

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, HLI 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, NATL2B), AF311220 (RCC264, TAK9803-2), AF311291 (WH7803), AF311292 (WH8018) and AF311293 (WH8103).

40° N–40° S latitudinal band (Partensky *et al.*, 1999). The remarkable range of depths colonized by these oxygenic photosynthetic prokaryotes is achieved by the existence of multiple ecotypes which are adapted for growth in high-light (HL) or low-light (LL) environments (Ferris & Palenik, 1998; Moore *et al.*, 1998; Urbach & Chisholm, 1998; West & Scanlan, 1999). Although only a single species, *Prochlorococcus marinus*, the nomenclatural type, has been described on the basis of strain SS120 (Chisholm *et al.*, 1992), the different light-dependent physiologies exhibited by this and numerous other isolates (Moore & Chisholm, 1999; Moore *et al.*, 1998) has led to the description of a new subspecies (Rippka *et al.*, 2000). This is in agree-

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 this genus as *Prochlorococcus* sp. Despite the relatively high similarity (97.3 %) of the most distantly related HL and LL *Prochlorococcus* sp. genotypes (Moore *et al.*, 1998), there are variable regions of the 16S rRNA which can be targeted by oligonucleotides in order to distinguish them. In a recent study (West & Scanlan, 1999), these genotype-specific probes were hybridized to PCR-amplified *Prochlorococcus* sp. 16S rDNA from natural samples. This confirmed the genetic distinctions between HL and LL ecotypes and revealed that nutrient concentrations and hydrological conditions may be important for their partitioning in surface and deep water, respectively.

Whilst the construction of 16S rRNA gene clone libraries, dot-blot hybridization and denaturing gradient gel electrophoresis (DGGE) are valuable techniques to analyse natural communities and to provide a picture of microbial diversity, the processes of DNA extraction and PCR amplification may introduce biases (von Wintzingerode *et al.*, 1997). Furthermore, the abundance of a particular genotype can only be expressed as a relative value. Whole-cell hybridization using 16S rRNA-targeted oligonucleotides avoids these potential biases, allows the actual abundance of the genotype to be evaluated and also permits visualization of the spatial relationships of the cells *in situ* (Amann *et al.*, 1997, 1995). For the majority of non-photosynthetic organisms, oligonucleotides monolabelled with a fluorescent dye are employed. However, for the hybridization of cyanobacteria, the signal from such probes has often been found to be insufficient to be visualized against the strong background of auto-fluorescence when viewed by epifluorescence microscopy (Schönhuber *et al.*, 1999). Fluorescently labelled probes have been used successfully for hybridization of *Synechococcus* and *Prochlorococcus* cells (Binder & Liu, 1998; Worden *et al.*, 2000) when used in combination with flow cytometry and using a general bacterial probe EUB338 which targets one of the more accessible regions of the 16S rRNA (Fuchs *et al.*, 1998). However, for hybridization of *Prochlorococcus* cells, it was necessary to use peptide nucleic acid (PNA) probes since DNA probes gave poor results (Worden *et al.*, 2000). Another method for improving the signal of hybridized cells is to use horseradish peroxidase (HRP)-labelled probes combined with a fluorescent detection system based on tyramide signal amplification (TSA) (Schönhuber *et al.*, 1997, 1999). Alternatively, hybridization with biotin-labelled probes and reaction with a streptavidin–HRP conjugate prior to detection

using TSA, a more indirect hybridization protocol successfully employed for bacteria (Lebaron *et al.*, 1997), should also overcome problems caused by auto-fluorescence. Here we report the use of a direct *in situ* hybridization method for members of the genus *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'-AGAGTTTGTATCMTGGCTCAG-3') and OXY1313R (5'-CTTACGTTAGGCGAGTTGCAGC-3'), and OXY107F (5'-GGACGGGTGAGTAACGCGTGR-3') and 1518R (5'-AAGGAGGTGATCCANCCRCA-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.

