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IDENTIFICATION OF THE CLASS PRYMNESIOPHYCEAE AND THE GENUS *PHAEOCYSTIS* WITH RIBOSOMAL RNA-TARGETED NUCLEIC ACID PROBES DETECTED BY FLOW CYTOMETRY[1]

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ABSTRACT

Target regions specific for the class Prymnesiophyceae and the genus Phaeocystis *(Har.) Lag. were identified from 18S ribosomal RNA coding regions, and two complementary probes were designed (PRYMN01 and PHAEO01). Detection of whole cells hybridized with these probes labeled with fluorescein isothiocyanate was difficult using epifluorescence microscopy because autofluorescence of the chlorophylls seriously interfered with the fluorescence of the probes. In contrast, flow cytometry proved very useful to detect and quantify the fluorescence of the hybridized cells. Hybridization conditions were optimized, especially with respect to formamide concentration. Both probes were tested on a large array of both target and nontarget strains. Positive and negative controls were also analyzed. Specificity was tested by adding a competing nonlabeled probe. Whereas probe PHAEO01 seems to have good specificity, probe PRYMN01 appeared less specific and must be used with stringent positive and negative controls.*

Key index words: epifluorescence microscopy; flow cytometry; oligonucleotide probes; Phaeocystis; *phytoplankton; Prymnesiophyceae*

Prymnesiophytes are important members of the marine phytoplankton. Many species are bloom-forming and contribute substantially to global carbon and sulphur cycles (Green and Leadbeater 1994). This algal class also figures predominantly in the pico- and nano-size fraction of the phytoplankton; thus, its representatives are not easy to identify (Green and Leadbeater 1994). *Phaeocystis* (Har.) Lag. is an important genus in the Prymnesiophyceae and is often regarded as a problematic, nuisance alga (Davidson 1985, Lancelot et al. 1987, Smith et al. 1991, Baumann et al. 1994). Its species can be difficult to separate because of the different morphologies associated with their colonial stages. Although physiological and biochemical differences among *Phaeocystis* species have been described (Jahnke 1989, Buma et al. 1991, Baumann et al. 1994, Vaulot et al. 1994), only electron microscopic investigations of the fine structure of the flagellated cell stage are used to confirm species identification unequivocally.

Recently, sequence data from the 18S ribosomal RNA (rRNA) gene have been used to support the species status of three colony-forming *Phaeocystis* species: *Phaeocystis globosa* from temperate/tropical waters, *Phaeocystis pouchetii* from the Arctic, and *Phaeocystis antarctica* from the Antarctic (Medlin et al. 1994). Also, a new species isolated from the Mediterranean Sea has been recognized by sequence data from the 18S rRNA coding region (Lange 1996), by ultrastructural features (Zingone, unpubl.), and by flow cytometric comparisons of its genome size (Vaulot et al. 1994). Furthermore, sequence data from the 18S rRNA coding region from six isolates of *Phaeocystis globosa* (Lange 1996) and comparisons of the genome size of many other *Phaeocystis globosa* isolates (Vaulot et al. 1994) suggest that *Phaeocystis globosa* is a species complex. In contrast, not all of the existing *Phaeocystis* records may be valid because *Phaeocystis*-like colonies in Antarctic sea-ice, for example, release flagellated cells belonging to other algal genera (Marchant and Thomsen 1994). These results indicate a need for a clear method to classify

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both the single flagellated stage and the colonial stage of *Phaeocystis* to the species level and to delimit *Phaeocystis* and prymnesiophytes from other algal genera.

Molecular techniques are being successfully applied to identify microorganisms and to solve problems in systematics and ecology (Stahl et al. 1988, Amann et al. 1990a, b, Ward et al. 1992, Muyzer et al. 1993, Simon et al. 1995). Among the algae, comparative sequence analyses of the 18S and 28S rRNA coding regions have been used to investigate taxonomic and phylogenetic problems (Chapman and Buchheim 1991, Bhattacharya et al. 1992, Maggs et al. 1992, Medlin et al. 1994, Ragan et al. 1994, Scholin et al. 1994, van Oppen et al. 1994). These coding regions contain both variable and conserved domains, which facilitate the design of short synthetic oligonucleotides that are highly specific against a complementary target sequence and can therefore serve as phylogenetic markers at a variety of taxonomic levels (Amann et al. 1995). The products of these coding regions, the rRNA molecules themselves, are ideally suited as targets for the oligonucleotide probes because their high abundance in each cell can yield a strong positive signal.

Probes targeted against variable regions enable identification at the species level, whereas probes complementary to more conserved regions can be used to characterize unknown species belonging to higher taxonomic groups (Giovannoni et al. 1988, Rehnstam et al. 1989). Still other regions of the rRNA molecules are perfectly conserved, and these regions have been used as targets for universal probes (Olsen et al. 1986, Weller and Ward 1989). Highly conserved regions have also been targeted by probes to determine total rRNA abundance in the environment (Stahl et al. 1988) or to assess differences in cellular rRNA content (Amann et al. 1990a).

Probes for single-cell identification can be labeled with radioisotopes (Giovannoni et al. 1988) or with fluorescent dyes (DeLong et al. 1989, Amann et al. 1990a, b). The application of several probes, each labeled with a different dye with different spectral properties, allows the detection of diverse groups of organisms in a single sample using epifluorescence microscopy (Spring et al. 1992). Oligonucleotide probes can also be linked with biotin or digoxigenin and visualized with enzyme-labeled antibodies that convert a colorless soluble substrate to a colored precipitate (Zarda et al. 1991). Techniques for fluorescence detection include epifluorescence microscopy and flow cytometry. Whereas the former is more commonly available in marine laboratories, the latter allies the advantages of speed, sensitivity, and quantification (Amann et al. 1992, Wallner et al. 1993, Simon et al. 1995).

Although rRNA probes are now commonly used in microbiology (Amann 1995), the application of this approach in discriminating unicellular marine algae is in its infancy. Recently, 18S rRNA–targeted oligonucleotide probes were successfully applied to discriminate chlorophyte from nonchlorophyte algae by flow cytometry (Simon et al. 1995). In this paper, we introduce and validate two probes to identify, respectively, members of the class Prymnesiophyceae and of the genus *Phaeocystis*.

