rDNA which is highly conserved within *Prochlorococcus* (~3% difference between the most divergent sequences) (Moore *et al*., 1998). Thus, while this study showed that the general depth distribution pattern of low and high B/A *Prochlorococcus* is consistent with predictions based on physiology, the abundance and distribution of all six ecotypes and their full range throughout the water column have not been explored.

Real-time quantitative polymerase chain reaction (PCR) techniques (Higuchi *et al*., 1992; Heid *et al*., 1996) enable sensitive quantitative detection of specific DNA sequences (Andreasson *et al*., 2002; Malinen *et al*., 2003). Real-time PCR has been successfully applied to quantify specific groups of microbes from both marine and freshwater environmental samples (Suzuki *et al*., 2000; Becker *et al*., 2004; Skovhus *et al*., 2004; Layton *et al*., 2005; Zhu *et al*., 2005). Because this method is rapid and high throughput it has proved useful for detailed observations of microbial population dynamics over various temporal and spatial scales (Suzuki *et al*., 2001a).

Prochlorococcus provides an excellent model system to quantify multiple ecotype abundances using real-time PCR because it has a unique flow cytometric signature (Chisholm *et al*., 1988; Olson *et al*., 1990) and can thus be enumerated in a sample – independently of molecular methods – to assess what fraction of the total population is being detected by real-time PCR. Here we present the development of real-time PCR assays to determine the abundance in field samples of the six *Prochlorococcus* ecotypes currently having cultured representatives. We targeted the more variable 23S rDNA and the 16S-23S ITS (Rocap *et al*., 2002) for these assays in order to distinguish between all six ecotypes.

Results and discussion

Nomenclature

The recent availability of several complete *Prochlorococcus* genome sequences (Dufresne *et al*., 2003; Rocap *et al*., 2003) has created 'type strains' within the six ecotype lineages about which an enormous amount of genetic information is now known. The genome sequences have revealed that the strains differ in hundreds of genes involved in functions such as utilization of different forms of organic nutrients, transport of small molecules, production of cell surface polysaccharides, regulation of gene expression and many others that remain unknown (Rocap *et al*., 2003). Thus, we think it prudent to adopt a new nomenclature for the six known ecotypes, consisting of the prefix, 'e', appended to the name of a type strain selected for each ecotype (Table 1). This nomenclature removes the emphasis on light adaptation as the defining characteristic of the ecotypes. Additionally, for novel genotypes discovered through molecular methods rather than from a cultured representative the use of a 'type sequence' will provide a uniform way to refer to lineages which do not yet have a phenotype attached to them.

Primer design

The design of specific primers and probes is critical to the success of real-time PCR as oligonucleotides that are not perfectly complementary to the target sequence can reduce the efficiency of the PCR (Klein *et al*., 1999; 2001; Tichopad *et al*., 2002) and cause inaccuracy in quantification. Primer sets specific for each of the six *Prochlorococcus* ecotypes currently present in culture were designed by targeting unique sequences in either the 23S rDNA (the eMIT9312 set) or the 16S-23S rDNA ITS region (all other sets) (Table 1). Primers were designed using a database of *Prochlorococcus* and *Synechococcus* sequences from cultured strains (Rocap *et al*., 2002) and cloned environmental DNA (Rocap, 2000; Suzuki *et al*., 2001b). We attempted to design primers to be perfectly complementary to all sequences of cultured strains within the ecotype and to have at the 3′ end two or more mismatches with other *Prochlorococcus* and *Synechococcus* ecotypes. In a few cases, these criteria could not be fully met. The forward primer for the eMIT9312 primer pair is also identical to eMED4 type sequences, although it has several mismatches with all other known *Prochlorococcus* and *Synechococcus* sequences, thus full specificity for this primer set comes from the reverse primer.

Table 1. Real-time PCR assay conditions for all six *Prochlorococcus* ecotypes.

Equivalent ecotype designations									
Based on type strain	As in Rocap *et al.* (2002)	Primers, 5′ to 3′ (forward, reverse)	Strains used[a]	Amplicon size (bp)	Primer concentration (nM)	Mg^{+2} (mM)	Cycling programme	Amplification efficiency	Limit of detection (cells ml^{-1})
eMED4[b]	Low B/A I	lowBAII2f, TACCTCCACTGAATACCACCTCT lowBAI2r, CGCACAAATAATAAATCTGCATCAT	**MED4** MIT 9515	88 88	50	2.5	95°C 45 s 58°C 45 s 72°C 30 s	0.52 ± 0.06	120
						4	95°C 45 s 58°C 45 s 72°C 30 s	0.84 ± 0.06	42
eMIT9312	Low B/A II	lowBAII3f, TCGGGGAGTTGGAAGCACA lowBAII2r, CCTATATATTCAGTTGGCCGTACAT	AS9601 **MIT 9312**	78 78	300	2.5	95°C 45 s 58°C 45 s 72°C 10 s	0.97 ± 0.02	200
eNATL2A	High B/A I	NATL3f, ACCTAGCTTCTTGTCATCTTTTTAT NATL2r, CATGAGATGCTTTATTCTTTCTAATC	**NATL2A**	120	100	4	95°C 30 s 56°C 30 s 72°C 10 s	0.89 ± 0.11	175
eSS120	High B/A II	SS1202f, AACTCAATTTGGAGATTATCGTT SS1205r, GCTTTGCTTAATAAGTAAATTTCAGT	**SS120**	241	500	4.5	95°C 30 s 56°C 45 s 72°C 20 s	0.80 ± 0.04	10
eMIT9211	High B/A III	92112f, GAACCTAGCTTCCTATTACCCTTT 92113r, GATGGTTATTAGACTTTTATAAATGAA	**MIT 9211**	268	200	5.5	95°C 30 s 56.4°C 40 s 72°C 20 s	0.79 ± 0.01	73
eMIT9313	High BA IV	93031f, CAACGAGCCAATGGTGAGAA 93133r, GGCTTCAATCTCAAACTTCTCC	MIT 9303 **MIT 9313**	421 420	200	4	95°C 20 s 59°C 20 s 72°C 20 s	0.75 ± 0.05	25

a. Type strains for each ecotype are listed in bold.
b. For eMED4 only, optimized conditions are given for use on both the LightCycler (upper row) and ICycler (lower row), conditions for all other ecotypes are presented for the Icycler.

