Closely related Prochlorococcus genotypes show remarkably different depth distributions in two oceanic regions as revealed by in situ hybridization using 16S rRNA-targeted oligonucleotides

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An in situ hybridization method was applied to the identification of marine cyanobacteria assignable to the genus Prochlorococcus using horseradish-peroxidase-labelled 16S rRNA-targeted oligonucleotide probes in combination with tyramide signal amplification (TSA). With this method very bright signals were obtained, in contrast to hybridizations with oligonucleotides monolabelled with fluorochromes, which failed to give positive signals. Genotype-specific oligonucleotides for high light (HL)- and low light (LL)-adapted members of this genus were identified by 16S rRNA sequence analyses and their specificities confirmed in whole-cell hybridizations with cultured strains of Prochlorococcus marinus Chisholm et al., 1992, Prochlorococcus sp. and Synechococcus sp. In situ hybridization of these genotype-specific probes to field samples from stratified water bodies collected in the North Atlantic Ocean and the Red Sea allowed a rapid assessment of the abundance and spatial distribution of HL- and LL-adapted Prochlorococcus. In both oceanic regions the LL-adapted Prochlorococcus populations were localized in deeper water whereas the HL-adapted Prochlorococcus populations were not only distinct in each region but also exhibited strikingly different depth distributions, HLII being confined to shallow water in the North Atlantic, in contrast to HLII, which was present throughout the water column in the Red Sea.

Keywords: cyanobacteria, Red Sea, oligonucleotide probes, 16S rRNA, TSA

INTRODUCTION

Members of the genus Prochlorococcus Chisholm et al., 1992 (Chisholm et al., 1992) are the most abundant photosynthetic organisms in the world’s oceans due to their high cell densities and wide distribution within the

Abbreviations: DAPI, 4',6-diamidino-2-phenylindole; HL, high light; HRP, horseradish peroxidase; LL, low light; TETON, 4-(1,4,7,10-tetra-oxadecyl)-1-naphthol; TSA, tyramide signal amplification.

The GenBank accession numbers for the sequences reported in this paper are AF311217 (RCC278, EQPAC1), AF311218 (RCC277, NATL1MIT), AF311219 (RCC280, NATL28), AF311220 (RCC264, TAK9803-2), AF311291 (WH7803), AF311292 (WH8018) and AF311293 (WH8103).
ment with comparative sequence analyses of 16S rRNA genes which demonstrated that HL- and LL-adapted representatives of the genus form three distinct sub-clusters (HLI, HLII and LL) in phylogenetic trees (Moore et al., 1998; Urbach et al., 1998; West & Scanlan, 1999). However, due to a lack of sufficient axenic strains no DNA–DNA hybridization studies are available to correlate 16S rRNA gene sequence divergence with the boundaries of species. Hence, with the exception of the type strain, we shall refer to all isolates or genotypes of Prochlorococcus, initially optimized on cells of cultured isolates and then applied to the analysis of natural Prochlorococcus communities sampled from the North Atlantic and the Red Sea. Using a suite of 16S rRNA-targeted oligonucleotides we extend previous work on the distribution and niche occupancy of specific Prochlorococcus genotypes and demonstrate that closely related high-light-adapted genotypes can show remarkably different depth distributions in these two oceanic regions.

METHODS

Strains and cultivation. The non-axenic Prochlorococcus marinus strain SS120 and Prochlorococcus sp. strains EQPAC1, TAK9803-2, NATL1MIT and NATL2B were obtained from the culture collection of the Station Biologique, Roscoff, France (culture collection numbers RCC156, RCC278, RCC264, RCC277 and RCC280, respectively). Strain PCC 9511 is axenic and was obtained from the Pasteur Culture Collection of Cyanobacteria (PCC) (Institut Pasteur, Paris, France). All strains were cultured in PCR-S11 medium (Rippka et al., 2000) as described previously (Scanlan et al., 1996). The axenic marine Synechococcus sp. strains WH7803 and WH8103, and the non-axenic marine Synechococcus sp. strain WH8018, were cultured in ASW medium (Wyman et al., 1985).

DNA sequencing and analysis. The 16S rRNA gene sequences of the Prochlorococcus sp. strains EQPAC1, TAK9803-2, NATL1MIT and NATL2B were obtained after PCR amplification of two overlapping fragments using two separate pairs of primers: 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and OXY1313R (5'-CTTCAGTTAGGAGTTGACGAG-3'), and OXY107F (5'-GGAGGTTGATCMTTGCTCAG-3') and 1518R (5'-AAGGGTGTTGACGGTGGCG-3'). Bidirectional sequencing was performed directly on the PCR products using an ABI 373A automated sequencer (Applied Biosystems). For the Synechococcus sp. strains WH7803, WH8018 and WH8103, 16S rRNA genes were amplified by the PCR using the primers 27F and 1518R and inserted into the pCRII TA vector (Invitrogen). For the non-axenic Synechococcus sp. strain WH8018, clones containing cyanobacterial sequences were screened by PCR amplification with the primers OXY107F and OXY1313R. Clones were bidirectionally sequenced as described for the Prochlorococcus 16S rRNA genes. Sequence alignment and phylogenetic analysis was carried out using the ARB sequence database tools (http://www.mikro.biologie.tu-muenchen.de/pub/ARB/). Initial analysis was carried out using the maximum-likelihood method, the maximum-parsimony method and evolutionary distance (Jukes and Cantor model for DNA) with almost full-length sequences, using 1364 positions after omitting positions where there were gaps, incomplete data or sequence ambiguity. Shorter sequences were then added in to each of the three resulting trees by the maximum-parsimony method. A consensus tree (based on the maximum-likelihood tree) was drawn to represent the combined results of the three trees and multifurcations were created when the branching order was not supported by all three methods.
In situ identification of Prochlorococcus genotypes