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

Specificities of the probes used in this study are illustrated by an alignment of the probe and target sequences against *Prochlorococcus marinus*, *Prochlorococcus* sp., *Synechococcus* sp. and other cyanobacterial 16S rRNA sequences. Probe positions correspond to *E. coli* numbering (Brosius *et al.*, 1981). Dots in the alignments indicate bases that are identical to those in the target sequence and dashes indicate a gap. Strains that are listed on the same line possess identical target sequences. GenBank accession numbers for 16S rRNA sequences from *Synechococcus* sp. Biwa-B0, *Synechococcus* sp. Biwa-G0, *Synechococcus* sp. Biwa-P0, *Oscillatoria neglecta* M-82, *Prochlorococcus* sp. MIT9211, '*Microcystis holsatica*' NIES43 and '*Microcystis elabens*' NIES42 are D50614, D50615, D50616, AB003168, AF115270, U40335 and U40336 respectively. Some of the sequences of *Prochlorococcus* sp. and marine *Synechococcus* sp., and the environmental clone sequences included in this study, have been reported elsewhere (Moore *et al.*, 1998; Rippka *et al.*, 2000; Urbach & Chisholm, 1998; West & Scanlan, 1999).

Probe 645HLI	3'-CTA GCC GAA CTC ATA CCA-5'	Probe 181HLI	3'-TAC GGC TAT CCA CTT TAC-5'
Target (<i>E. coli</i> numbering 645-662)	5'-GAU CGG CUU GAG UAU GGU-3'	Target (<i>E. coli</i> numbering 181-198)	5'-AUG CCG AUA GGU GAA AUG-3'
<i>Prochlorococcus</i> sp. PCC 9511/EQPAC1 (HLI)	...	<i>Prochlorococcus</i> sp. PCC 9511/EQPAC1	...
ENATL1 environmental clone (HLI)	...	ENATL1 environmental clone	...
ENATL4 environmental clone (LL)	...	ENATL4 environmental clone	...
<i>Prochlorococcus</i> sp. NATL1MIT/NATL2B (LL)	...	<i>Prochlorococcus</i> sp. NATL1MIT/NATL2B	...
<i>Prochlorococcus</i> sp. TAK9803-2 (HLII)	...	/MIT9303/MIT9313	...
<i>Prochlorococcus</i> sp. MIT9303/MIT9313 (LL)	...	<i>Prochlorococcus</i> sp. MIT9211	...
<i>Prochlorococcus</i> sp. MIT9211 (LL)	...	<i>Prochlorococcus marinus</i> SS120	...
<i>Prochlorococcus marinus</i> SS120 (LL)	...	<i>Prochlorococcus</i> sp. TAK9803-2	...
<i>Synechococcus</i> sp. WH7803/WH8103	...	<i>Synechococcus</i> sp. WH7805/WH7803/WH8018	...
<i>Synechococcus</i> sp. WH8018	...	<i>Synechococcus</i> sp. WH8103	...
<i>Synechococcus</i> sp. WH7805	...	<i>Synechococcus</i> sp. WH8101	...
<i>Synechococcus</i> sp. WH8101	...	Probe 181LL	3'-TAC GGC TCT CCA CTT TAC-5'
Probe 645HLII	3'-CTT TCC GAA CTC ATA CCA-5'	Target (<i>E. coli</i> numbering 181-198)	5'-AUG CCG AGA GGU GAA AUG-3'
Target (<i>E. coli</i> numbering 645-662)	5'-GAA AGG CUU GAG UAU GGU-3'	<i>Prochlorococcus</i> sp. NATL1MIT/NATL2B	...
<i>Prochlorococcus</i> sp. TAK9803-2	...	<i>Prochlorococcus</i> sp. MIT9303/MIT9313	...
<i>Prochlorococcus</i> sp. PCC 9511/EQPAC1	...	<i>Prochlorococcus marinus</i> SS120	...
ENATL1 environmental clone	...	ENATL4 environmental clone	...