Additionally, one of the 18 eMIT9312 isolate sequences has one mismatch (A-G) in the middle of the reverse primer. The primers for the eMED4 primer set contain one mismatch at the 3′ end of the forward primer (C-T) and two mismatches in the middle of the reverse primer (C-T) to a cloned environmental sequence belonging to this ecotype (Suzuki *et al*., 2001b). Both the eSS120 and eMIT9211 primer sets are based on the single available sequence for each of these ecotypes.

Sensitivity and specificity of assays

In contrast to many other studies which use plasmids as DNA standards, the use of standards made from known amounts of cultured cells has several advantages. First, absolute quantification of cell abundance is possible, as long as extraction efficiencies are similar between cells present in standard and unknown samples. This approach also takes into account potential differences in genome rDNA copy number (assuming rDNA copy number is consistent within cells of the same ecotype). Controlling for this issue when using plasmid DNA as standards requires *a priori* knowledge of the genome rDNA copy number for each ecotype.

Real-time PCR assays were developed and optimized for each primer set. Lower limits of detection for each assay were set by first determining the lower limit at which DNA standards amplified reliably and above no-template negative controls. Cultured standards were tested at concentrations down to 20–40 cells per reaction. The lower limit of precise amplification was selected by picking the least concentrated standard which still fell on the line defined by the more concentrated standards and for which there was no significant difference in the error of cycle threshold (C_T) values for triplicate reactions between the least concentrated standard and all other standards of higher concentration.

Next, the specificity of the assays was investigated by testing each primer set with non-target template from each of the other five *Prochlorococcus* ecotypes and representative marine *Synechococcus* strains at concentrations of at least 2×10^5 cells per reaction. This corresponds to a concentration of 1×10^5 cells ml^{-1} for detection from field samples, comparable to the maximum reported abundance of natural populations of both *Prochlorococcus* and marine *Synechococcus* (Olson *et al*., 1990; Crosbie and Furnas, 2001). For most of the assays, non-target DNA did not amplify or amplified below the level of the least concentrated standard, so the lower limit of detection for these sets was placed at the precision limits determined above (Table 1). For the eMIT9312 primer set and the eMED4 primer set on the ICycler, *Synechococcus* WH 8103 template produced numbers slightly higher than the limit of precise quantification set from above. In these cases, the lower limits of specific detection were set at twice the average value apparently given by WH 8103 (Table 1).

Within ecotype comparisons

We investigated possible bias in quantification using different strain templates as standards. Some bias could arise from either small differences in the amplification efficiency between sequences (Klein *et al*., 2001) or minor disparities in the fluorescence of amplicons detected with SYBR Green I. Genomic standards were created and quantified with real-time PCR for two strains each of the eMED4, eMIT9312 and eMIT9313 ecotypes. Standard curves were compared for each ecotype assay (Fig. 1), and in all cases, there was no significant difference in the slopes of standard curves (Table 2). Melting curves where also compared between pairs of cultured standards, and there were no statistically significant differences in the melting curve peaks except for the eMIT9313 strains, MIT 9303 and MIT 9313. Despite a difference of 0.70°C in the peaks of MIT 9303 and MIT 9313 products, there was no bias in quantification between these strains. Thus, moderate variability in amplicon sequences does not bias quantification.

Mixed culture sets

Mixtures of genomic DNA of several closely related ecotypes can introduce a bias in amplification in real-time PCR reactions (Becker *et al*., 2000) through increased likelihood of the amplification of non-specific products and enhancement or inhibition of amplification efficiency. We tested for such biases by comparing MED4 standards to mixed culture samples containing both MED4 and MIT 9313 cells (Fig. 2). There was no difference between the amplification efficiency of standards and mixed samples (Table 2). There was a significant difference in the intercepts of the standard curves of 4.0. The slightly lower C_T values for the mixed samples resulted in an overestimation of cell counts ranging from 20% to 154%. In contrast, when extracted DNA was serially diluted in both 10 mM Tris and 10 mM Tris with 0.8 μg ml^{-1} herring sperm DNA added, no significant difference was seen in the amplification of the two DNA sets (data not shown), suggesting that the presence of additional non-specific DNA in the reaction does not enhance amplification efficiency. Thus, overestimation in the mixed culture set may be caused by a DNA carrier effect (Mygind *et al*., 2003) in the extraction of mixed samples, whereby the added MIT 9313 cells increases the yield of MED4 genomic DNA, especially at low cell numbers. For silica column-based extraction methods as used here, addition of carrier DNA is recommended to increase extraction yield for samples with low