Table 1. Group- and genotype-specific 16S rRNA oligonucleotide probes targeting marine cyanobacterial strains

<table>
<thead>
<tr>
<th>Probe</th>
<th>Specificity</th>
<th>Accession Numbers</th>
</tr>
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<tbody>
<tr>
<td>Probe 645HII</td>
<td>3′-CTA GCC CGG CCG GAA GAA CCA CCA-5′</td>
<td>EUB338 (5′-GCTGCCTCCGAGTGTGAT-3′) (Amann et al., 1990)</td>
</tr>
<tr>
<td>Probe 18HII</td>
<td>3′-TAC GGC CTA CTA TAC TAC-5′</td>
<td>EUB338 (5′-GCTGCCTCCGAGTGTGAT-3′) (Amann et al., 1990)</td>
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Probes. The ARB sequence database tools were used for probe design and probe matching. The probe sequences and their specificities are shown in Table 1. The bacterial probe EUB338 (5′-GCTGCCTCCGAGTGTGAT-3′) (Amann et al., 1990) and the oligonucleotide NON338 (5′-ACTCCTACGGGAGCGCC-3′) (Wallner et al., 1993), which served respectively as positive and negative controls, were monolabelled with the fluorochromes CY3 and BODIPY-FL (Interactiva).

For hybridizations carried out with HRP-labelled probes, probe CYA664 (5′-GGATTTGCTCCTGCGGC-3′) (Schönhuber et al., 1999) and the oligonucleotide NON338 were employed as positive and negative controls, respectively. Probe CYA664 targets all known marine Synechococcus sp. and Prochlorococcus sp., which fall into the marine pico-phytoplankton clade (Urbach et al., 1998), several genera of filamentous cyanobacteria (Schönhuber et al., 1999) and some chloroplasts. Oligonucleotides with a C6-aminolink (Interactiva) were labelled with HRP as described previously (Amann et al., 1992; Urdea et al., 1988).

Cell fixation of cultured isolates. Prochlorococcus and Synechococcus cells (approx. 2 ml of liquid cultures) were pelleted by centrifugation (10000 × g, 5 min) and resuspended in 200 µl PBS
(130 mM sodium chloride, 10 mM sodium phosphate buffer, pH 7–2). Fixation was performed either by mixing immediately with 1 vol. ethanol (96%, v/v) or by adding paraformaldehyde (4%, w/v, in PBS) to a final concentration of 3% (w/v) and incubation for 1 h on ice. After fixation with paraformaldehyde, the cells were washed once with PBS, resuspended in 1% (w/v) ethanol, and then mixed with 1 vol. ethanol (96%, v/v). The fixed samples were stored at −20 °C.

**Sampling and fixation of natural samples.** Water samples from the North Atlantic Ocean were collected from discrete depths (3, 20, 30, 40, 60, 80 and 100 m) on 14 July (36°25′ N, 19°24′ W; Station 25) and 15 July (36°63′ N, 19°24′ W; Station 26) 1996 with a rosette of 25-litre Go-Flo bottles during a Natural Environment Research Council, Plankton Reactivity in the Marine Environment (PRIME) cruise, aboard the RRS *Discovery*. Aliquots (1 ml) were immediately fixed by adding glutaraldehyde to a final concentration of 0.1% (v/v) prior to freezing in liquid nitrogen.

Samples from the Red Sea were also taken from discrete depths (10, 30, 50, 80 and 100 and 150 m) on 18 October 1999 at Station A (29°28′ N, 34°55′ E) (Lindell & Post, 1995) using a hydrographic cube with 30-litre Niskin bottles. Samples were fixed in two different ways: (i) 100 ml seawater (for depths 10, 30, 50 and 80 m) or 150 ml seawater (for depths 100 and 150 m) were vacuum-filtered onto polycarbonate membranes (GTTP 4700, 0.2 µm pore size, Millipore) using a filter tower with 2 ml PBS. Further treatment was as described for cultured strains. (ii) 50 ml seawater was fixed with glutaraldehyde (0.1%, v/v, final concentration) for 10 min at 4 °C. The paraformaldehyde-fixed filters and the 50 µl glutaraldehyde-fixed liquid samples were then stored at −20 °C.

Flow cytometric data for *Prochlorococcus*, *Synechococcus* and heterotrophic bacteria cell numbers were obtained as described previously (Jacquet et al., 1998).

**In situ hybridization**

**Preparation of cells of cultured *Prochlorococcus* and *Synechococcus* strains.** Fixed cells of cultured *Prochlorococcus* and *Synechococcus* strains were spotted into wells on microscope slides (Paul Marienfeld KG, Germany), dried at 45 °C for 5 min and then dehydrated in an ethanol series, 50%, 80% and 96% (v/v), for 3 min each. The slides were then air-dried and the cells permeabilized by adding 30 µl lysozyme solution [1 or 5 mg ml−1 of lysozyme (109000 U mg−1; Fluka)] dissolved in 100 mM Tris/HCl (pH 8.0), 50 mM EDTA for 15 min at 37 °C. After rinsing the slides with distilled water, the cells were dehydrated as described above.

**Preparation of natural samples.** For in situ hybridization of the North Atlantic samples, aliquots (400 µl) of the glutaraldehyde-fixed samples were filtered onto polycarbonate filters (GTTP 2500; 0.2 µm pore size; Millipore) using a filter tower with a diameter of 5 mm. The filters were then washed once with 1 ml PBS and divided into quarters. For the Red Sea glutaraldehyde-fixed samples, each sample (50 ml) was filtered onto a single polycarbonate filter, but using a filter tower with a diameter of 20 mm. After filtration, the filters were washed with 2 ml PBS. Further treatment was as described for cultured strains except that the first ethanol dehydration step was omitted.