ENATL4 environmental clone	...	<i>Synechococcus</i> sp. PCC 6307	...
<i>Prochlorococcus</i> sp. NATL1MIT/NATL2B	...	<i>Synechococcus</i> sp. WH7805/WH7803/WH8018	...
<i>Prochlorococcus</i> sp. MIT9303/MIT9313	...	<i>Synechococcus</i> sp. Biwa-B0/Biwa-G0/Biwa-P0	...
<i>Prochlorococcus</i> sp. MIT9211	...	<i>Oscillatoria neglecta</i> M-82	...
<i>Prochlorococcus marinus</i> SS120	...	<i>Synechococcus</i> sp. WH8103	...
<i>Synechococcus</i> sp. WH7803/WH8103	...	<i>Prochlorococcus</i> sp. MIT9211	...
<i>Synechococcus</i> sp. WH8018	...	<i>Prochlorococcus</i> sp. PCC 9511/EQPAC1	...
<i>Synechococcus</i> sp. WH7805	...	ENATL1 environmental clone	...
<i>Synechococcus</i> sp. WH8101	...	<i>Prochlorococcus</i> sp. TAK9803-2	...
Probe 645LL	3'-CTA ACC GAA CTC ATA CCA-5'	<i>Synechococcus</i> sp. WH8101	...
Target (<i>E. coli</i> numbering 645-662)	5'-GAU UGG CUU GAG UAU GGU-3'	Probe 405PRO	3'-ACT CCC TGC TTC CGG AGA-5'
<i>Prochlorococcus</i> sp. NATL1MIT/NATL2B	...	Target (<i>E. coli</i> numbering 405-422)	5'-UGA GGG ACG AAG GCC UCU-3'
ENATL4 environmental clone	...	<i>Prochlorococcus</i> sp. PCC 9511/EQPAC1/TAK9803-2/	...
<i>Prochlorococcus</i> sp. MIT9303/MIT9313	...	NATL1MIT/NATL2B	...
<i>Prochlorococcus</i> sp. MIT9211	...	ENATL1/ENATL4 environmental clones	...
<i>Prochlorococcus</i> sp. TAK9803-2	...	<i>Prochlorococcus marinus</i> SS120	...
<i>Prochlorococcus</i> sp. PCC 9511/EQPAC1	...	<i>Prochlorococcus</i> sp. MIT9303/MIT9313/MIT9211	...
ENATL1 environmental clone	...	<i>Synechococcus</i> sp.	...
<i>Prochlorococcus marinus</i> SS120	...	WH7803/WH7805/WH8103/WH8018/WH8101	...
<i>Synechococcus</i> sp. WH7803/WH8103	...	Probe 405SYN	3'-ACT CCC TAC TTC CGG AGA-5'
<i>Synechococcus</i> sp. WH8018	...	Target (<i>E. coli</i> numbering 405-422)	5'-UGA GGG AUG AAG GCC UCU-3'
<i>Synechococcus</i> sp. WH7805	...	<i>Prochlorococcus marinus</i> SS120	...
<i>Synechococcus</i> sp. WH8101	...	<i>Prochlorococcus</i> sp. MIT9303/MIT9313/MIT9211	...
Probe 1445NATL	3'-ATT GGG AAC ACC TCT TCC-5'	<i>Synechococcus</i> sp.	...
Target (<i>E. coli</i> numbering 1445-1462)	5'-UAA CCC UUG UGG AGA AGG-3'	WH7803/WH7805/WH8103/WH8018/WH8101	...
<i>Prochlorococcus</i> sp. NATL1MIT/NATL2B	...	<i>Synechococcus</i> sp. Biwa-B0/Biwa-G0/Biwa-P0	...
ENATL4 environmental clone	No sequence data available	<i>Synechococcus</i> sp. PCC 6307	...
<i>Prochlorococcus</i> sp. PCC 9511/EQPAC1/TAK9803-2	...	' <i>Microcystis holsatica</i> ' NIES43/	...
<i>Prochlorococcus</i> sp. MIT9303/MIT9313/MIT9211	...	' <i>Microcystis elabens</i> ' NIES42*	...
<i>Synechococcus</i> sp. WH8018/WH8101	...	<i>Prochlorococcus</i> sp. PCC 9511/EQPAC1/TAK9803-2/	...
<i>Synechococcus</i> sp. WH7803/8103	...	NATL1MIT/NATL2B	...
<i>Prochlorococcus marinus</i> SS120/	No sequence data available	ENATL1/ENATL4 environmental clones	...
<i>Synechococcus</i> sp. WH7805	...		