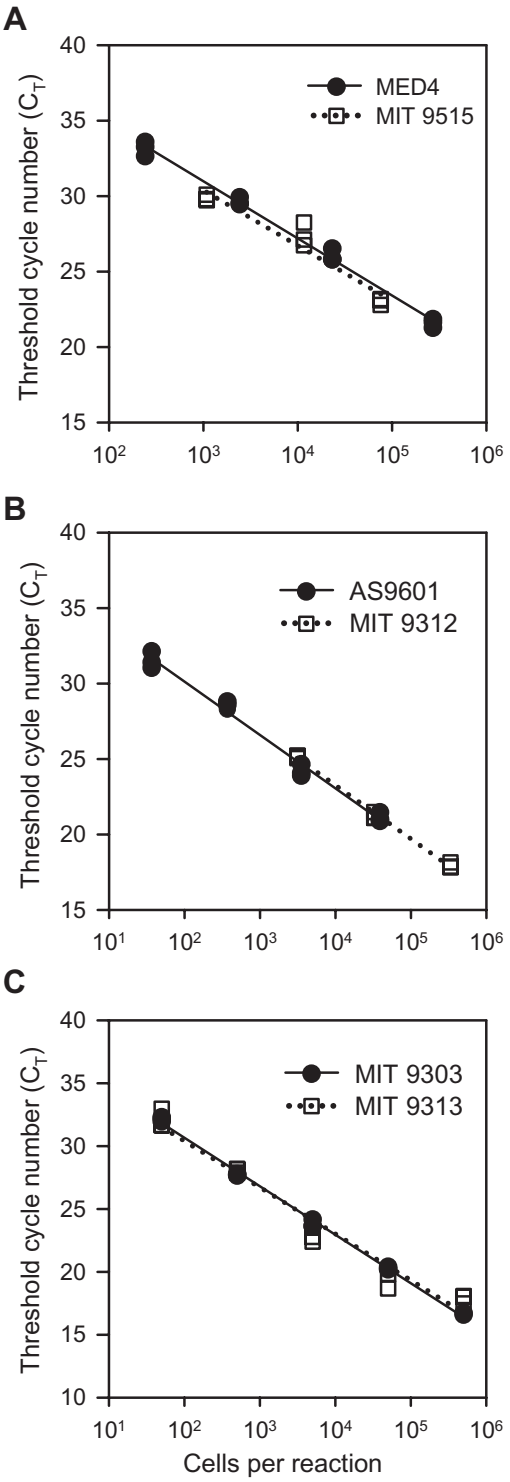

Fig. 1. Threshold cycle number (C_T) as a function of amount of template added (cells per reaction) as measured by real-time PCR. The standard curves (cells per reaction versus C_T) for genomic DNA standards are compared between two strains within an ecotype: (A) strains MED4 and MIT 9515 from ecotype eMED4, (B) strains AS9601 and MIT 9312 from ecotype eMIT9312, (C) strains MIT 9303 and MIT 9313 from ecotype eMIT9313. Points represent triplicate reactions at each concentration tested, and linear regressions of each set of standards are plotted.

amounts of genomic DNA (<10 ng) (Qiagen, Valencia, CA).

Mock field samples

These methods were tested to see whether they were transferable to accurately quantify cells from marine water samples, which might contain substances or additional non-specific bacterial DNA that could affect PCR amplification (von Wintzingerode *et al*., 1997; Becker *et al*., 2000; Stubner, 2002). We compared standards made from cultured MED4 cells with simulated field samples created by spiking cultured MED4 cells into filtered coastal marine water (Fig. 3). There was a statistical difference in the amplification efficiencies between the standards and mock field samples as determined from the slope of individual reactions (Table 2). The amplification efficiencies of mock field samples were only slightly more efficient on average than standards (Table 2), but there was no statistical difference in amplification efficiency using the slopes of standard curves, indicating that there is no bias in quantification over the concentration range tested. There was a significant difference of 1.31 between the intercepts of standard curves (Table 2) resulting in overestimation of cell counts ranging from 6.8% to 98.4%, with the highest error at the lowest cell concentrations. The most likely explanation for this overestimation is that the additional biomass in the coastal seawater enhances extraction by a DNA carrier effect, as was seen in the mixed sample experiment.

In order to enumerate *Prochlorococcus* using these assays, we must assume that the extraction efficiency is equal across environmental samples. We believe this is a fair assumption, even given the potential issues at very low cell concentrations observed above, as bias seen in the mixed and mock environmental samples is minimal when more than 5×10^6 cells were extracted (corresponding to 1×10^5 cells per reaction in Figs 2 and 3). Bacterial counts in the Sargasso Sea are typically $2–5 \times 10^5$ cells ml^{-1} in the upper 200 m (Gundersen *et al*., 2001), thus 100 ml samples provide a background level of DNA well above the threshold at which carrier effects were observed. However, other sources of variation between samples could also potentially affect quantification, such as the presence of PCR inhibitors or variable lysis efficiency of cells in particular physiological states (e.g. stationary phase). To more fully account for such variability between samples, these methods could be further improved by spiking specially modified cells into each environmental sample as an internal standard.

Field samples

The six real-time PCR assays were applied to water col-

Table 2. Results of statistical analyses (two-tailed *t*-tests) performed on control experiments.

		Standard curve		Amplification efficiency of individual rxns	Melting curve
Control experiment	Strains or samples tested	Slope different?[a]	Intercept different?[a]	Average of efficiencies different?[a]	Peaks different?[a]
Within eMED4	MED4 versus MIT 9515	N (0.98)	N (0.09)	N (0.21)	N (0.40)
Within eMIT9312	AS9601 versus MIT 9312	N (1.00)	N (0.37)	N (0.59)	N (0.37)
Within eMIT9313	MIT 9303 versus MIT 9313	N (0.98)	N (1.00)	N (0.69)	Y (<0.001) [–0.70°C]
Mixed sample	MED4 versus MED4 + MIT 9313	N (0.09)	Y (<0.001) [3.99]	N (0.44)	N (0.24)
Mock environmental samples	MED4 versus MED4 + coastal water	N (0.99)	Y (<0.001) [1.31]	Y (0.04) [–0.07]	N (0.20)

a. *P*-values are given in parentheses. If a statistical difference was detected the difference observed is given in brackets.

lected from the Sargasso Sea in September 2001 to quantify *Prochlorococcus* ecotypes in natural populations. First we established that there was no statistical difference between the amplification efficiencies of the water column samples and cultured standards (data not shown).

We also compared the melting curves of the Sargasso Sea samples with those of cultured standards to verify that each primer set was amplifying the correct target sequence. Amplicons belonging to the same ecotype will have similar or indistinguishable melting curves while amplicons having a significantly different sequence from the cultured standards are expected to have different melting curves. Products amplified from the Sargasso Sea by the eMED4 primer set all had a primary peak which was indistinguishable from MED4 standards as well as one or two smaller secondary peaks at lower temperatures, one of which was similar to peaks seen in no-template negative controls (Fig. 4A). The eMIT9312 assay produced peaks that were indistinguishable from both AS9601 and MIT 9312 standards (Fig. 4B).