**Hybridization with fluorescently labelled probes.** Hybridizations with fluorochrome-labelled probes were performed as described previously (Mantz et al., 1992). The fluorochrome-labelled probes EUB338 and NON338 were hybridized in buffer containing 20% (v/v) formamide.

**Hybridization with HRP-labelled probes.** The hybridization procedure was based on those described previously (Amann et al., 1992; Schönhuber et al., 1999). Each well on the microscope slide or filter portion was overlaid with 10 µl hybridization buffer [0.9 M NaCl, 20 mM Tris/HCl (pH 7.2), 0.01% (w/v) SDS and 2% blocking reagent (Boehringer Mannheim)] which contained 50 ng of an HRP-labelled probe and different concentrations of formamide depending on the probe used. The formamide concentrations used in hybridizations with the HRP-labelled probes were as follows: 40%PRO, 40%SYN and 1445NATL, 60%; 645HLI, 181HLI, 181LL and CYA664, 55%; 645HLII, 645LL and NON338, 50%. Probe NON338 was always used as a negative control in hybridizations with cultured *Prochlorococcus* and *Synechococcus* strains and with environmental samples. Slides were incubated in humid chambers at 35 °C for 2 h and then washed at the same temperature for 30 min in 50 ml wash buffer [20 mM Tris/HCl (pH 7.2), 5 mM EDTA, 0.01% (w/v), SDS] containing NaCl at molarities equivalent to the formamide concentrations present in the hybridization buffer [0.019 M, 0.013 M and no NaCl for formamide concentrations of 50%, 55% and 60%, (v/v), respectively]. Hybridization of HRP-labelled probes was detected initially by incubation with the non-fluorescent substrate TETON [4-[(1,4,7,10-tetraoxaacycl-1-naphthol; Boehringer Mannheim] as described previously (Schönhuber et al., 1999). For detection with the fluorescent substrate fluorescein–tyramide (Schönhuber et al., 1997, 1999), slides were first equilibrated in TNT buffer [0.1 M Tris/HCl (pH 7.5), 0.15 M NaCl, 0.05% (v/v), Tween 20] for 15 min at room temperature. After drying around each well/filter portion, 10 µl fluorescein–tyramide substrate solution [1:1 ratio of amplification diluent (a constituent of the TSA kit) and 40% (w/v), dextran sulphate with 1/50 vol. fluorescein–tyramide] was added to each well/filter and then incubated at room temperature in the dark for 10–60 min. The wells or filters were then carefully rinsed with distilled water and washed in TNT buffer at 55 °C for 10 min. After a final rinse in distilled water, the slides were dried and mounted in a 4:1 mixture respectively of Citifluor (Agar Scientific) and Vectashield (Vector Laboratories). Filters were stained with 4',6-diamidino-2-phenylindole (DAPI) prior to mounting (see ‘Counting on filters’ section). Slides were examined using either a Zeiss Axioptost2 microscope (Carl Zeiss) equipped for epifluorescence with a high-pressure mercury bulb (100 W) and specific filter sets 01 (excitation 365/12 nm, emission LP 397 nm) and 09 (excitation BP450–490, emission LP 520 nm) (Carl Zeiss) and Chroma HQ 41007 (Chroma Tech.), or an Olympus BX-60 microscope (Olympus Optical) equipped with a high-pressure mercury bulb (100 W) and specific filter sets U-MWU (excitation BP330–385 nm, BA420 nm) and U-MNB (excitation BP470–490 nm, BA515 nm) (Olympus Optical). Epifluorescence micrographs and phase-contrast images were captured onto Kodak film P1600X. The epifluorescence micrographs of cells hybridized with a positive or negative probe were taken at the same exposure in order to provide a fair comparison.

**Counting on filters.** To obtain total bacterial counts filters were counterstained with DAPI following hybridization. Filters were stained for 7 min with a solution (about 5 µl) of DAPI at a concentration of 1 µg ml−1 in water and then rinsed briefly by dipping in sterile distilled water, followed by 96% (v/v) ethanol. Filters were air-dried and placed onto glass slides in the mounting medium described above. For the North
Atlantic samples, 20 randomly chosen fields of view were counted using the DAPI filter set to enumerate total cells and the fluorescin filter set for the probe-specific cell counts. For each microscope field, probe-specific counts were expressed as a percentage of the total cells visualized by DAPI and the mean abundance and standard deviation calculated from these values. The total numbers of cells counted per filter ranged from 360 to 1280. For the Red Sea samples, for which greater volumes of sample were available, 10 randomly chosen fields of view were counted in the same way as for the North Atlantic samples and the total number of cells counted per filter was at least 1400. Filters for which no signals were observed after inspecting at least 20 random fields of view were deemed negative and were not counted.

