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A *Synechococcus* serotype is found preferentially in surface marine waters

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Abstract

In marine ecosystems, gradients of light, temperature, and nutrients occur horizontally (coastal to offshore) and vertically. The extent to which microorganisms acclimate or speciate in response to these gradients is under active investigation. Strain isolation data (e.g., site or depth), environmental DNA clone libraries, and preliminary physiology experiments have indicated that marine *Synechococcus* strain CC9605 might be adapted to the surface oligotrophic ocean. In the present work, we used an immunofluorescent approach to detect the CC9605 serotype in the California Current during September 1998. At two offshore stations, samples were collected along vertical profiles. The relative abundance of the CC9605 serotype was significantly higher in shallow depths within the mixed layer than in deeper depths at the two stations, with maximum values (± standard deviation) of 10.3% ± 6.4 and 28.7% ± 9.5. Surface samples along an offshore–inshore transect showed higher abundance in the most oligotrophic site (8% ± 3), compared with almost 1% inshore, but one coastal site also had high relative abundance of the CC9605 serotype (7% ± 0.5). These data indicate that *Synechococcus* strains are not uniformly distributed and that some strains, such as CC9605, are more abundant in the mixed layer of the euphotic zone than below the mixed layer.

In marine ecosystems, environmental clines exist in temperature, light intensity, spectral composition, and nutrient availability. One striking example of these clines and how they affect marine organisms can be observed in comparing the coastal, highly productive regions with the oligotrophic open ocean. In a transect away from the California coast, for example, in situ surface chlorophyll *a* concentrations have been observed to begin at 2.39 μg L^{-1} and decrease to values of 0.13 μg L^{-1}, an 18-fold difference (Scripps Institution of Oceanography 1999) that is likely driven by nutrient availability.

Vertical gradients are also significant. In depth profiles at oligotrophic sites off the coast of California, temperature drops by ~5°C between the surface and the bottom of the euphotic zone at 150 m. Silicate and phosphate increase almost fourfold, whereas nitrate and nitrite are undetectable at the surface and reach concentrations of 8 and 0.02 μmol L^{-1}, respectively, at 150 m (Scripps Institution of Oceanography 1999). Light intensity was also shown to decrease by three orders of magnitude in a similar profile (Eppley 1986).

As a result of these strong horizontal or vertical environmental gradients, it seems likely that an evolutionary adaptation of phytoplankton to specific ecological niches would occur, resulting in structured communities. One piece of evidence for these niches would be the lack of a uniform distribution of different strains or species of phytoplankton. Although physiological and genetic evidence are also needed to prove niche adaptation, without evidence of a differential distribution of strains or species, the existence of niches based on ecological clines would be much less likely.

For eukaryotic phytoplankton, there is evidence of niche-adapted species and niche-adapted strains within the same species. Venrick (1990, 1998) reported the persistence of distinct species clusters in shallow (0–100 m) or deep (100–200 m) depths in the central North Pacific. It was found that, over 12 yr, samples from within the same depth were more similar to each other than were deep and shallow samples collected at the same time. Distinct eukaryotic phytoplankton communities associated with either inshore or offshore regimes have also been reported (Venrick 1998).

In bacteria, speciation with depth may occur. For example, distinct subgroups of the major bacterial group SAR11 have been found as a function of depth (Field et al. 1997). One of the best studied cases to date is the marine cyanobacterium *Prochlorococcus*, in which niche-adapted groups have been clearly identified, specifically surface–high-light– and deep–low-light–adapted lineages (Partensky et al. 1999). These two groups have been identified by physiological responses of isolates (Moore et al. 1995, 1998) and the genetic relatedness between strains from similar depths, regardless of the geographic origin of isolation (Scanlan et al. 1996). In situ detection confirmed the distributions of at least two distinct genetic groups along vertical profiles (Ferris and Palenik 1998; Urbach and Chisholm 1998; West and Scanlan 1999; West et al. 2001).