* See Results for contradictory identification of these strains.

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'-GCTGCCCTCCCGTAGGAGT-3') (Amann *et al.*, 1990) and the oligonucleotide NON338 (5'-ACTCCTACGGGA-GGCAGC-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'-GGAATTCCTCTGCCCC-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 picoplankton 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 the same buffer 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 (36.82° 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, 100 and 150 m) on 18 October 1999 at Station A (29.28° N, 34.55° E) (Lindell & Post, 1995) using a hydrographic cable 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 a diameter of 40 mm and the cells were fixed by overlaying the membranes for 1 h with 5 ml paraformaldehyde (1%, w/v). The fixative was then drawn through the membrane and washed with 5 ml PBS. (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 ml 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] and incubating for 30 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 de-

scribed previously (Manz *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: 405PRO, 405SYN 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-tetraoxadecyl)-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 Axiophot2 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 $\mu\text{g ml}^{-1}$ 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 fluorescein 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-*

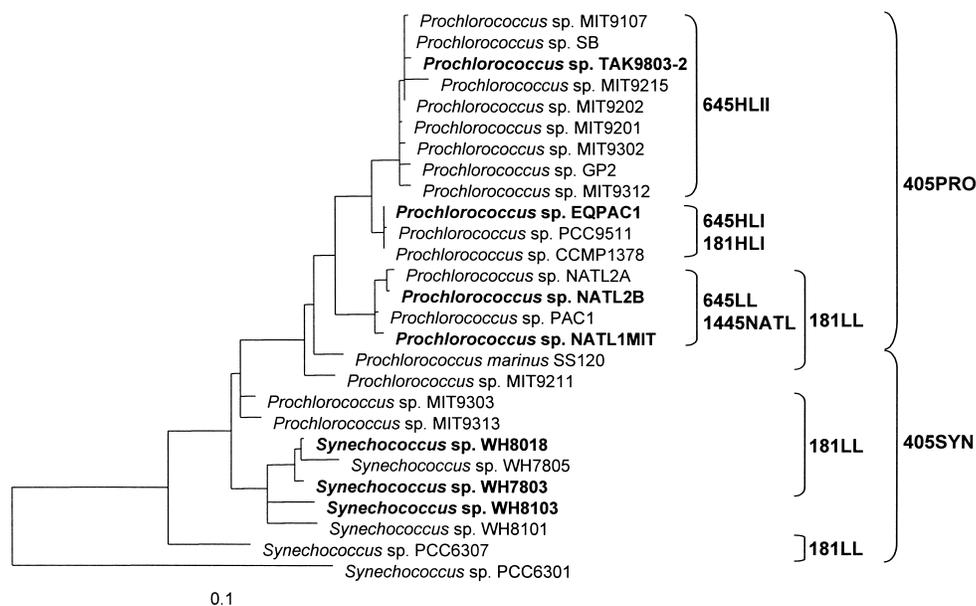


Fig. 1. Consensus tree showing the phylogenetic relationships amongst *Prochlorococcus* sp. and *Synechococcus* sp. The initial analysis was based on 1364 positions, and shorter sequences were subsequently added by the maximum-parsimony method. The scale bar represents 10% estimated sequence divergence. The 16S rRNA sequences obtained from strains in this study are shown in bold. Strains targeted by the probes are bracketed.