Melting curves for the high B/A ecotypes demonstrated more variation than the low B/A assays. In the eNATL2A assay, two distinct peaks were seen in the 70 m sample, one at 80.0°C and one at 82.5°C, the same temperature as the NATL2A standards (Fig. 4C). This bimodal melting curve is consistent with predicted melting temperatures of amplicons within this ecotype, which split the sequences into two distinct groups, one including NATL2A and a second having a lower temperature (data not shown). Thus, the broad peaks seen at other depths (e.g. at 90 m, Fig. 4C) probably consist of a mixture of the peaks seen at 70 m and represent the sequence (and hence melting

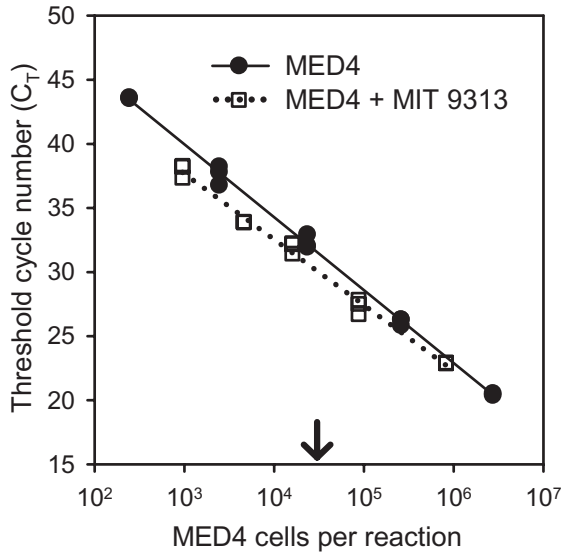

Fig. 2. Comparison of real-time PCR standard curves of MED4 standards (MED4) with mixed samples (MED4 + MIT 9313) containing MED4 cells at varying concentrations and approximately 10 000 MIT 9313 cells per reaction. Arrow indicates the approximate concentration of MIT 9313 cells present in all of the mixed samples. Standard curves of triplicate reactions with linear regressions are shown.

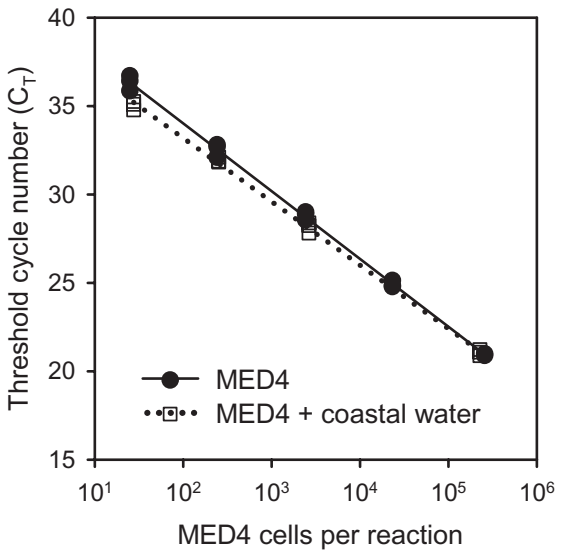

Fig. 3. Comparison of real-time PCR standard curves of MED4 standards (MED4) to MED4 cells spiked into water collected off of the dock at Woods Hole, MA (MED4 + coastal water). Standard curves of triplicate reactions with linear regressions are shown.