MATERIALS AND METHODS

A large collection of marine algal strains was selected for the hybridization experiments (Table 1). Depending on the strain, cells were grown in either f/2 or WC medium (Guillard 1975), K medium (Keller et al. 1987), or MLH medium (Tuttle and Loeblich 1975) or in an enriched seawater medium with major nutrients diluted by half or 50 times (von Stosch and Drebes 1964). Culture conditions were either 17° C and 50 μmol photons·m^{-2}·s^{-1} continuous white light or 12:12 h LD or 20° C and 100 μmol photons·m^{-2}·s^{-1} 12:12 h LD. Light was provided by Sylvania Daylight fluorescent bulbs.

A secondary structure of the 18S rRNA molecule for the *Phaeocystis globosa* complex (Fig. 1) was designed using a secondary structure model for 18S rRNA adapted from Neefs et al. (1991) (Fig. 1) and 18S rRNA sequence data from *Phaeocystis globosa* strains listed in Table 1.

18S rRNA gene sequences from 50 eukaryotic algae including *14 Phaeocystis* strains were aligned with the ARB program package (Ludwig and Strunk, pers. commun.) or the Olsen sequence editor (Larsen et al. 1993). Regions of the gene that were unique for 1) the class Prymnesiophyceae and 2) the genus *Phaeocystis* were identified using the ARB program. These regions and their corresponding oligonucleotide probe sequences are shown in Table 2. Chlorophyte (CHLO01) and nonchlorophyte- (NCHLO01) specific probes were used according to Simon et al. (1995) (Table 2). Oligonucleotides were synthesized and labeled with fluorescein isothiocyanate (FITC) following the protocol of Amann (1995) or were purchased labeled from Eurogentec (Angers, France).

The hybridization procedure for epifluorescence microscopy was adapted from Amann (1995). Cultures were harvested in exponential growth phase, and 3 μL cell suspension was spread onto a gelatin-coated slide over an area of about 5 mm in diameter and dried at 37° C. The cells were dehydrated by passages through 50, 80, and 98% ethanol washes for 1–2 min each. After drying, a mixture of 8 μL hybridization buffer (0.9 M NaCl, 0.01% sodium dodecyl sulphate [SDS], 20 mM Tris-HCl, pH 7.2) and 1 μL fluorescent probe (25 ng·μL^{-1}) was spread over the spot of fixed cells. The hybridization was carried out in a moisture chamber (50-mL polypropylene screw-top tube, Sarstedt, Germany) at 46° C for 1.5 h. The hybridization was stopped by carefully rinsing the probe from the slides with prewarmed (46° C) hybridization buffer. The slides were then transferred to a 50-mL polypropylene screw tube filled with 50 mL hybridization buffer prewarmed to 46° C and incubated 20 min at 46° C. Salts were removed by dipping slides 5 s into sterile water.

Slides were mounted in Citifluor (Citifluor Ltd., London), and fluorescence was detected with a Zeiss Axioplan microscope (Zeiss, Oberkochen, Germany) fitted for epifluorescence microscopy with a 50-W Mercury high-pressure bulb and Zeiss filter Nr. 09 for fluorescein. Color photomicrographs were taken with Kodak Ektachrome P1600 color reversal film.

The whole-cell hybridization procedure for flow cytometry was adapted from Simon et al. (1995). Cells from 50-mL cultures were harvested in exponential growth phase, fixed in fresh paraformaldehyde to a final concentration of 1% and stored at 4° C for 1 h. The cells were centrifuged (3 min, 4000 × *g*) and resuspended in a cold (−80° C) mixture of ethanol and phosphate-buffered saline (PBS, Sigma, St. Louis, Missouri, 120 mM NaCl, 2.7 mM KCl in 10 mM phosphate buffer, pH 7.4, 70:30). The cells were centrifuged again and resuspended in 200 μL hybrid-

TABLE 1. *Marine strains used in this study. AWI = Alfred Wegener Institute, Bremerhaven, Germany; Roscoff = Station Biologique, Roscoff, France; Plymouth = Plymouth Culture Collection, Plymouth Marine Laboratory, Plymouth, United Kingdom; Bigelow = Provasoli-Guillard Culture Center for Marine Phytoplankton, Bigelow Laboratory for Ocean Sciences, West Boothbay Harbor, Maine. a = 18S rRNA gene (Medlin et al. 1994); b = 18S rRNA gene (Medlin et al., unpubl.).*

Class	Species	Culture facility	Strain number	Geographic origin	Used for hybridization	Used for 18S rRNA structure
Prymnesiophyceae	*Phaeocystis antarctica* Karst.	AWI	SK 22	Antarctica	+	
	P. antarctica	AWI	SK 23	Antarctica		
	Phaeocystis globosa Scherff.	AWI	SK 35	North Sea		a
	P. globosa	Plymouth	PLY 64	English Channel		
	P. globosa	Bigelow	CCMP 1524	Thailand		b
	P. globosa	Bigelow	CCMP 1528	Galapagos		b
	P. globosa	Bigelow	CCMP 627	Gulf of Mexico	+	b
	P. globosa	Bigelow	CCMP 628	Surinam	+	b
	P. globosa	AWI	P 162	South Africa		b
	P. globosa	Plymouth	PLY 540	East Atlantic	+	b
	Phaeocystis sp.	Roscoff	Med NS2	Mediterranean Sea	+	
	Phaeocystis sp.	Roscoff	Naples 2	Mediterranean Sea	+	
	Phaeocystis sp.	Roscoff	OLI 26 SF	Equatorial Pacific	+	
	Pleurochrysis carterae (Braar. & Fag.) Christ.	Bigelow	CCMP 645	Woods Hole, U.S.A	+	
	Pavlova lutheri (Droop) Green	Plymouth	PLY 75	Finland	+	
	Emiliania huxleyi (Lohm.) Hay & Mohl.	Plymouth	Texel B	North Sea	+	
	Chrysochromulina hirta Mant.	Roscoff	C. hi-2	Bergen, Norway	+	
	Prymnesium patelliferum Green, Hibb. & Pien.	Plymouth	PLY 527	Dorset, U.K.	+	
	Imantonia sp.	Plymouth	PLY 1856L1	Northeast Atlantic	+	
Dinophyceae	*Amphidinium carterae* Hulb.	Bigelow	CCMP 1314	Falmouth, U.S.A.	+	
	Crypthecodinium cohnii Biech.	Roscoff	Whd	Woods Hole, U.S.A.	+	
Chrysophyceae	*Ochromonas danica* Prings.	Plymouth	PLY 493	Kent coast, U.K.	+	
Pelagophyceae	*Pelagomonas calceolata* And.	Bigelow	CCMP 1212	Great Barrier Reef, Australia	+	
Bacilliariophyceae	*Thalassiosira weissflogii* (Grun.) Fryx. & Hasle	Bigelow	CCMP 1336	Long Island, U.S.A.	+	
Chlorophyceae	*Chlamydomonas coccoides* Butch.	Plymouth	PLY 494	Northeast Atlantic Roscoff, France	+	
Prasinophyceae	*Micromonas pusilla* (Butcher) Mant. & Parke	Bigelow	CCMP 490	Woods Hole, U.S.A.	+	