chlorococcus 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 holsatica*' 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 obtained 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 for flow cytometric analysis. Nevertheless, preliminary hybridizations with selected samples from different depths of Station 26 in the North Atlantic yielded very bright signals when using probes CYA664 and 405PRO. For the genotype-specific probes the signals were lower and the order of brightness was subjectively assessed as: 181LL > 645HLI > 645LL. Since hybridization of probe 181HLI to the filters resulted in a high background that masked positive signals, this probe was not used further. No positive signals were observed by hybridization of probe 645HLII to all of the samples from Stations 25 and 26. Some larger cells were observed after hybridization to probe CYA664 and these cells were believed to be representatives of *Synechococcus*. The number of these cells was very low, consistent with flow cytometric data for the days sampled at either side of Stations 25 and 26, which indicated that *Prochlorococcus* sp. accounted for approximately 80–97 % of the total picophytoplankton (West & Scanlan, 1999).

The first results of enumerating hybridizing cells as the fraction (mean \pm SD) of total bacterial counts by DAPI staining from different depths (3, 20 and 40 m) of Station 25 in the North Atlantic Ocean are shown in Table 2. The mean fraction of cells collected from 20 and 40 m and hybridizing with the two general probes CYA664 and 405PRO were very similar. This suggests, in agreement with previous data (West & Scanlan, 1999), that the cyanobacterial community in these water

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. 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 to allow estimation of the *Synechococcus* sp. cell abundance. 1445NATL and 405SYN were then used together with 645HLI, 645HLII, 181LL, 405PRO and CYA664 for hybridization with the Red Sea samples. 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