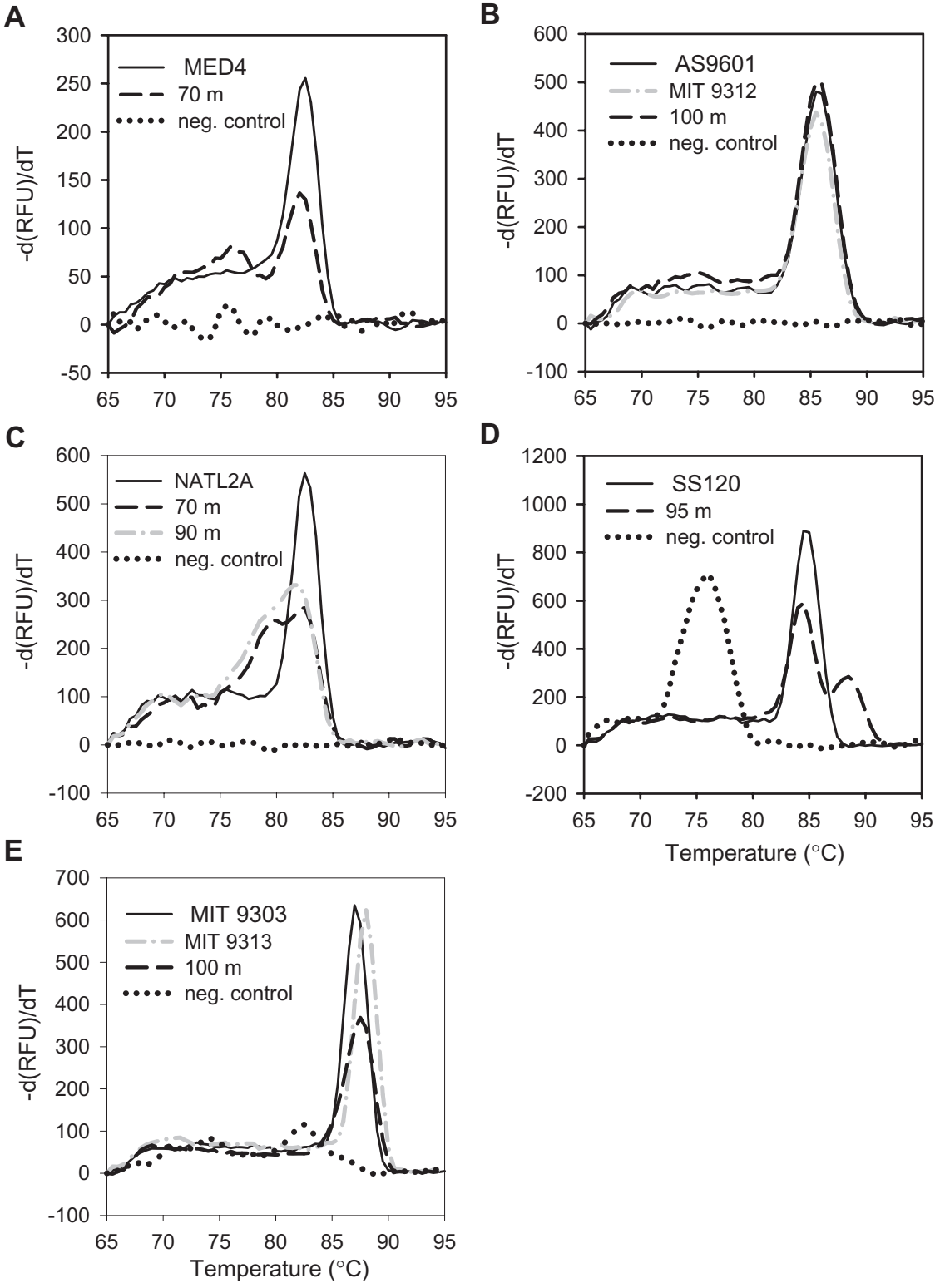

Fig. 4. Representative melting curves of real-time PCR products for standards (solid or dash-dot lines), no-template negative (neg.) controls (dotted lines) and field samples (dashed or dash-dot lines) from the North Atlantic at given depths. The *y*-axis is the negative of the first derivative of relative fluorescence units (RFU) versus temperature (T). (A) eMED4, (B) eMIT9312, (C) eNATL2A, (D) eSS120, (E) eMIT9313.

property) diversity within the eNATL2A amplicons. In the eSS120 assay, the melting curves of environmental samples also had two peaks (Fig. 4D). One peak was similar (0.42°C lower) to the SS120 standards while the other peak was on average 4.0°C higher. This higher peak likely represents a sequence from a genotype other than eSS120 as it is significantly different from the SS120 standards. eMIT9313 assays produced one distinct peak that lies in between the MIT 9303 and MIT 9313 standards (Fig. 4E). Thus, the majority of the assays produced melting peaks that were indistinguishable or similar to the peaks of cultured standards, confirming that the sets are amplifying the correct target sequence. The main exception was the additional peak seen in the eSS120 (Fig. 4D), suggesting this primer set may be amplifying a novel *Prochlorococcus* type in addition to eSS120 cells. Finally, no eMIT9211 cells were detected at any depth.

The field samples used in this study were collected from the Sargasso Sea in September when the water column was well stratified (Fig. 5A). The surface mixed layer extended to ~60 m, and there was a deep chlorophyll maximum at ~100 m. Total *Prochlorococcus* abundance (as determined by flow cytometry) ranged from 2×10^4 to 1×10^5 cells ml^{-1} (Fig. 5B), while *Synechococcus* cell concentrations were an order of magnitude lower, as is typical for this location and time of year (DuRand *et al*., 2001). The sum of all ecotype abundances from real-time PCR was compared with total *Prochlorococcus* concentrations determined by flow cytometry to determine what fraction of the total population was captured by the real-time assays (Fig. 5B). There is good agreement between the two measures throughout most of the water column. At 130 m, however, the sum of real-time PCR numbers is only 25% of the flow cytometrically determined concentrations, suggesting that either our primers are not picking up all of the strains belonging to each of the six ecotypes, or there are additional ecotype clusters not currently represented in culture. Nonetheless, it is remarkable that there is such good agreement between total real-time and flow cytometry counts despite all of the potentially complicating factors of extraction efficiency discussed above.

The absolute abundances of each ecotype in the depth profile demonstrate that *Prochlorococcus* does partition the water column and different ecotypes within the major light physiology groups have different distributions. Surface waters were completely dominated by low B/A *Prochlorococcus*, as only eMED4 and eMIT9312 type cells were detected above 60 m. However, peak abundances for both of these types occurred at 80 m (Fig. 5C) and the relative contribution of eMED4 cells to the total *Prochlorococcus* population was highest at 90 m (Fig. 5D). High B/A cells were detected only deeper in the water column, with eNATL2A cells present at 60 m and below and eSS120 and eMIT9313 type cells detected only below 90 m. Both eSS120 and eMIT9313 type cells exhibit two maxima, at 95 m and 130 m. Interestingly, the 95 m maxima coincides with a local minima in concentration of eNATL2A cells. Plotting the fraction of each ecotype to the sum of real-time PCR cell counts clearly reveals that eMIT9312 cells dominate the *Prochlorococcus* population in the upper depths while eNATL2A and eMIT9313 cells make up the majority of the detectable population below 110 m (Fig. 5D).

The depth distributions of low and high B/A ecotypes agree well with previous light physiology experiments on cultured strains (Moore *et al*., 1998; Moore and Chisholm, 1999). Growth versus light irradiance curves show that for high B/A strains the lower limit of growth, maximal growth rate and photoinhibition occur at light levels lower than that of low B/A strains. Our measurements of low B/A abundances as a function of depth in the field show that they drop significantly at 130 m while high B/A cells are still found at significant numbers at and below this depth. The light level at 130 m is approximately 7 μmol Q m^{-2} s^{-1}, similar to the lower limit of growth for low B/A strains (Moore and Chisholm, 1999). Low B/A ecotypes are often referred to as 'high-light adapted' strains. While it is true that they grow at light intensities that are inhibitory to high B/A strains, and thus are relatively high-light adapted, light physiology data suggest that these cells are capable of significant growth rates at all but the very bottom of the euphotic zone (Moore and Chisholm, 1999). This is confirmed here by the dominance of the low B/A cells at depths as deep as 120 m.