RESULTS

Probe design

The new 16S rRNA sequences determined for four Prochlorococcus and three Synechococcus strains were compared with other sequences available for these genera and the results presented as a phylogenetic tree (Fig. 1). For the genus Prochlorococcus, strain EQPAC1 clustered with HLI genotypes, whereas TAK9803-2 was assignable to the HLII cluster and NATL1MIT and NATL2B proved to be LL genotypes. The three Synechococcus isolates (WH7803, WH8018 and WH8103) clustered as expected with two other marine members of this genus (Fig. 1). The strains targeted by each probe are indicated by the brackets to the right of the tree (Fig. 1). Genotype-specific probes for members of the genus Prochlorococcus were mainly targeted to two regions of the 16S rRNA corresponding to Escherichia coli positions 181–198 and 645–662, which allow discrimination between the HL- and the LL-adapted phylogenetic clusters within this genus (Urbach et al., 1998; West & Scanlan, 1999). The probes designated 645 (Table 1) are in part based on the sequence positions previously employed for specific identification (West & Scanlan, 1999), but are shifted 11 nucleotides towards the 3'-end of the 16S rRNA gene, a region potentially more accessible in whole-cell hybridizations (Fuchs et al., 1998). The genotype-specific probes targeted to the 645 region of the 16S rRNA are fully complementary to isolates assignable to phylogenetic subclusters (HLI, HLII or LL) (Fig. 1), with the exception of 645LL, which excludes some LL Prochlorococcus strains (Table 1). For the 181 target region, 181HLI targets specifically all HLI strains, whereas probe 181LL, although fully complementary to all LL-adapted strains, also recognizes a few other cyanobacterial sequences (Table 1). However, in the marine environments inhabited by representatives of the genus Prochlorococcus only a few other cyanobacterial species are known to coexist, notably Synechococcus spp. (Partensky et al., 1999) and Trichodesmium spp. (Capone et al., 1997). The latter species can be readily distinguished from the genus Prochlorococcus by their filamentous morphology. Synechococcus sp. cells are generally larger than those of Prochlorococcus and so in environments where Synechococcus is also abundant, it would be necessary to discriminate subjectively between Prochlorococcus and Synechococcus cells on the basis of size when hybridizing with 181LL. Consequently in this case, it is important to compare the counts obtained following hybridization with all of the genotype-specific probes in order to specifically enumerate the Pro-

![Fig. 1. Consensus tree showing the phylogenetic relationships amongst Prochlorococcus sp. and Synechococcus sp. The initial analysis was based on 1364 positions, and shorter sequences were subsequently added by the maximum-parsimony method. The scale bar represents 10% estimated sequence divergence. The 16S rRNA sequences obtained from strains in this study are shown in bold. Strains targeted by the probes are bracketed.](image-url)
Synechococcus LL genotype. The advantage of probe 181LL over probe 645LL is that it is fully complementary to all known LL-adapted isolates of the genus Prochlorococcus presently in culture. An additional probe, 1445NATL, was designed to target the same cluster of LL genotypes as recognized by 645LL. This region is advantageous since the target region is probably more accessible (Fuchs et al., 1998). Based on the comparison of the almost full-length 16S rRNA gene sequences available for Prochlorococcus and bacteria, probe 1445NATL is specific for the LL genotypes. However, sequence data for this specific region is relatively limited and thus this probe should be used in parallel with the other LL-targeted probes. Probe 405PRO is fully complementary to the target sites of the majority of Prochlorococcus sp. strains sequenced to date but has a single mismatch with respect to those of Prochlorococcus marinus SS120, Prochlorococcus sp. MIT9303, MIT9313 and MIT9211 (all LL genotypes), (Moore et al., 1998; West & Scanlan, 1999) and to all known marine Synechococcus sp. (see Table 1). Probe 405SYN is the Synechococcus counterpart to 405PRO and targets the majority of marine Synechococcus strains and a few freshwater Synechococcus strains, but also the Prochlorococcus strains Prochlorococcus marinus SS120, MIT9303, MIT9313 and MIT9211. Although 405SYN targets two strains designated as ‘Microcystis bolutica’ NIES43 and ‘Microcystis elabens’ NIES42, there is evidence to suggest that they should be reclassified as Synechococcus sp. since 16S rRNA comparisons have placed them within one of the Synechococcus clusters (Neilan et al., 1997) and they also lack gas vesicles (Otsuka et al., 1998). Probe 405PRO was used in parallel with probe CYA664 (Schönhuber et al., 1999) to estimate the total number of cells representative of the genus Prochlorococcus in the natural samples (Tables 2 and 3).

Whole-cell hybridization with fluorescently labelled oligonucleotides

Since all representatives of the genus Prochlorococcus exhibit dim autofluorescence compared to those of the marine Synechococcus genus, whole-cell hybridizations with cultured isolates of Prochlorococcus were initially carried out using oligonucleotides EUB338 and NON338 monolabelled with the fluorochromes CY3 and BODIPY-FL. Although these fluorochromes are amongst the brightest that are currently available (Glöckner et al., 1996), no appreciable difference was observed between cells hybridized with a positive and negative probe despite permeabilization with variable concentrations of lysozyme. The heterotrophic bacteria present in the non-axenic Prochlorococcus and Synechococcus cultures, however, did give good signals with the CY3-labelled probe EUB338 and hence acted as positive controls for the hybridization constituents and conditions (data not shown).

In order to improve the positive: negative probe signal ratio, HRP-labelled oligonucleotides were used in hybridizations with Prochlorococcus cells. With this approach the hybridization signal is amplified by the activity of the probe-conferred enzyme, leading to a deposit of multiple substrate molecules in the cell.

Whole-cell hybridization using HRP-labelled oligonucleotides

Probe CYA664, which is complementary to 16S rRNA gene sequences of all known Prochlorococcus and marine Synechococcus strains and to some chloroplasts (Schönhuber et al., 1999), was initially employed as an alternative to probe EUB338 to test the utility of HRP-labelled oligonucleotides for whole-cell hybridizations with cultured strains. As for EUB338, its target region in the 16S rRNA of *E. coli* is readily accessible (Fuchs et al., 1998). Hybridization was detected using either the non-fluorescent substrate TETON or the fluorescent substrate fluorescein–tyramide. With the former, revealing hybridization by production of a blue precipitate, no positive results were obtained. In contrast, a 10 min incubation with the fluorescein–tyramide substrate yielded extremely bright green single-cell hybridization signals with all strains examined (see for example Fig. 2e, left panel).