TABLE 2. *Oligonucleotide target positions and their corresponding probes based on an 18S rDNA dataset, which includes 50 eukaryotic algae; positions refer to those of* Phaeocystis globosa *in Figure 1 and to the corresponding position in* E. coli, *but helix numbers are used according to Neefs et al. (1991).*

Probe designation	Target positions	Helix number	Oligonucleotide sequence	
Prymnesiophyte-specific PRYMN01	Position: 946–963	24	Target:	5'AGCAUUUGCCAGGGAUGU 3'
	E. coli pos. 728		Probe:	3'TCGTAAACGGTCCCTACA 5'
Phaeocystis-specific PHAEO01	Position: 1488–1505	44	Target:	5'ACGAGUCCACCUCGACCG 3'
	E. coli pos. 1251		Probe:	3'TGCTCAGGTGGAGCTGGC 5'
Chlorophyte-specific CHLO01	Position: 1159–1176	From 30 to 32	Target:	5'CACCACCAGGCGUGGAGC 3'
	E. coli pos. 930		Probe:	3'GTGGTGGTCCGCACCTCG 5'
Nonchlorophyte-specific NCHLO01	Position: 1159–1176	From 30 to 32	Target:	5'CACCACCAGGAGUGGAGC 3'
	E. coli pos. 930		Probe:	3'GTGGTGGTCCTCACCTCG 5'

ization buffer (0.9 M NaCl, 20 mM Tris-HCl pH 7.8, 0.01% SDS). Cells were either used immediately or deep-frozen in liquid nitrogen and stored at −80° C. The latter treatment did not interfere with hybridization (L.G., unpubl.). Twenty-microliter aliquots of the cell suspension were incubated in the presence of deionized formamide (varying formamide concentrations from 0 to 30% were tested initially [see Results and Discussion]) at 46° C for 3 h as follows: 1) without probe, to measure autofluorescence, 2) with FITC-labeled probe, or 3) in combination with equal concentrations of labeled and unlabeled probe as a control for site-specific hybridization. The PRYMN01 and PHAEO01 probes were used with 10% formamide at a final concentration of 1.25 ng·μL^{-1}. The CHLO01 and NCHLO01 probes (Table 2, Simon et al. 1995) served as positive and negative controls in order to assess specific and nonspecific hybridization. The FITC-labeled CHLO01 and NCHLO01 probes were incubated with 25% formamide, at a final probe concentration of 2.5 ng·μL^{-1} and after the addition of the nonfluorescent NCHLO01 and CHLO01 probes, respectively, as competitors (Simon et al. 1995). Hybridization was stopped by adding 500 μL cold PBS, pH 9.0. Samples were stored in the dark at 4° C until analysis with flow cytometry within 24 h.

A standard FACSsort flow cytometer (Becton Dickinson, San Jose, California) was used for analysis. Excitation was achieved with a laser emitting at 488 nm. Green fluorescence of FITC was detected through a 525-nm bandpass filter. Cell mean fluorescence values were normalized to that of 0.95-μm beads (Polysciences Inc., Warrington, Pennsylvania) using CYTOPC software (Vaulot 1989).

RESULTS AND DISCUSSION

Oligonucleotide probes. Two class-specific and four genus-specific regions in the 18S rRNA coding region have been identified (Lange 1996, data not shown). Of these, two were selected to design oligonucleotide probes for whole-cell hybridization (Table 2). The position of the target regions complementary to the probes corresponded to the numbering of 18S rRNA coding region from *Phaeocystis globosa* (Fig. 1).

The class-specific probe, PRYMN01, matched 100% with all available prymnesiophyte sequences including *Phaeocystis* spp., *Emiliania huxleyi, Pavlova* cf. *salina, Prymnesium patelliferum, Coccolithus pelagicus,* and *Chrysochromulina polylepis* (Table 3). Only a single mismatch was found within the target region of probe PRYMN01 in the dinoflagellate *Crypthecodinium cohnii* and the prasinophyte *Mantoniella squamata.* More generally, sequences available to date indicate that probe PRYMN01 presents only a single mismatch with most dinoflagellates as well as with some Chlorophyceae and Prasinophyceae and at least two or more mismatches with all other species (Table 3).

The genus-specific probe, PHAEO01, matched its target region 100% in 18S rRNA coding regions of 14 *Phaeocystis* strains. *Phaeocystis globosa* was selected as a representative of the genus (Table 3). Several mismatches were found in the target regions of *Emiliania huxleyi, Pavlova* cf. *salina, Coccolithus pelagicus, Prymnesium patelliferum,* and *Chrysochromulina polylepis* (Table 3).

Epifluorescence microscopy. Epifluorescence microscopy was initially used to test the feasibility of using oligonucleotide probes with marine phytoplankton. Typical colony morphologies for *P. pouchetii* and *P. globosa/antarctica* are shown in Figure 2A, B. Probe hybridization to intact colonies was not difficult, but best resolution of probe hybridization was obtained with individual cells (Fig. 2C). Whole cells of *Phaeocystis antarctica,* strain SK 23, were hybridized with the FITC-labeled *Phaeocystis*-specific probe, PHAEO01 (Fig. 2C). Under optimal growth conditions, green fluorescence of the *Phaeocystis* cell was detected indicating hybridization of probe PHAEO01 to its 18S rRNA target region, whereas bacteria cells were not stained by probe PHAEO01 (compare position of bacteria in Fig. 2D to their absence in Fig. 2C). However, cell preparation for hybridization induced chlorophyll degradation that resulted in yellow autofluorescence, which often seriously interfered with the green fluorescence of probe PHAEO01 (data not shown). The detection of *Phaeocystis* cells with FITC-labeled probes using epifluorescence microscopy was therefore limited and may be difficult. One to four chloroplasts can occupy most of the cell volume of *Phaeocystis,* and their autofluorescence can mask the green fluorescence of the probe, which is limited to the cell cytoplasm.