The dominance of the majority of the water column by eMIT9312 cells is similar to observations using FISH methods in the Red Sea in October 1999 where they were dominant in the upper water column and were present down to 100 m (West *et al*., 2001) and eMED4 cells were not detected. In contrast, in the Eastern North Atlantic in June 1996 eMED4 cells were the most abundant at 3 and 40 m and eMIT9312 cells were not detected (West *et al*., 2001). In our sample from the Sargasso Sea (Western North Atlantic) both low B/A ecotypes were present with eMIT9312 dominating the upper water column, suggesting that the FISH method was not sensitive enough to detect MED4 in these waters. It is more difficult to directly compare results from the high B/A ecotypes as probes used in the Eastern North Atlantic did not discriminate among the high B/A ecotypes (West *et al*., 2001). In the Red Sea the use of a more specific probe revealed that the majority of cells at 100 m are comprised of eNATL2A cells whereas we see both eNATL2A and eMIT9313 cells predominating in our samples.

Although differences in efficiency of light utilization can explain the general trend of water column partitioning of low and high B/A cells, clearly there are other factors shaping patterns in ecotype abundance, particularly

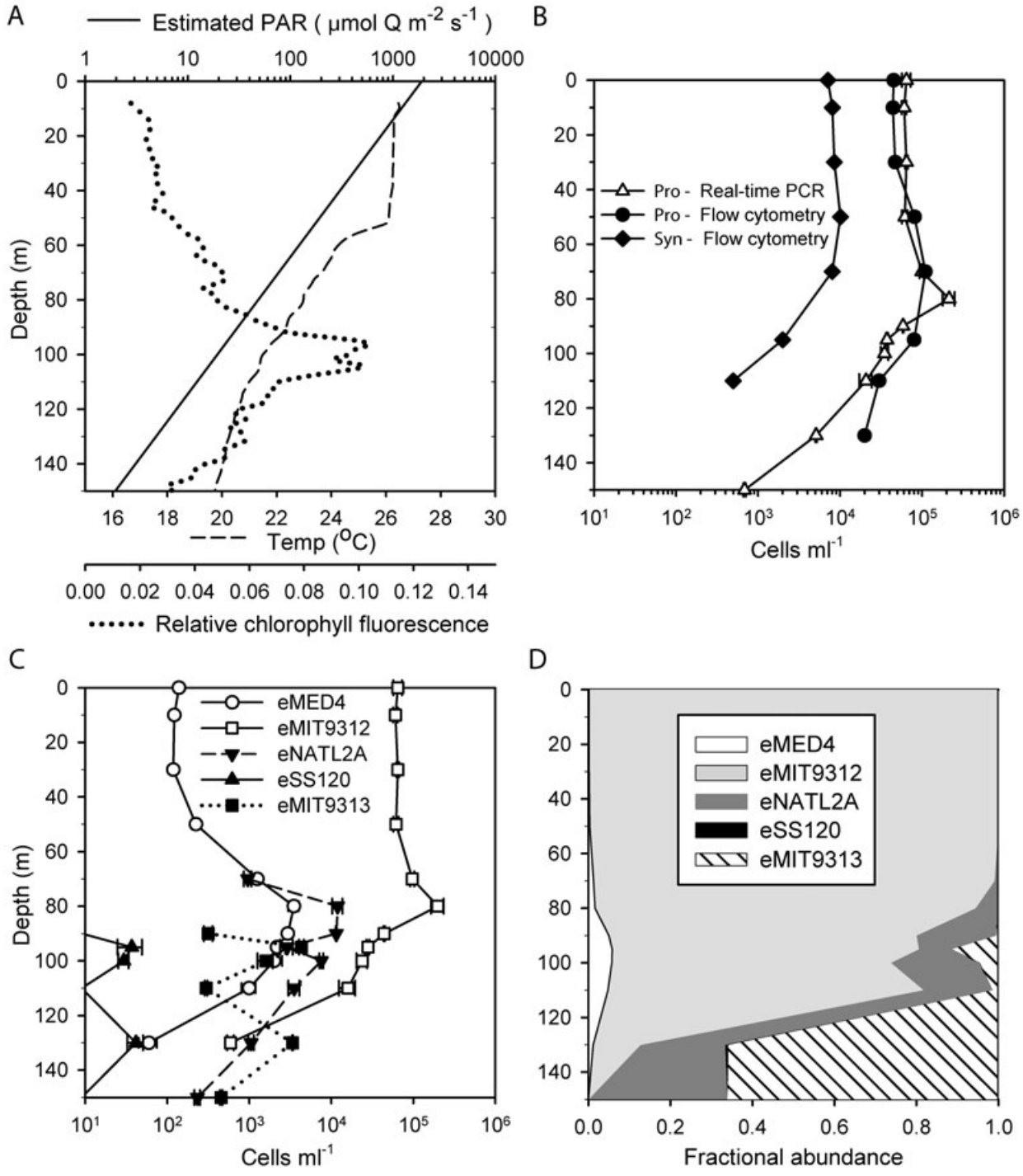

Fig. 5. Depth profiles of hydrographic data and *Prochlorococcus* abundance from a stratified water column in the Sargasso Sea, September 2001.
A. Water column properties.
B. Total *Prochlorococcus* (Pro) and *Synechococcus* (Syn) abundance determined by flow cytometry and total *Prochlorococcus* determined by summing ecotype counts from real-time PCR. Flow cytometry counts were not determined at 80 and 150 m.
C. Abundance of *Prochlorococcus* ecotypes determined by real-time PCR. Points represent averages of triplicate reactions and error bars are standard error of triplicate reactions. No eSS120 cells were seen above the detection limit (10 cells ml^{-1}) at 110 m.
D. Relative abundance of each ecotype to the total concentration of *Prochlorococcus* as determined by real-time PCR counts. Note that the relative abundance of eSS120 cells from 110 to 130 m is less than 1%, thus on this graph its contribution can only be seen as a small black line lying between the plots of eNATL2A and eMIT9313 cells near 130 m.