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For marine *Synechococcus*, niche-adapted groups are beginning to be defined using an analysis of cultivated strains and environmental DNA clone library data. In addition, one physiological trait used for the definition of groups is the pigment composition, in particular the ratio of the chromophores attached to phycoerythrin (PE): phycourobilin (PUB) and phycoerythrobilin (PEB). According to the current paradigm, strains with high PUB : PEB ratios are well adapted to harvest the blue light common in the lower layers of the euphotic zone at oligotrophic sites (Wood et al. 1985; Olson et al. 1988). Conversely, populations with low PUB are more prevalent at shallow depths (Olson et al. 1990) and coastal environments (Olson et al. 1988; Wood et al. 1999). Populations lacking PUB altogether are restricted to coastal waters that often contain particulate or dissolved organic matter of terriginous or benthic origin that is not recently derived from phytoplankton (Wood et al. 1998). PUB : PEB ratios are not always coherent within a genetic lineage, however, and therefore do not simply reflect phylogenetic relatedness of strains (Toledo et al. 1999). In addition, it has been recently found that some strains can change their PUB : PEB ratios in response to blue light (Palenik 2001). PUB : PEB ratios thus do not provide enough information to uniquely identify niche-adapted groups.

Another approach to identifying and characterizing *Synechococcus* groups is by the use of whole-cell polyclonal antiserum and the immunofluorescence (IF) technique (Shapiro et al. 1989; Shapiro and Campbell 1998). This technique allows the clustering of isolates with similar antigenic characteristics, and these are presumably closely related. For example, strains with low PUB : PEB ratio cross-reacted with an anti-WH7803 serum (a low PUB strain), but no strains with high PUB cross-reacted with this antiserum (Campbell and Iturriaga 1988). Most strains with swimming motility cross-reacted with an anti-WH8113 serum developed against a motile strain (Campbell 1988). These common traits among serogroups indicate that they may represent genetically related strains, although this has never been shown. Conversely, genetic diversity may exist within the WH7803 serogroup (Wood and Townsend 1990).

To understand whether niche-adapted groups of *Synechococcus* occur in marine environments, strains have been isolated from various depths and sites in the California Current (Toledo and Palenik 1997; Toledo et al. 1999; Palenik et al. unpubl. data). One very interesting isolate was *Synechococcus* CC9605. This strain is a member of a phylogenetic cluster based on RNA polymerase (*rpoC1*) sequence data in which most isolates have been cultivated from samples collected at offshore stations in the CalCOFI sampling grid and in the Sargasso Sea (Palenik et al. unpubl. data). This cluster has been reisolated from the California Current in subsequent years from relatively shallow depths (strain CC9705). Environmental clone libraries from shallow depths in the oligotrophic edge of the California Current (Ferris and Palenik 1998) and the Sargasso Sea (Palenik 1994) show sequences that are phylogenetically closely related to CC9605, and this group has been referred to as *Synechococcus* clade 2. Recent analysis of a similar group of isolates using internal transcribed spacer data also suggest that this is a clade of related bacteria (Rocap et al. 2002). These pieces of evidence lead us to propose the hypothesis that CC9605 would be found at higher relative abundance in surface and offshore waters. A whole-cell polyclonal antiserum was developed for this strain and applied to samples collected to assess its relative abundance in the California Current during September 1998.

Table 1. List of axenic (underlined) and unialgal strains and growth conditions for cross-reactivity tests with CC9605 antiserum.

Strain	Light intensity (μmol quanta m^{-2} s^{-1})	Temp. (°)	Source or reference	Origin	Cross-reactivity
CC9311	28	21	1	California Current	−
CC9311	28 (blue)	20	1		−
CC9305-3	57	19	1		−
CC9317	57	19	1		−
CC9318	57	19	1		−
CC9605	28	21	3		+
CC9605	28 (blue)	20	3		+
CC9616	57	19	3		−
CC9617	9.5	19	3		−
CC9701	57	19	3		−
CC9702	57	19	2		−
CC9703	9.5	19	2		−
CC9704	57	19	3		−
CC9705	57	19	3		+
C129	28	21	A.Post, D.Lindell	Red Sea	−
SS9401	57	19	3	Sargasso Sea	−
SS10U3	57	19	B.Palenik		−
SS10U5	57	19	3		−
SS10N1	57	19	B.Palenik		−
SS10N2	57	19	B.Palenik		−
SS30N1	57	19	B.Palenik		−
SS70N1	57	19	3		−
WH7803	28	21	4	Atlantic Ocean	−
WH7805	28	21	4		−
WH8103	28	21	4		−