Flow cytometry. Flow cytometry proved more useful than epifluorescence microscopy to detect the fluorescence induced by the probes. For example, target cells labeled with the probe PRYMN01 could be completely separated from unlabeled cells, whereas

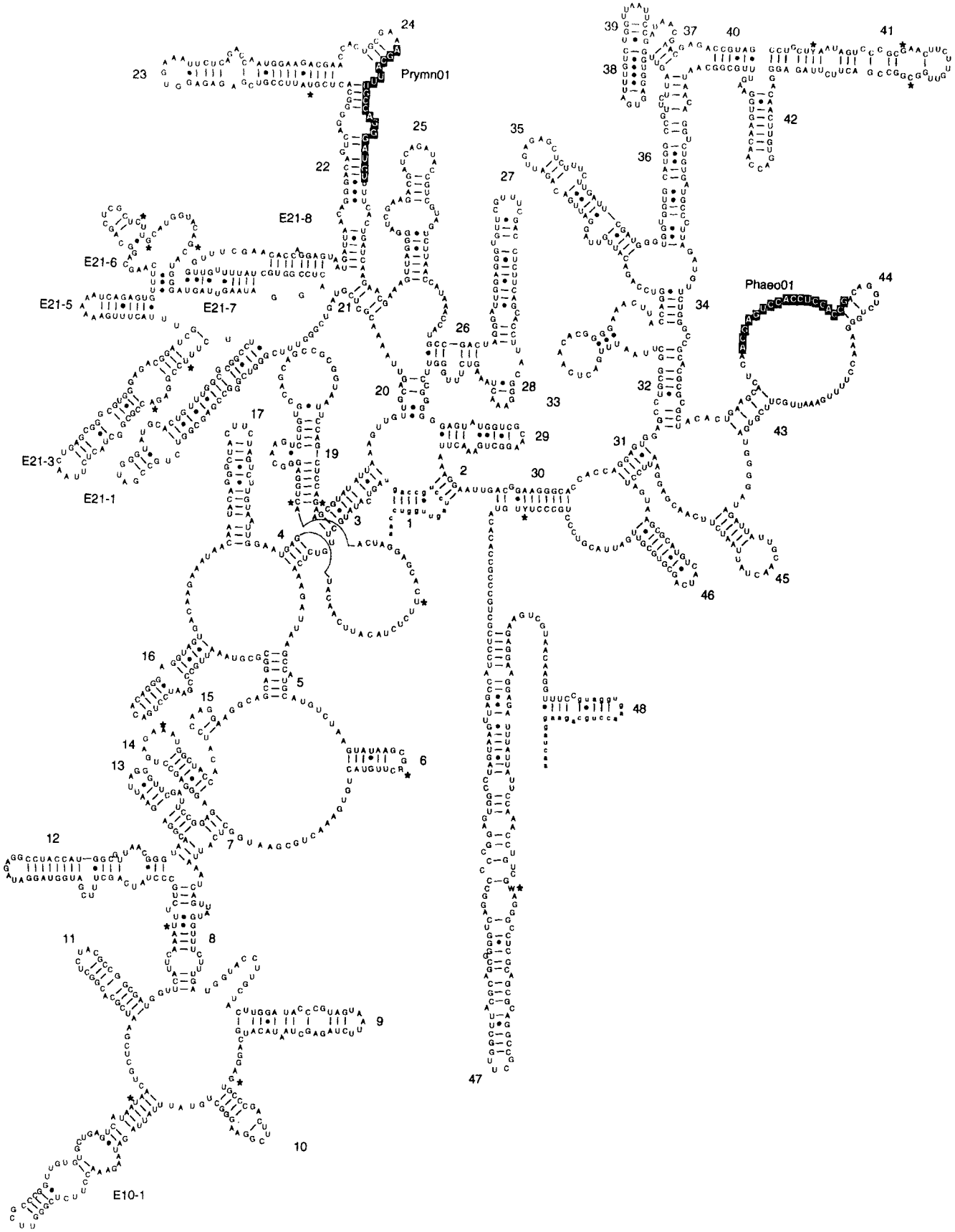

FIG. 1. Secondary structure model of the SSU rRNA molecule from the *Phaeocystis globosa* complex. Class- and genus-specific regions are highlighted in black. Asterisks indicate base substitutions and/or ambiguities within the *Phaeocystis globosa* complex.

TABLE 3. *Number of mismatches or insertions / deletions from selected algal species using the* Phaeocystis globosa *18S rRNA coding region as a reference. Target positions of probes are shown in Table 2 and Figure 1. Asterisks indicate target positions with 10 or more mismatches including insertions / deletions.*

Organism	Prymnesiophyte-specific PRYMN01	*Phaeocystis*-specific PHAEO01	Non-chlorophyte-specific NCHLO01	Chlorophyte-specific CHLO01	Sequence source
Phaeocystis globosa Scherff.	0	0	0	1	X77476
Chrysochromulina polylepis Mant. & Parke	0	3	0	1	B. Edvardsen, unpubl.
Coccolithus pelagicus (Wall.) Schill.	0	5	0	1	G. Barker, unpubl.
Emiliania huxleyi (Lohm.) Hay & Mohl.	0	4	0	1	M87327
Pavlova cf. *salina* (N. Cart.) Green	0	7	1	0	L34669
Prymnesium patelliferum Green, Hibb. & Pien.	0	4	0	1	L34670
Crypthecodinium cohnii (Sel.) Chatt.	1	*	0	1	M34847
Skeletonema costatum (Grev.) Cl.	2	*	1	2	M54988
Fucus distichus L.	2	*	0	1	X53987
Nannochloropsis salina Hibb.	2	7	0	1	M87328
Guillardia θ	3	8	0	1	X57162
Ochromonas danica Prings.	2	7	0	1	M32704
Porphyra umbilicalis (L.) J. Ag.	5	*	4	4	X53500
Pelagomonas calceolata And. & Saund.	7	7	0	1	U14389
Chlamydomonas rheinhardii Dang.	2	6	1	0	M32703
Mantoniella squamata (Mant. & Parke) Des.	1	8	1	0	X73999
Myrmecia israelensis (Chant. & Bold) Friedl	3	5	1	0	M62995