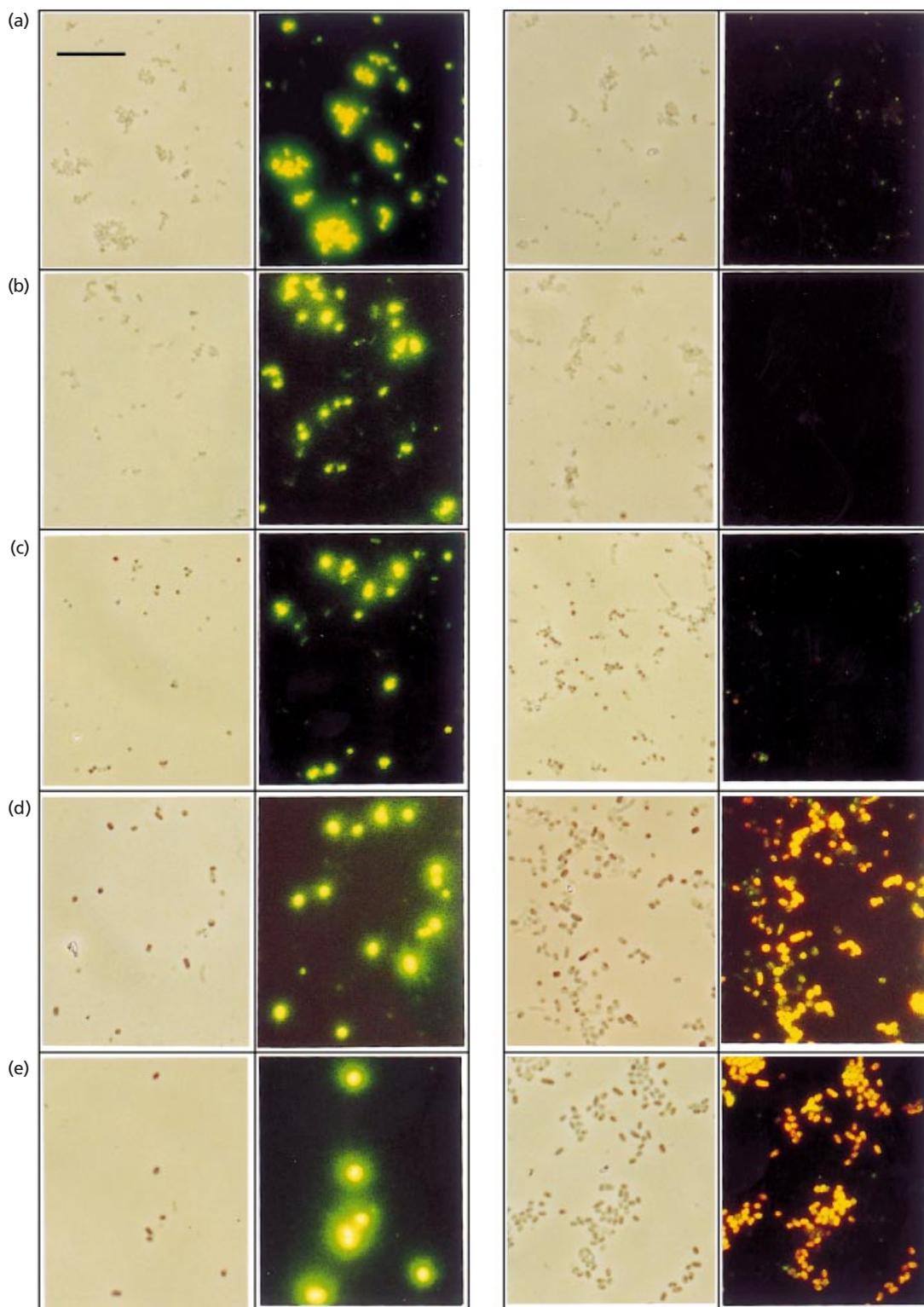


Fig. 2. Colour micrographs of *Prochlorococcus* and *Synechococcus* strains after hybridization with a positive probe (left panels) or a negative probe (right panels). Each panel shows a phase-contrast image with its corresponding epifluorescence image (filter set U-MNB) to the right. (a) *Prochlorococcus* 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 .

Table 2. Relative proportions of HLI and LL *Prochlorococcus* sp. genotypes and total *Prochlorococcus*, as a percentage of total bacteria for two depth profiles in the North Atlantic Ocean

PRIME Station 25, 14 July 1996, 36.82° N, 19.24° W				
Sample depth (m)	Fraction (%) of total bacteria (mean ± SD) detected with each probe*			
	645HLI	645LL	405PRO	CYA664
3	22 ± 9 (950)	NC	ND	24 ± 9 (1048)
20	13 ± 9 (470)	NC	27 ± 6 (359)	26 ± 7 (561)
40	8 ± 4 (940)	3 ± 3 (958)	25 ± 6 (1055)	26 ± 7 (983)
PRIME Station 26, 15 July 1996 36.63° N, 19.24° W				
Sample depth (m)	Fraction (%) of total bacteria (mean ± SD) detected with each probe*			
	645HLI	181LL†	405PRO	CYA664
80	NC	10 ± 5 (756)	ND	14 ± 4 (983)
3	21 ± 5 (1283)	0.5 ± 0.8 (647)	ND	23 ± 10 (444)
40	18 ± 7 (864)	0.7 ± 1.2 (551)	ND	22 ± 11 (383)
80	NC	13 ± 6 (825)	ND	13 ± 5 (933)

* 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

Station A, 18 October 1999, 29.28° N, 34.55° E			
Sample depth (m)	Fraction (%) of total bacteria (mean ± SD) detected with each probe*		
	645HLII	181LL	1445NATL
10	12 ± 3 (1863)	0.2 ± 0.4 (1637)	0 ± 0.1 (1972)
50	8 ± 3 (2199)	0.2 ± 0.3 (2287)	0.1 ± 0.2 (2236)
100	8 ± 3 (1780)	2 ± 1 (2034)	2 ± 1 (1742)
405PRO 405SYN CYA664			
10	15 ± 5 (1846)	0.7 ± 0.5 (1840)	17 ± 4 (1971)
50	18 ± 4 (2280)	0.7 ± 0.6 (2287)	18 ± 3 (2335)
100	14 ± 5 (1652)	0.7 ± 0.6 (1450)	17 ± 4 (1850)

* Numbers in parentheses indicate the total DAPI counts.