The conditions of optimal pre-treatment for successful hybridizations of whole cells in members of the genus Prochlorococcus and Synechococcus were determined by varying the fixation procedures and by incubating the fixed cells with different concentrations of lysozyme. The brightest signals were observed when cells were fixed with paraformaldehyde and treated with lysozyme (1 or 5 mg ml$^{-1}$), although the latter treatment was not strictly necessary with cultured isolates. However, in order to obtain the best signals, subsequent hybridizations on cultured isolates were all carried out on cells fixed with paraformaldehyde and treated with 5 mg lysozyme ml$^{-1}$ following fixation.

Evaluation of probe specificities

The optimal hybridization conditions were determined for all of the genotype-specific probes listed in Table 1 by hybridizing each of them to cells of all of the cultured Prochlorococcus and Synechococcus strains (see section above) at different formamide concentrations (30, 40, 50, 55 and 60%). Although the target 16S rRNA sequence among the strains examined often differed by a single mismatch only, specific hybridization for each probe was clearly observed at formamide concentrations of 50, 55 or 60% (see Methods for probe-specific formamide concentrations). Hybridizations were performed at the optimal formamide concentrations at least twice and the specificity of each probe was consistently observed. The clear difference between a positive and negative hybridization signal is shown in Fig. 2(a–e), where three Prochlorococcus strains representative of HLI, HLII and LL genotypes respectively and two Synechococcus strains were each hybridized with a positive (left panel) and negative probe (right panel).
The autofluorescence of the *Prochlorococcus* cells hybridized with the negative probe was very low (Fig. 2a–c, right panel) in contrast to *Synechococcus* cells, which showed significant yellow/orange autofluorescence (Fig. 2d and e, right panel). Despite the strong autofluorescence of the *Synechococcus* cells, the difference between a negative signal (yellow/orange) and a positive signal (green) could clearly be observed. Each epifluorescence photomicrograph has been presented beside the corresponding phase-contrast image to demonstrate the efficient labelling of all cells. The signal intensities observed for each probe were very similar, in spite of potentially more difficult accessibility of the 645 target site compared to that of the 181 region of the 16S rRNA (Fuchs et al., 1998). Figs 2(a) and 2(c) (right panels) demonstrate the specificity of the 181LL and 645HLI probes respectively, which both distinguish between *Prochlorococcus* sp. PCC9511 and NATL1MIT by only a single mismatch in the respective rRNA target sites (Table 1). Furthermore, probe 181LL did not hybridize to *Synechococcus* sp. strain WH8103 despite there also being only a single mismatch between the 16S rRNA sequence and the probe (Fig. 2e). The 16S rRNA sequences of some marine *Synechococcus* strains are targeted by probe 181LL (see Table 1) and give a positive hybridization signal as illustrated for strain WH8018 (Fig. 2d).

**Application of probes to identify *Prochlorococcus* sp. genotypes in the North Atlantic and the Red Sea**

The seawater samples available to us from the North Atlantic had originally been fixed with glutaraldehyde or paraformaldehyde, to determine the optimum fixation conditions for natural samples. In an attempt to improve the signals with the genotype-specific probes, subsequent substrate incubation times were extended to 1 h. Additionally, probe 181LL was selected for hybridizations in preference to probe 645LL, since it gave higher signals (see above). With these changes, the mean fraction of total *Prochlorococcus* sp. detected by CYA664 and the sum of genotype-specific fractions were in much better agreement. This is shown in Table 2, both for a sample from 80 m at Station 25 and for all samples of Station 26. The typical signals observed by hybridization with probes 645HLI and 181LL, following the improved protocol, are shown in Fig. 3(a) and Fig. 3(b). For the 80 m sample of Station 25, probe 181LL detected 70% of the mean fraction of cells identified by the CYA664 probe. The results for the samples from Station 26 showed an even better agreement between the enumerations made with the two genotype-specific probes, which together accounted for 87–95% of the mean fraction determined by hybridizations with probe CYA664. A comparison of the hybridization results and enumerations made by DAPI staining indicate that at both stations HLI-adapted *Prochlorococcus* sp. dominated the surface water, representing approximately 25% of the total bacterial counts, whereas at 80 m, the LL-adapted genotypes were dominant and the proportion of both genotypes together decreased to about 14% of total counts. Additionally, at a depth of 40 m at Station 25, both HLI- and LL-adapted *Prochlorococcus* sp. genotypes were detected.