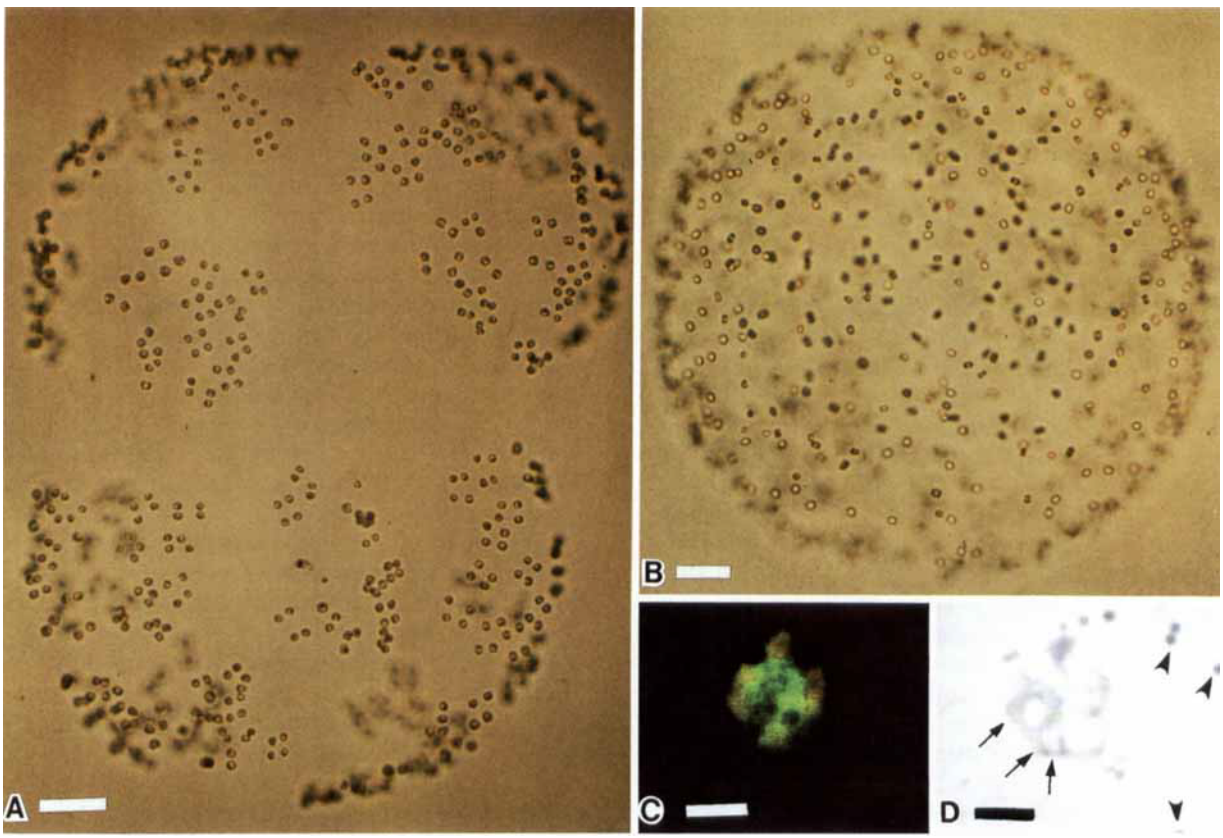

FIG. 2. Light and epifluorescent microscopy of *Phaeocystis* colonies and individual cells. A) Typical colony morphology for *P. pouchetii*, LM. Scale bar = 100 μm. B) Typical colony morphology for *P. globosa* and *P. antarctica*, LM. Scale bar = 100 μm. C) Individual cell of *Phaeocystis antarctica*, strain SK 23 hybridized with probe PHAEO01 viewed by epifluorescence microscopy showing chlorophyll (yellow) and probe (green). D) Individual cell in C viewed just before probe hybridization, LM phase contrast. Scale bar = 4 μm. Cell outline marked with arrows for clarity because the cells have been preserved and subsequently dehydrated prior to probe hybridization. Note bacteria (arrowheads) in the culture that do not react with the PHAEO01 probe visualized in C. Scale bar = 4 μm. Photographs A and B courtesy of Dr. M. E. M. Baumann.