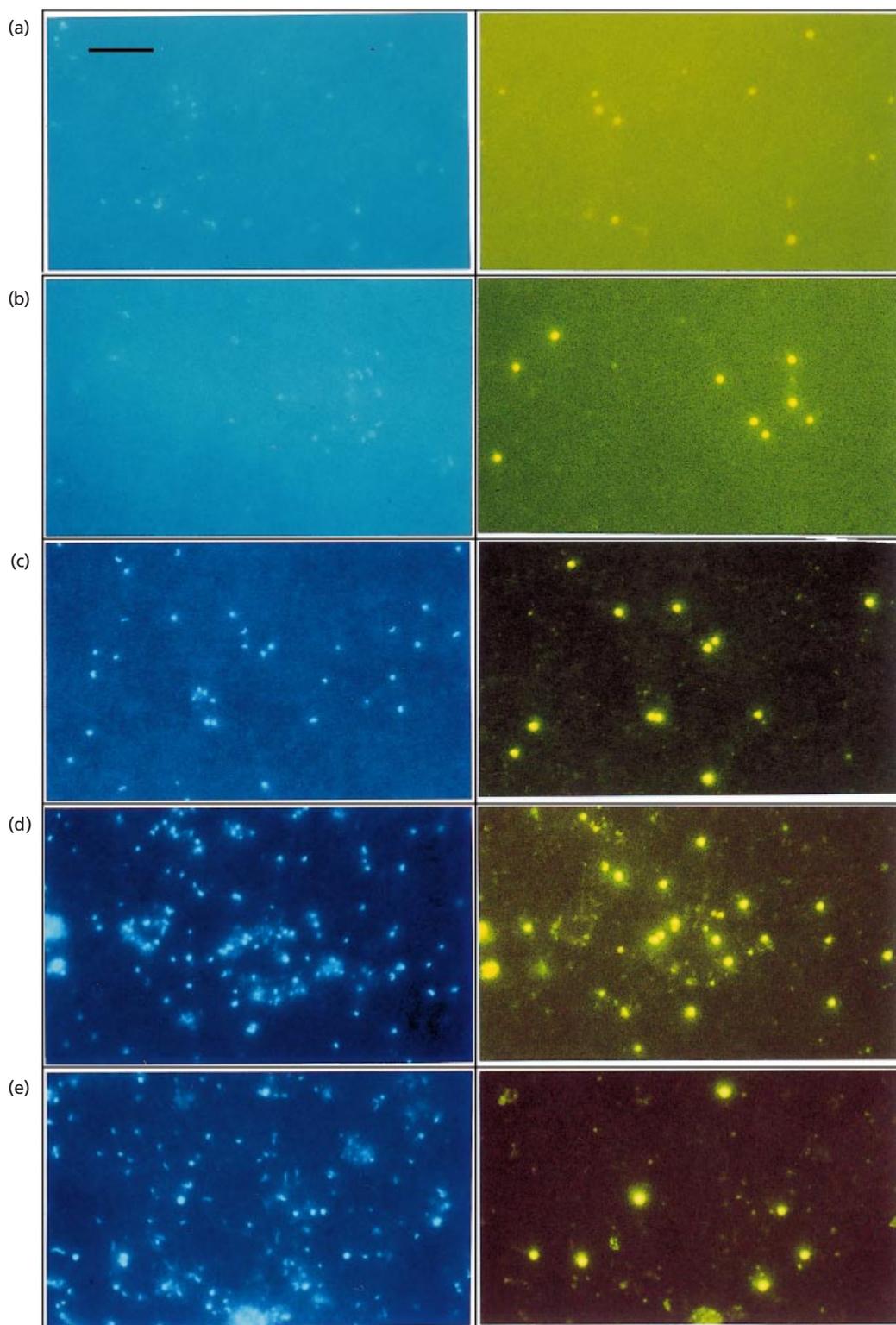


Fig. 3. Colour micrographs of natural samples from the North Atlantic Ocean and the Red Sea after hybridization. (a, b), samples from 3 m (a) and 80 m (b) from the North Atlantic Station 26, hybridized to 645HLI and 181LL respectively, and shown by DAPI staining using filter set 01 (left) and by epifluorescence 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 μ m.