Following the analysis of the North Atlantic samples, two further probes were designed: 1445NATL and 405SYN to allow enumeration of both genotypes. The seawater samples had been fixed in two different ways, with paraformaldehyde or glutaraldehyde, to determine the optimum fixation conditions for natural samples. Test hybridizations showed that consistently good hybridization signals were obtained with seawater columns is almost entirely composed of members of the genus *Prochlorococcus*, which are all recognized by probe 405PRO. For the 3 m sample, there was also good agreement between the mean fraction of cells hybridizing with probe 645HLI compared to probes CYA664 and 405PRO, suggesting that the genotypes detected were almost entirely assignable to the HLI cluster. However, for the deeper samples (20 and 40 m) the sum of cells detected by the 645HLI and 645LL genotype-specific probes only accounted for about 50% of the total mean fraction of *Prochlorococcus* sp. determined with probes CYA664 and 405PRO, one or both of the two genotypes evidently being underestimated. Indeed, the signal intensities observed in samples from deeper depths with both genotype-specific probes were lower than those obtained for the 3 m sample, which no doubt contributed to the lower counts. These initial hybridizations on filters were carried out using a fluorescein–tyramide incubation time of 30 min.
Fig. 2. Colour micrographs of Prochlorococcus and Synechococcus strains after hybridization with a positive probe (left panels) or a negative probe (right panels). Each panel shows a phase-contrast image with its corresponding epifluorescence image (filter set U-MNB) to the right. (a) Prochlorococcus sp. strain PCC 9511 (HLI genotype) hybridized with probe 645HLI (left panel) and 181LL (right panel). (b) Prochlorococcus sp. TAK9803-2 (HLII genotype) hybridized with probe 645HLII (left panel) and 181LL (right panel). (c) Prochlorococcus sp. NATL1MIT (LL genotype) hybridized with probe 181LL (left panel) and 645HLI (right panel). (d) Synechococcus sp. strain WH8018 hybridized with probe 181LL (left panel) and 645HLI (right panel). (e) Synechococcus sp. strain WH8103 hybridized with probe CYA664 (left panel) and 181LL (right panel). Bar, 10 µm.
suggesting that this population was dominated by Prochlorococcus genotypes identical or similar to NATL1MIT rather than other LL representatives (such as strains MIT9303, MIT9313, MIT9211, Prochlorococcus marinus sp. SS120) which branch more deeply in the phylogenetic tree (Fig. 1). Synechococcus cell

<table>
<thead>
<tr>
<th>Sample depth (m)</th>
<th>Fraction (%) of total bacteria (mean ± SD) detected with each probe*</th>
<th>645HLI</th>
<th>645LL</th>
<th>405PRO</th>
<th>CYA664</th>
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<tr>
<td>3</td>
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<td>ND</td>
<td>24 ± 9 (1048)</td>
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<tr>
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<td>27 ± 6 (359)</td>
<td>26 ± 7 (561)</td>
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<tr>
<td>40</td>
<td>8 ± 4 (940)</td>
<td>3 ± 3 (958)</td>
<td>25 ± 6 (1055)</td>
<td>26 ± 7 (983)</td>
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<tr>
<td>645HLI</td>
<td>181LL†</td>
<td>405PRO</td>
<td>CYA664</td>
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<td>10 ± 5 (756)</td>
<td>ND</td>
<td>14 ± 4 (983)</td>
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* For the Station 25 samples from 3, 20 and 40 m, the fluorescein–tyramide incubation was carried out for 30 min whereas the incubation time was extended to 1 h for the other samples. Numbers in parentheses indicate the total DAPI counts. NC, not counted. ND, not determined.
† For the 80 m sample of Station 25 and for all depths at Station 26, probe 181LL was used since it gave a higher signal than that of probe 645LL.

samples fixed with glutaraldehyde whereas this was not the case with paraformaldehyde-fixed samples. Thus, subsequent hybridizations and counting were performed with glutaraldehyde-fixed samples. No signals were observed for any of the three samples following hybridization with probe 645HLI. However, good signals were obtained with the other six probes (see above) and the fraction of cells (mean ± SD) hybridizing with each probe as a percentage of total bacteria is shown in Table 3. As for the North Atlantic samples, all of the group-specific probes (CYA664, 405PRO, 405SYN) gave very bright signals and the order of signal brightness for the genotype-specific probes was subjectively assessed as 181LL > 1445NATL > 645HLII. Typical signals obtained with probes 405PRO, 645HLII and 181LL are shown in Fig. 3(c–e). For probe 645HLII, the degree of labelling was more variable – some cells seemed only partly labelled, whereas for the other probes, the cells were more uniform in appearance due to even labelling. Despite the variability in labelling, observation of the hybridized filter revealed that HLII Prochlorococcus genotypes were very abundant at all depths and this was reflected in the counts obtained for each depth (Table 3). Interestingly, LL genotypes were also present but were confined to the deepest depth and were also much less abundant than the HLII genotypes. Both LL Prochlorococcus probes (181LL and 1445NATL) gave similar estimations of LL genotypes, suggesting that this population was dominated by Prochlorococcus genotypes identical or similar to NATL1MIT rather than other LL representatives (such as strains MIT9303, MIT9313, MIT9211, Prochlorococcus marinus sp. SS120) which branch more deeply in the phylogenetic tree (Fig. 1). Synechococcus cell

Table 3. Relative proportions of HLII and LL Prochlorococcus sp. genotypes and total Prochlorococcus and Synechococcus as a percentage of total bacterial counts for the depth profile in the Red Sea

<table>
<thead>
<tr>
<th>Sample depth (m)</th>
<th>Fraction (%) of total bacteria (mean ± SD) detected with each probe*</th>
<th>645HLII</th>
<th>181LL</th>
<th>1445NATL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>12 ± 2 (1863)</td>
<td>0 ± 0.4 (1637)</td>
<td>0 ± 0.1 (1972)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>8 ± 2 (2199)</td>
<td>0 ± 0.3 (2287)</td>
<td>0 ± 0.2 (2236)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>8 ± 2 (1780)</td>
<td>2 ± 1 (2034)</td>
<td>2 ± 1 (1742)</td>
<td></td>
</tr>
<tr>
<td>405PRO</td>
<td>405SYN</td>
<td>CYA664</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>15 ± 5 (1846)</td>
<td>0 ± 0.5 (1840)</td>
<td>17 ± 4 (1971)</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>18 ± 4 (2280)</td>
<td>0 ± 0.6 (2287)</td>
<td>18 ± 3 (2335)</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>14 ± 5 (1652)</td>
<td>0 ± 0.6 (1450)</td>
<td>17 ± 4 (1850)</td>
<td></td>
</tr>
</tbody>
</table>