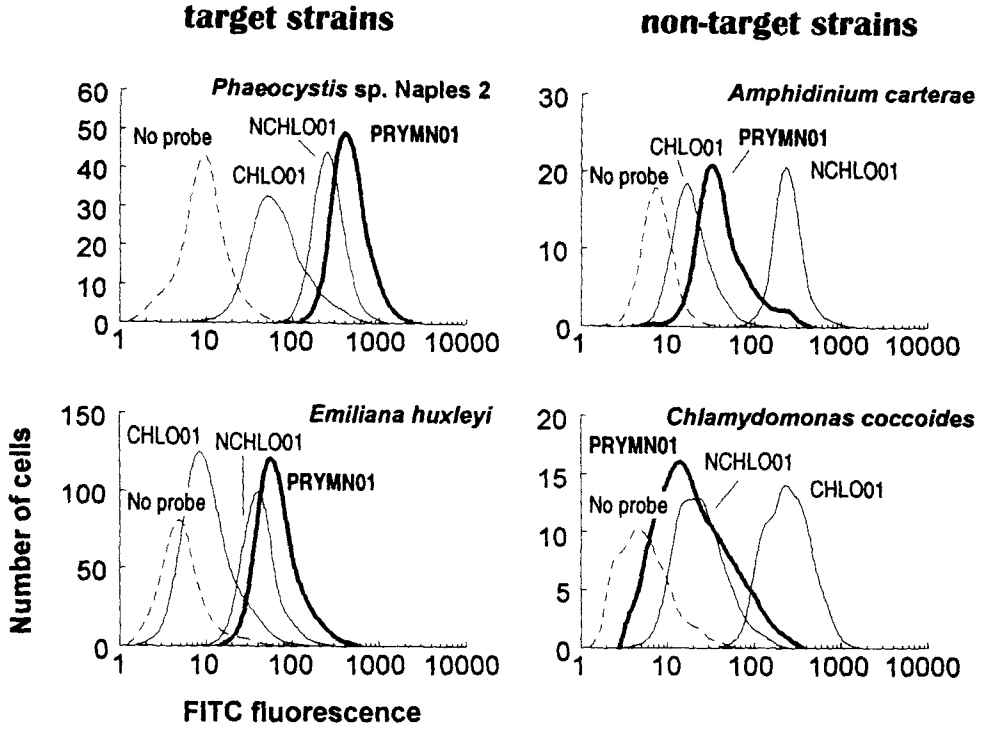

FIG. 3. Flow cytometric histograms of green fluorescence for cell populations hybridized with FITC-labeled probes. Cells were either not hybridized (no probe = autofluorescence) or hybridized with the following probes: PRYMN01, CHLO01, and NCHLO01 (Table 2). On the left are target strains for the PRYMN01 probe and on the right nontarget strains. The x axis corresponds to fluorescence intensity, and the y axis corresponds to cell number.

nontarget cells only exhibited marginal fluorescence increase (Fig. 3). This allowed us to test extensively both probes on a variety of target and nontarget strains. We employed the following positive and negative controls: 1) A sample without probe was used to record autofluorescence (Fig. 3). All other fluorescence values were rationed to autofluorescence. 2) The NCHLO01 and CHLO01 probes (Table 2) were used as positive controls for nonchlorophyte and chlorophyte cultures, respectively (Fig. 3). 3) Conversely, the CHLO01 and NCHLO01 probes were used as negative controls for nonchlorophyte and chlorophyte cultures, respectively (Fig. 3). 4) An additional specificity control was performed by mixing FITC-labeled and unlabeled probe in equal or varying proportions. If the probe is really specific, then 18S rRNA target sites should be blocked by the unlabeled probe, and green fluorescence should decrease as the proportion of unlabeled probe is increased. Such controls are important because cells, which grow under suboptimal conditions, may have fewer ribosomes and therefore show reduced fluorescence signals (Simon et al. 1995). Differences in the cell permeability for probes among different species could be another reason for unequal labeling of cells.

Prymnesiophyte-specific probe PRYMN01. The first step was to optimize hybridization conditions. In practice, the stringency factor that is most easily varied is the formamide concentration. Formamide concentration had little effect on the fluorescence intensity from nontarget cells (Fig. 4). However, for target cells, fluorescence increased at 5% formamide and then decreased at 25% (Fig. 4). Moreover, as formamide concentration increased, cell loss also increased (data not shown). Therefore, a 10% formamide concentration seemed a good compromise and was used in all subsequent experiments. For probe concentrations over 1 $\mu g \cdot L^{-1}$, fluorescence leveled off (data not shown), as demonstrated in Wallner et al. (1993). Therefore, a concentration of 1.25 $\mu g \cdot L^{-1}$ was selected.

Specificity of the probe was clearly demonstrated on the coccolithophorid *Pleurochrysis carterae* by mixing FITC-labeled and nonfluorescent probes (Fig. 5). As the concentration of the former remained fixed and the concentration of the later increased, fluorescence gradually decreased to a level obtained with a nonspecific probe (CHLO01), thus demonstrating that all target sites were blocked by the unlabeled probe.

The PRYMN01 probe labeled with FITC hybridized positively when tested against a large number of prymnesiophytes strains (Fig. 6, top). The fluorescence obtained was roughly of the same intensity as that obtained with a positive probe (usually NCHLO01, specific for nonchlorophyte) and always much stronger than that obtained with a negative

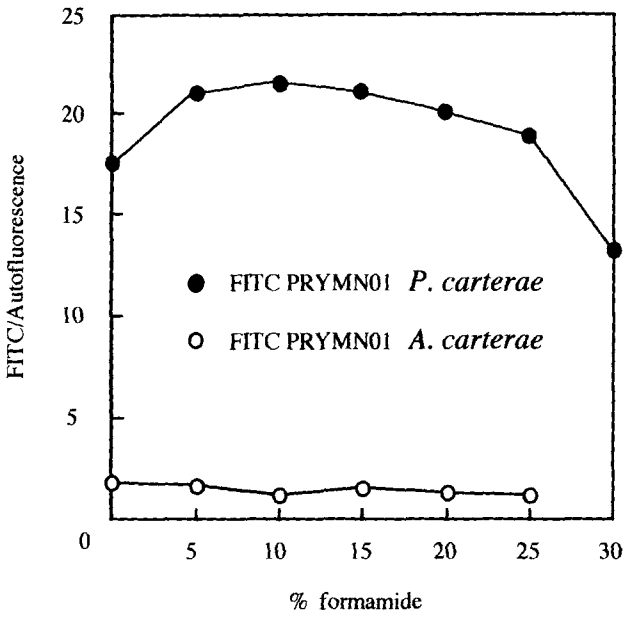

FIG. 4. Effect of formamide concentration on the ratio of probe FITC fluorescence to autofluorescence (no probe) for the PRYMN01 probe hybridized to a target strain (the prymnesiophyte *P. carterae*) and a nontarget strain (the dinoflagellate *A. carterae*). Fluorescence was measured by flow cytometry.

probe (usually CHLO01). Note, however, that *Pavlova lutheri* did not hybridize with NCHLO01 but with CHLO01, as expected from the available sequence (Table 3). Specificity was nevertheless confirmed by the reduction in fluorescence observed when the FITC-labeled PRYMN01 probe was made to compete with the unlabeled probe (e.g. for *E.*