among the different low and high B/A subtypes. Differences in nutrient utilization (e.g. nitrite or organic phosphorus) that have been observed between the ecotypes (Moore *et al*., 2002; 2005) temperature optima, or trace metal availability or toxicity (Mann *et al*., 2002) may control growth rates. Alternatively, different mortality rates may also contribute to the observed differences in standing stock. For example, nanoflagellates have been shown use prey cell surface hydrophobicity to selectively feed on *Prochlorococcus*, and this property varies with depth in field populations of *Prochlorococcus* (Monger *et al*., 1999). Whole genome sequence analysis of the strains MED4 and MIT 9313 (Rocap *et al*., 2003) demonstrates they carry different sets of genes for lipopolysaccharide biosynthesis and therefore may have significant variability in cell surface proteins which could drive preferential grazing.

A second potential cause of differential mortality is dissimilar rates of phage lysis. The same water samples used for real-time PCR assessment of ecotype abundance in this study have also been used to determine titres of phage infecting particular *Prochlorococcus* and *Synechococcus* strains (Sullivan *et al*., 2003), so we wondered whether there was any relationship between the ecotype abundances and the abundance of phage that would infect their type strains. Significant phage titres were only found when strains MED4 and NATL2A were used as host cells. Assays employing MIT 9312, SS120, MIT 9211 or MIT 9313 as host cells yielded no phage. Particularly surprising is the absence of MIT 9312 infecting phage as this represents the dominant ecotype in this water column (Fig. 5C and D). Although intriguing, the ecological significance of the observations is difficult to assess because many phage are capable of infecting more than one ecotype and in some cases different host strains within the same ecotype yield significantly different titres (Sullivan *et al*., 2003). Unravelling this predator–prey system will require the development of specific assays for detecting infective phage–host pairs in the field, a topic largely unexplored in natural systems.

The design of specific primers is critical to the success of the *Prochlorococcus* real-time PCR method and obtaining more sequences, particularly for the high B/A ecotypes, would be very desirable in order to refine the existing primer sets. Furthermore sequencing of other loci (*cpeB*, *psbA*) from field samples suggests that there are groups of as yet uncharacterized *Prochlorococcus* genotypes that fall within both the low and high B/A lineages (Steglich *et al*., 2003; Zeidner *et al*., 2003). The additional non-specific peak in eSS120 assay amplicons we observed here in the Sargasso Sea may represent a closely related as yet uncharacterized *Prochlorococcus* ecotype. As there is excellent agreement between real-time PCR and flow cytometry numbers here, such additional ecotypes likely represent a small minority of the population in this water column, with the exception of 130 m.

Microdiversity and the role it plays in partitioning of the water column are emerging common themes in other marine microbial populations (Field *et al*., 1997; Beja *et al*., 2000). From the distributions of *Prochlorococcus* ecotypes documented here for the Sargasso Sea, it is clear that high and low B/A cells partition the water column, consistent with predictions based on laboratory studies (Moore and Chisholm, 1999). However, light physiology is not the only factor influencing cell distributions, as the genetically distinct lineages within the high and low B/A groups have very different abundances, confirming the ecological relevance of these genetic distinctions. The development of real-time PCR assays to enumerate the multiple lineages of *Prochlorococcus* may identify how particular environmental parameters act as evolutionary forces in shaping these populations. The comparison of genomes from three strains (belonging to the eMED4, eSS120 and eMIT9313 ecotypes) has revealed they possess different gene complements that convey distinctive, ecologically important physiologies (Dufresne *et al*., 2003; Rocap *et al*., 2003). Further genomic sequencing of strains from the remaining ecotypes may reveal genome-level adaptations that contribute to the patterns of distribution we are now able to observe.

Experimental procedures

Cyanobacterial cultures

For each of the six ecotypes, one or two physiologically characterized strains of *Prochlorococcus* were selected for creating sets of genomic DNA standards (Table 1). Strains representing multiple ecotypes of closely related marine *Synechococcus* were selected for use as negative controls: WH 8017, WH 8020, WH 8103, WH 8012, WH 7803, WH 8101 and MIT S9220. All cultures were grown at 20°C in a 14:10 light:dark cycle under cool-white fluorescent lamps at irradiances ranging from 10 to 40 mol Q m^{-2} s^{-1}. Cultures were grown in sterile (0.2 μm filtered, autoclaved) Sargasso Sea water supplemented with 800 μM NH_4Cl, 50 μM NaH_2PO_4 and trace metal mix described in Moore and Chisholm (1999).

Cultured genomic DNA standards

Sets of genomic DNA standards were made by purifying DNA from known numbers (ranging over at least three orders of magnitude) of cultured cells. Cultures were harvested in late exponential phase as determined by monitoring with a fluorometer (Turner model 10-AU, Turner Designs, Sunnyvale, CA). Because *Prochlorococcus* exhibits tight synchronicity of cell division (Vaulot *et al*., 1995; Vaulot and Marie, 1999), cultures were harvested in the morning before the start of DNA replication to ensure that all cells contained one

genome copy per cell. Harvested cells were serially diluted with sterile sea water and 3 ml of each dilution was filtered in triplicate onto 25 mm, 0.2 μm polycarbonate membranes (Osmonics, Minnetonka, MN) and chased with 3 ml of preservation solution (10 mM Tris-HCl pH 8.0, 100 mM EDTA, 0.5 M NaCl) (West and Scanlan, 1999) using 10 inches Hg of vacuum or less. Filters were placed in 2 ml bead-beating tubes (BioSpec Products, Bartlesville, OK) and frozen and stored in liquid nitrogen. For cell counts, 1 ml of each dilution was preserved with 0.125% glutaraldehyde and stored in liquid nitrogen. All cell counts were determined by flow cytometry using a modified Becton-Dickinson FACScan (Dusenberry and Frankel, 1994).