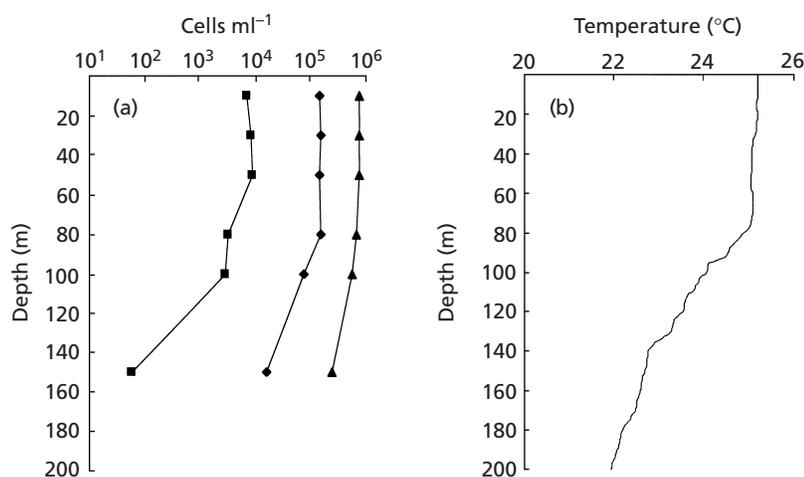


Fig. 4. (a) Flow cytometric data of *Prochlorococcus* (◆), *Synechococcus* (■) and heterotrophic bacteria (▲) cell numbers, and (b) the temperature profile for the water column on 18 October 1999 at Station A in the Red Sea.

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.

Depth (m)	Flow cytometry data (cells ml ⁻¹)			% <i>Prochlorococcus</i> (as a proportion of total bacteria)		% <i>Prochlorococcus</i> + <i>Synechococcus</i> (as a proportion of total bacteria)	
	<i>Prochlorococcus</i>	<i>Synechococcus</i>	Total bacteria	Flow cytometry	ISH* (405PRO)	Flow cytometry	ISH* (CYA664)
10	144835	7124	799771	18.1	15 ± 5	19.0	17 ± 4
50	144387	8787	771098	18.7	18 ± 4	19.9	18 ± 3
100	78988	2945	592609	13.3	14 ± 5	13.8	17 ± 4

* ISH, *in situ* hybridization.

numbers which were estimated following hybridization with probe 405SYN were extremely low at all depths in agreement with the flow cytometry data (Fig. 4a). In this stratified water column at Station A (Fig. 4b), *Prochlorococcus* dominated the picophytoplankton, with cell numbers of 1.4×10^5 ml⁻¹ at depths of 10 m and 50 m and 0.79×10^5 cells ml⁻¹ at 100 m. *Prochlorococcus* thus accounted for 93–94% of the picophytoplankton population, whereas *Synechococcus* contributed 3.5–5.7%.

The combined mean abundance of *Prochlorococcus* and *Synechococcus* as a percentage of total bacteria (estimated by hybridization with probe CYA664) was consistent throughout the water column at 17–18%. The estimation of *Prochlorococcus* abundance by hybridization with probe 405PRO was slightly lower than this value, except for the sample taken at 50 m. At all depths, the combined abundance of *Prochlorococcus* genotypes was less than estimated with either probe 405PRO or CYA664, but this was particularly marked at 50 m.

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 \pm 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 NATL1MIT/NATL2B isolates seem to be ubiquitous, having been detected only at depth, but 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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