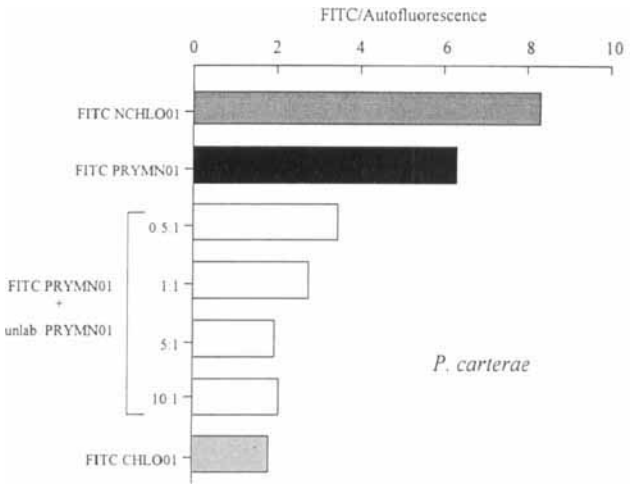

FIG. 5. Effect of the addition of a nonfluorescent competitor probe on the ratio of probe fluorescence to autofluorescence (no probe) for the PRYMN01 probe hybridized to a target strain (the prymnesiophyte *P. carterae*). The FITC-labeled PRYMN01 probe was kept at 1.25 ng·μL^{-1}, whereas the unlabeled PRYMN01 probe was varied at 0.75, 1.25, 6.25, and 12.5 ng·μL^{-1}. Probes NCHLO01 and CHLO01 were used, respectively, as positive and negative controls. Fluorescence was measured by flow cytometry.

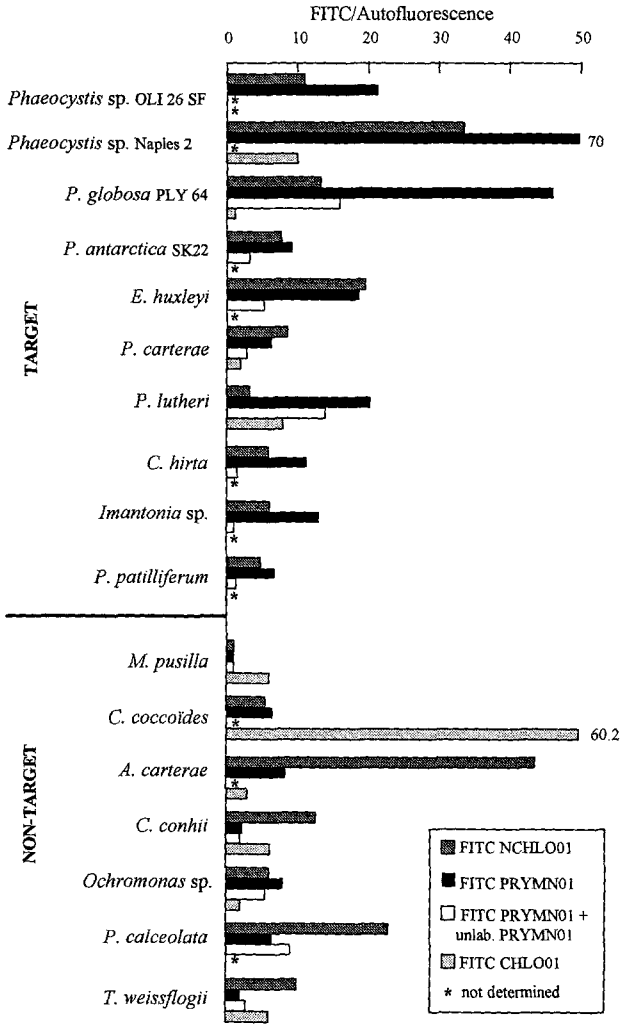

FIG. 6. Hybridization data for the prymnesiophyte-specific probe (PRYMN01). Probes NCHLO01 and CHLO01 were used as positive and negative controls according to strains. In some cases, unlabeled PRYMN01 was added to the FITC-labeled PRYMN01 to check probe specificity. FITC fluorescence was measured by flow cytometry and divided by autofluorescence. *n.d. = not determined.

huxleyi, P. lutheri, Phaeocystis globosa PLY 64). For nontarget strains (Fig. 6, bottom), the PRYMN01 probe always induced weaker fluorescence than a positive probe (either NCHLO01 or CHLO01, depending on the strain). Even for the dinoflagellate *C. cohnii*, which displayed a single mismatch with PRYMN01, the fluorescence of the PRYMN01 probe was much weaker than that induced by a positive probe. Moreover, in each case in which we added the nonlabeled competitor, fluorescence was not reduced. This was in contrast to that observed with target species. The absence of a decrease in fluorescence when a nonlabeled competitor was used suggests that the weak hybridization observed in the nontarget strains was nonspecific. Of all the nontarget strains tested, only *Ochromonas danica* hybridized with PRYMN01 to a level close to that obtained

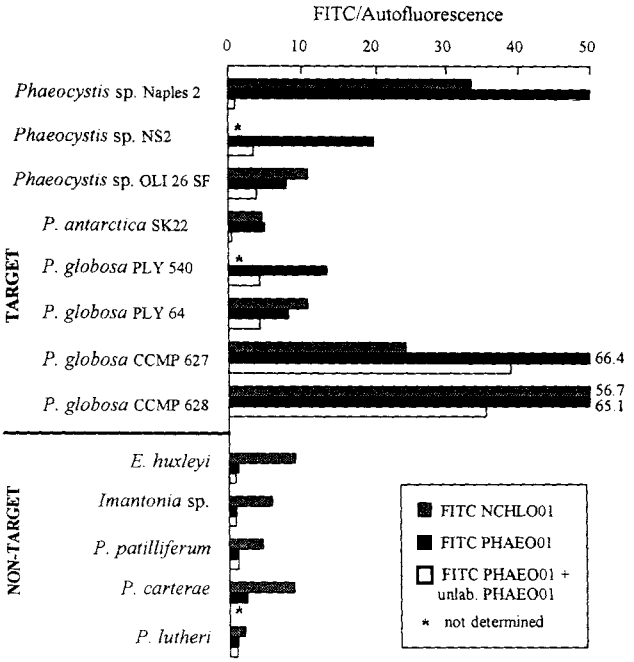

FIG. 7. Hybridization data for the *Phaeocystis* specific probe (PHAEO01). Probes NCHLO01 and CHLO01 were used as positive and negative controls according to strains. In some cases, unlabeled PHAEO01 was added to the FITC-labeled PHAEO01 to check probe specificity. FITC fluorescence was measured by flow cytometry and divided by autofluorescence. *n.d. = not determined.

with the positive NCHLO01 probe, although its rRNA has two mismatches to PRYMN01 (Table 3).