DNA extraction

Genomic DNA was extracted from filters using a modified lysozyme, Proteinase K extraction protocol. Filters were thawed and shredded with TE in a bead beater without beads for 30 s at maximum speed (50 000 r.p.m.) (BioSpec Products). After incubations with lysozyme and Proteinase K, DNA was purified via phenol chloroform extractions. A predetermined volume of aqueous phase was collected at each step to maintain consistency across extractions. Genomic DNA from the aqueous phase was then purified and concentrated using a Stratagene PCR Purification kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. DNA was eluted with 10 mM Tris and stored at –20°C. For all samples except the environmental samples (see below), equal volumes of DNA sample from the triplicate filters were pooled and subsequently used in real-time PCR assays.

Real-time PCR reactions

Real-time PCR assays were optimized for use on both the Roche Biochemicals LightCycler (Roche Diagnostics, Indianapolis, IN) and the Bio-Rad Icycler iQ real-time PCR detection system (Bio-Rad Life Science, Hercules, CA). For both machines, the reaction volume was 20 μl, and assays were performed with 2 μl of template DNA. On the ICycler, the DNA Master SYBR Green I Kit (Roche Diagnostics) was used according to manufacturer's instructions. Assays performed on the Icycler contained 0.5 U of HotStarTaq (Panvera LLC, Madison, WI), provided polymerase buffer, 10 nM fluorescein, 2 μl of a 25 000-fold dilution of SYBR Green I (Molecular Bioprobes, Eugene, OR), and various primer, dimethyl sulfoxide (DMSO), and $MgCl_2$ concentrations (Table 1). DMSO was used at 0.25% for the eMIT9313 assay and at 1% for all other ICycler assays. No-template negative controls (2 μl of 10 mM Tris-HCl pH 8.0) were analysed with each assay to monitor any primer-dimer formation or contamination. Polymerase chain reaction conditions and thermocycling programmes (Table 1) for each ecotype assay were optimized for high amplification efficiency of specific product (see below) and minimal amplification of primer-dimer products in no-template controls. All cycling programmes consisted of an initial step at 95°C for 1 min. Because the SYBR Green I method detects all PCR products formed, a melting curve analysis was performed at the end of each assay to determine whether the correct target sequence was amplified. In the initial optimization stage, products were visualized and sized using gel electrophoresis to confirm amplification of specific product. For quantification, standards and unknown samples were always run in triplicate. Non-target template used in specificity tests were run in either duplicate or triplicate. All data presented here came from assays performed on the ICycler except for the mixed culture experiment which was performed on the LightCycler.

Data analysis for real-time PCR assays

Quantification of ecotype cell numbers was performed using the data analysis software provided with the respective machines. For both machines, the background fluorescence readings of initial cycles before amplification were used to normalize all of the samples in each run. Cycle threshold (C_T) values were obtained by selecting a fluorescence threshold which crossed all amplification plots in the middle of their exponential ranges. Outliers of triplicate reactions were identified as samples having an elevated standard error among triplicate C_T values of ≥3%.

Theoretical amplification efficiency was determined from the slope of the standard curve – efficiency is $10^{-1/\text{slope}} - 1$, where 100% efficiency represents perfect doubling of products for each cycle (Rasmussen, 2001). For some assays, the amplification efficiency for each individual sample was calculated by determining the slope of the exponential phase of amplification, where efficiency is 10^{slope} using the software LinRegPCR (Ramakers *et al*., 2003) with fitting options selected to search for four to six points with the maximum regression coefficient.

Mixed culture sets

DNA samples containing mixtures of two ecotypes were made to investigate possible bias that might occur from amplification of one ecotype template in the presence of another, non-specific template. Mixed genomic DNA samples were created from cultures of MED4 and MIT 9313 following the same procedures as for the standard DNA sets. Dilutions contained a range of concentrations of MED4 cells and between 9000 and 11 000 MIT 9313 cells. Cells were filtered and DNA was extracted in the same manner as the standards.

Mock field samples

To verify that the real-time PCR assays were transferable and comparable for detection of cells collected from the ocean, mock field samples were created by spiking known amounts of MED4 cells into natural seawater samples. Water was collected off the main dock at the Woods Hole Oceanographic Institution (Falmouth, MA) and sequentially filtered with 20 μm and 2 μm filters (Osmonics) in order to better mimic the amount of biomass of the open ocean. MED4 cells were serially diluted and spiked into filtered dock water, and mixtures were collected onto triplicate filters for genomic DNA extraction. The 2 μm filtered dock water lacked *Prochlorococcus* but contained a natural *Synechococcus* population of approximately 300 cells ml^{-1}.

Field samples

Samples were taken from the western Sargasso Sea (34°24′N, 72°03′W) on 22 September 2001 (13:00 local time). Standard hydrographic data and water samples were collected using a SBE19 Seabird Electronics CTD (conductivity-temperature-depth) mounted on a 12-bottle, 12 l SBE Carousel. On deck photosynthetically available radiation (PAR) was measured as 1908 μmol Q m^{-2} s^{-1}. Only relative PAR values were available from the CTD data, so absolute PAR through the water column was estimated from the on-deck value by applying an exponential decay function using an attenuation coefficient of $k = 0.0459$ m^{-1} determined from the relative PAR data. For each depth 100 ml of water was filtered onto 25 mm, 0.2 μm polycarbonate membranes (Osmonics). Filters were chased with 3 ml of preservation solution, frozen in liquid nitrogen and later stored at −20°C. Samples of the 2 μm filtered water were preserved with 0.125% glutaraldehyde and total *Prochlorococcus* cell counts via flow cytometry. Ecotypes were quantified using real-time PCR on DNA extracted from a single filter. MED4, both AS9601 and MIT 9312, and MIT 9303 genomic standards were used with the ecotype eMED4, eMIT9312 and eMIT9313 assays respectively. For the filtering and elution volumes used here, the value of quantified cells per reaction is equivalent to twice the concentration (cells ml^{-1}).

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