* Numbers in parentheses indicate the total DAPI counts.
Fig. 3. Colour micrographs of natural samples from the North Atlantic Ocean and the Red Sea after hybridization. (a, b), samples from 3 m (a) and 80 m (b) from the North Atlantic Station 26, hybridized to 645HLI and 181LL respectively, and shown by DAPI staining using filter set 01 (left) and by epifluorescence using filter set 09 (right). (c) Sample from 50 m from the Red Sea Station A, hybridized to 405PRO and shown by DAPI staining using filter set U-MWU (left) and by epifluorescence using filter set U-MNB (right). (d, e), sample from 100 m from the Red Sea Station A, hybridized to probes 645HLII and 181LL respectively, and shown by DAPI staining using filter set U-MWU (left) and by epifluorescence using filter set U-MNB (right). Bar, 10 µm.
In situ identification of Prochlorococcus genotypes

Fig. 4. (a) Flow cytometric data of Prochlorococcus (●), Synechococcus (■) and heterotrophic bacteria (▲) cell numbers, and (b) the temperature profile for the water column on 18 October 1999 at Station A in the Red Sea.

Table 4. Relative proportions of Prochlorococcus, and Prochlorococcus + Synechococcus, expressed as a percentage of total bacteria as determined by in situ hybridization using probes 405PRO and CYA664 respectively or by flow cytometry for the depth profile in the Red Sea.

The abundance of Prochlorococcus, Synechococcus and total bacteria (Prochlorococcus + Synechococcus + heterotrophic bacteria) as determined by flow cytometry is also presented.

<table>
<thead>
<tr>
<th>Depth (m)</th>
<th>Flow cytometry data (cells ml⁻¹)</th>
<th>% Prochlorococcus (as a proportion of total bacteria)</th>
<th>% Prochlorococcus + Synechococcus (as a proportion of total bacteria)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prochlorococcus</td>
<td>Synechococcus</td>
<td>Total bacteria</td>
</tr>
<tr>
<td>10</td>
<td>144835</td>
<td>7124</td>
<td>799771</td>
</tr>
<tr>
<td>50</td>
<td>144387</td>
<td>8787</td>
<td>771098</td>
</tr>
<tr>
<td>100</td>
<td>78988</td>
<td>2945</td>
<td>592609</td>
</tr>
</tbody>
</table>

*ISH, in situ hybridization.

The mean fraction of Prochlorococcus, or Prochlorococcus plus Synechococcus, as a percentage of total bacteria as assessed by in situ hybridization was then compared to that estimated by flow cytometry (Table 4). The two independent methods gave similar results although the estimates by in situ hybridization were often slightly lower than those obtained by flow cytometry, except for the 100 m sample, where the converse was observed.

DISCUSSION

In view of their ecological importance (Partensky et al., 1999), the development of a whole-cell hybridization technique was attempted for members of the genus Prochlorococcus. However, the signals conferred by monolabelled fluorescent probes were not sufficient to be visualized in these bacteria. A poor positive:negative probe signal ratio was also observed when flow cytometry was used to analyse Prochlorococcus cells.
hybridized with fluorescently labelled DNA oligonucleotides (Worden et al., 2000).

Although initially it seemed possible that the permeability of the cells was inadequate, the success of the HRP-labelled probes combined with the TSA detection system showed this not to be the case. In contrast, the alternative detection system offered by the non-fluorescent, precipitable substrate TETON, although successful in hybridizations with some cyanobacteria (Schönhuber et al., 1999), was not suitable for use in Prochlorococcus. It is possible that, as a consequence of a low rRNA content together with the small cells (less than 0.8 µm in diameter) typical for members of this genus, the number of probe-conferred HRP molecules is too low to allow the formation of sufficient oxidized substrate for the production of a visible precipitate (Schönhuber et al., 1999). For a given tyramide incubation time, the fluorescent signals from cells of cultured Prochlorococcus marinus and Prochlorococcus sp. isolates were significantly brighter than those obtained in situ, particularly in deep-water samples. An explanation for the lower hybridization signals observed in cells of Prochlorococcus sp. from natural samples may be a low rRNA content per cell due to slow growth. Indeed, cell cycle analysis of natural Prochlorococcus sp. populations in the equatorial Pacific Ocean has revealed that deep populations grow more slowly than surface populations (Vaulot et al., 1995). However, since surface populations may exhibit growth rates equivalent to those observed in cultured isolates (about one doubling per day) (Moore & Chisholm, 1999), there must be additional factors which may contribute to the reduced hybridization signals observed in natural samples. It is possible that there are structural or physiological differences between cultured isolates and natural populations that, in the latter, decrease the permeability of the cells or perhaps reduce the accessibility of the 16S rRNA target sites.

For the initial enumerations made at Station 25, the mean fraction of cells detected by the genotype-specific probes 645HL and 645LL was too low to account for the total number of Prochlorococcus sp. determined either with CYA664 or 405PRO. However, the replacement of probe 645LL by probe 181LL, and the extension of the fluorescein–tyramide incubation time, enhanced the signals so that the mean fraction of cells hybridizing with the genotype-specific probes was raised to the level determined with the CYA664 probe. Thus it is most likely that in the earlier enumerations a proportion of the hybridizing cells gave signals that fell below the detection threshold, rather than the discrepancies in the analyses being due to the existence of genotypes other than those that can be recognized by the three genotype-specific probes employed. The dominance of Prochlorococcus sp., the observed transition of HLI- to LL-adapted genotypes from surface to deep waters respectively, and the absence of HLII genotypes from these particular sampling stations in the North Atlantic Ocean, are entirely consistent with previous molecular data (West & Scanlan, 1999). The degree of variation of the data as observed by the mean ± sd values was relatively high due to the low numbers of cells and their uneven distribution on the filters. Nevertheless, there was good agreement between the mean fractions of total Prochlorococcus as a percentage of total bacteria as assessed by probes 405PRO and CYA664 compared to the sum of the two Prochlorococcus genotypes.