Phaeocystis-specific probe PHAEO01. Formamide concentrations had relatively little effect on probe fluorescence (data not shown) in contrast to that demonstrated for the PRYMN01 probe (Fig. 4). Nevertheless, we used the same formamide concentration (10%) as for the testing of the PRYMN01 probe to avoid varying the hybridization conditions between probes. When hybridized to target *Phaeocystis* strains (Fig. 7, top), the PHAEO01 probe induced, in general, stronger fluorescence than the positive NCHLO01 probe. Addition of the nonlabeled PHAEO01 probe always reduced fluorescence, demonstrating specificity (see earlier). *Phaeocystis* strain to strain variability in labeling intensity likely reflect differences in intracellular rRNA concentrations, which may result from suboptimal growth conditions (Simon et al. 1995). For nontarget prymnesiophyte strains (Fig. 7, bottom), the PHAEO01 probe induced fluorescence levels virtually indistinguishable from background fluorescence, and these levels did not change with the addition of the nonlabeled probe as a competitor.

CONCLUSIONS

The fluorescence of phytoplankton cells hybridized with FITC-labeled probes appeared to be difficult to detect with standard epifluorescence microscopy. Although labeling of *Phaeocystis* with probe PHAEO01 could be observed, green probe fluorescence was often totally masked by autofluorescence of the cell, resulting from chlorophyll degradation. For microscopy, whole-cell hybridization with a nonfluorescent probe detection system that includes the formation of a dye (Zarda et al. 1991, Amann et al. 1992) could constitute a better alternative to fluorescent labeling, although brighter fluorochromes and removal of chlorophyll fluorescence (e.g. by detergent) could also be tested.

In contrast, flow cytometry was well suited for detecting and quantifying probe fluorescence. It allowed us to test extensively the specificity of two probes, PRYMN01 directed against prymnesiophytes and PHAEO01 directed against *Phaeocystis*. For such tests, both positive and negative controls are extremely important. The PHAEO01 probe appears to have the best specificity because it did not label any nontarget strains. This was expected from the large number of mismatches it contained respective to the sequences of nontarget species. In contrast, and again as expected from sequence data, the PRYMN01 was not as specific because it labeled, albeit weakly, nontarget strains. However in such cases, PRYMN01 fluorescence was still much lower than the fluorescence induced by a positive probe. Moreover, it was reduced when nonfluorescent PRYMN01 was added as a competitor. Therefore, PRYMN01 can be used as a prymnesiophyte probe, provided that proper controls are performed.

The two probes described here should be useful to provide a rapid assessment of the taxonomic nature of unknown isolates. As more sequence data become available for new prymnesiophytes and *Phaeocystis* species, it will be necessary to verify the specificity of these regions. Other prymnesiophyte- and *Phaeocystis*-specific probes are available that match their target regions perfectly (data not shown): they should be tested in future work and could be used to corroborate identifications obtained with these results.

Ultimately, we want to use these probes on natural samples to identify phytoplankton populations. However, certain problems must be overcome before this is possible. Necessary improvements to these techniques include 1) developing better methods for cell permeabilization, 2) amplifying the weak fluorescence signal generated from low concentrations of intracellular rRNA, 3) concentrating those cells whose low natural abundances induce very long analysis time with flow cytometry, 4) minimizing cell loss during sample preparation, and 5) reducing interference from marine particles (see Simon et al. 1995 for discussion). Moreover, proper controls, such as those used in this study, will need to be performed with a large array of probes targeted at various taxonomic levels to ensure specificity. However, once these problems are solved, whole-cell hybridization coupled with flow cytometry should prove

more useful than bulk rRNA hybridization (e.g. Giovanonni et al. 1990), because it will afford an estimation of the contribution of each taxon, in terms not only of cell abundance but also of carbon biomass (Campbell et al. 1994).

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ANALYSIS OF THE EXPRESSION OF GENES FOR P-TYPE ATPASE IN THE HALOTOLERANT ALGA *DUNALIELLA SALINA* (CHLOROPHYCEAE)[1]

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ABSTRACT

A partial complementary DNA (cDNA) (DSA8) for a P-type ATPase was obtained from the halotolerant alga Dunaliella salina *(Dunal) Teod. (Chlorophyceae). The cDNA exhibited greater than 90% homology to the cDNA for a* H^+*-ATPase in* D. bioculata *Butcher. The expression of the gene that corresponded to DSA8 was decreased strongly by increases in NaCl concentration. The expression of a gene that corresponded to another ATPase (DSA1; possibly for a* Ca^{2+}*-ATPase) from* D. salina *did not show the same decrease as did the DSA8. However, increased osmotic pressure due to glycerol resulted in the same decrease in the DSA8 gene. Under salt or osmotic stress, the activity of a* H^+*-ATPase from microsomes of this alga also decreased. We suggest that expression of the gene for the plasma membrane* H^+*-ATPase of* D. salina *is regulated by osmotic pressure rather than by the concentration of NaCl.*

Key index words: ATPase; cDNA; Chlorophyta; Dunaliella salina; *gene expression; osmotic pressure; P-type ATPase; sodium chloride*

Dunaliella salina is a species of unicellular alga that lacks a cell wall, and it is found naturally in habitats of medium to extreme salinity. However, the internal content of Na^+ ion in the cells is very low compared with the high external concentration of salt (Ehrenfeld and Cousin 1984, Pick et al. 1986, Bental et al. 1988). For example, in *D. salina* cells cultured at 0.25 to 3 M NaCl, the calculated internal concentration of Na^+ ions was 10–30 mM (Pick et al. 1986). Thus, the cells must exploit efficient mechanisms for the elimination and regulation of intracellular Na^+ ions. At high salt concentrations, *D. salina* is able to synthesize glycerol as an osmolyte (Avron 1986, 1992), but there are no reports of how *D. salina* eliminates Na^+ ions. Plants and fungi have a vanadate-sensitive H^+-ATPase in their plasma membrane, which provides the driving force for the active transport of ions. *Dunaliella salina* also has a H^+-ATPase on the plasma membrane (Weiss et al. 1989). Some reports suggest that the H^+-ATPase is involved in the osmoregulatory mechanism of this alga (Oren-Shamir et al. 1989, 1990). These reports showed that orthovanadate and diethylstylbestrol (DES) each inhibited the rapid recovery from the shock of transfer to high salt medium. These re-

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