In situ hybridization of Prochlorococcus cells sampled from the Red Sea revealed a remarkably different community structure, both in the genotypes present and in their vertical distribution. Despite the stratification of the water column (Fig. 4b), HLII genotypes were dominant at all depths and even outnumbered LL genotypes by around 4 times at 100 m, the only depth at which LL genotypes were observed in significant numbers. The dominance of HLII genotypes in this oceanic region is further supported by the retrieval of environmental sequences of the ntcA gene (encoding a cyanobacterial-specific transcriptional activator involved in global nitrogen control) since they were found to cluster together with the ntcA genes of HLII cultured Prochlorococcus strains (Lindell, 2000). The ability of HLII genotypes to colonize the euphotic zone from the surface to a depth below the 1% light level is remarkable and differs from the Prochlorococcus community structure in the eastern North Atlantic Ocean, where the HL- and LL-adapted populations were clearly partitioned (West & Scanlan, 1999) (see Table 2). The data for the Red Sea are consistent with flow cytometric observations on samples from the same water column in November 1998 that contained dimly fluorescent (HL-adapted) Prochlorococcus cells from surface water down to 120 m. This depth distribution partly overlapped with that of strongly fluorescent (LL-adapted) cells that were observed from 80 m down to 175 m (A. F. Post, unpublished results). Photophysiological studies of HL-adapted Prochlorococcus strains have not indicated that the HLII genotype strains have greater photoacclimative abilities than HLI genotype strains (Moore & Chisholm, 1999). However, these studies also revealed that at light levels corresponding to a depth of 118 m, some HL- and LL-adapted strains had similar growth rates, which may help explain the coexistence of HLII and LL Prochlorococcus at 100 m in the Red Sea. It is possible that in deeper layers the HLII cells are present but are growing slowly. Therefore it would be very interesting to carry out an in situ hybridization study in parallel with flow cytometric cell cycle analysis (see Vaulot et al., 1995) in order to estimate the growth rates of cells at different depths.

Despite the use of a tyramide incubation time of 1 h, hybridization of the genotype-specific probes to the Red Sea samples gave a lower estimation of Prochlorococcus cell numbers compared to the group-specific probes. This may be explained by an underestimation of HLII genotypes due to the lower signal conferred by this probe, or could also be due to the presence of genotypes not targeted by our probes. Since the HLII probe targets a region of the 16S rRNA that may be less accessible to oligonucleotides (Fuchs et al., 1998), it is worth testing
the use of unlabelled helper oligonucleotides which are designed to bind to regions adjacent to that of the target probe and were shown to improve the hybridization signals in E. coli (Fuchs et al., 2000). Future retrieval and sequencing of further Prochlorococcus environmental 16S rDNA clones will be required in order to verify that all of the genotypes present are indeed targeted by the probes described here. The statistical variation observed within the Red Sea data was less than that of the North Atlantic samples due to a higher density of cells on the filters, which allowed a greater number of cells to be counted. Despite this variation, the fact that the mean fractions of Prochlorococcus assessed by in situ hybridization agreed so well with Prochlorococcus abundance determined by flow cytometry (Table 4) lends strong credence to the use of this in situ hybridization protocol in quantitative analyses.

The original examination of 16S rRNA sequences obtained for a number of Prochlorococcus strains isolated from surface and deep waters from different oceanic regions demonstrated a clear correlation with their depth of isolation (Scanlan et al., 1996) whilst closely related strains were found in different geographical locations such as the Atlantic and Pacific Oceans (Urbach et al., 1998). These findings are extended in this study, which showed that LL genotypes comparable to the NATLIMIT/NATL2B isolates seem to be ubiquitous, having been detected only at depth, in both the Red Sea and the North Atlantic. However, for the HL genotypes the picture is more complicated. The results presented here, and other preliminary data which show a dominance of HLI in the Mediterranean Sea and HLII in the Sargasso (N. West & D. Scanlan, unpublished data), indicate that the relative abundance of the two types of HL populations and their vertical distribution may differ significantly with geographical location. This may reflect other more subtle features of the water column itself such as salinity, temperature, prevailing oceanic circulation or the source of the Prochlorococcus ‘seed’ population.

Our results demonstrate that an in situ hybridization method can be used to rapidly assess the community structure of Prochlorococcus sp. in the natural environment. Moreover, using this technique, estimation of Prochlorococcus abundance, as a proportion of total bacteria, can be directly correlated with flow cytometric data for Prochlorococcus and bacterial cell numbers, and the two independent methods have been shown to compare favourably. The fixation of samples is simple and the analysis does not require time-consuming DNA extraction procedures and is free of the potential biases of the PCR. The counting of cells using epifluorescence microscopy is the most labour-intensive part of the method and this step may be improved in the future by using a laser scanning cytometer for the counting of cells on a filter (Parthuisot et al., 2000) or by optimizing the hybridization method in liquid for analysis by flow cytometry. Both of these methods would allow the rapid counting of large numbers of cells, either at the genus level or of particular genotypes, thus providing very precise estimates of population dynamics. Moreover, by combining in situ hybridization analyses using 16S rRNA-targeted oligonucleotides with single-cell immunofluorescence assays for protein markers of nutrient stress (Scanlan et al., 1997), it should be possible to determine the specific physiological state of individual Prochlorococcus sp. genotypes. Such a multiple-probing approach will be essential to elucidate the nutritional adaptation and functional importance of the various ecotypes of Prochlorococcus sp. within natural communities.

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