1: Toledo and Palenik (1977), 2: Toledo et al. (1999), 3: Palenik et al., unpubl. data, 4: Waterbury et al. (1986).

Methods

Organisms and culture conditions—Axenic or unialgal *Synechococcus* strains (Table 1) were cultivated in SN medium (Waterbury et al. 1986) for cross-reactivity tests. Typical light levels ranged between 9.5 and 57 μmol quanta $m^{-2}s^{-1}$ of white light provided by a cool-white fluorescent lamps, and temperatures between 19°C and 21°C. Additionally, strains CC9605 and CC9311 were also grown at 20°C under blue light at 28 μmol quanta $m^{-2}s^{-1}$ provided by an halogen lamp and blue filter screens. For the preparation of antiserum, axenic cultures of CC9605 were fixed with 0.6% formaldehyde. Polyclonal antiserum against CC9605 was obtained from a rabbit using standard commercial protocols (Covance).

IF labeling with laboratory cultures—A protocol for indirect IF labeling of the SwmA protein from swimming marine *Synechococcus* strains was developed by B. Brahamsha and D. Ratliff (unpubl. data) based on a recommended protocol from B. Ward (pers. comm.). We followed their protocol for our study. In brief, a nylon filter mesh of 11 μm pore size (Millipore) was used as backing filter and a few drops of Tween-20 (5% v/v) were placed on top of the filter, to avoid aggregates and potential clogging. Above the backing filter, glutaraldehyde fixed cells (0.25% v/v) were applied to a polycarbonate filter membrane (25 mm, 0.22 μm; Osmonics) at a concentration of 1×10^5 cells ml^{-1}. Blue autofluorescence from the glutaraldehyde-treated cells was not a significant problem for this work. The cells were washed with 20 ml of 0.22 μm prefiltered phosphate-buffered saline (PBS; 2.68 mmol L^{-1} KCl, 8.10 mmol L^{-1} Na_2HPO_4, 1.47 mmol L^{-1} KH_2PO_4, and 137 mmol L^{-1} NaCl [pH 7.5]). To block the samples and prevent unspecific antibody binding, we substituted the recommended gelatin (2% w/v) with 2.5 ml of 1% γ-globulin (bovine, from Cohn fraction II and III; Sigma) in PBS and incubated the cells for 30 min. Then, CC9605 antiserum was diluted to 1 : 50 in a final volume of 2.5 ml, pre-filtered through a 0.22-μm polycarbonate filter membrane, applied to the sample, and incubated for 45 min. We found this dilution to be the best—higher dilutions tested (1 : 100, 1 : 150, and 1 : 200) resulted in lower percentages of positive labeled cells, and lower dilutions tested (1 : 25 and 1 : 10) did not significantly increase the proportion of positive cells. After washing with 40 ml of PBS, a secondary antibody was applied for 40 min. We used goat anti-rabbit antiserum with a conjugated chromophore (Alexa 488; Molecular Probes) at a dilution of 1 : 150 (final volume, 2.5 ml) in blocking solution. After washing with 40 ml of PBS, the filter was mounted on microscope slides with the ProLong Antifade kit (Molecular Probes) and covered with a coverslip. Cells were visualized with a fluorescence microscope (Axioskop; Zeiss) equipped with a 200-W mercury arc lamp. CC9605-positive cells fluoresce green under blue excitation filter set (Zeiss catalog 487909) because of the Alexa 488 chromophore (excitation at 495 nm, emission at 519 nm) conjugated to the secondary antibody. CC9605-negative cells show orange fluorescence (because of their PE composition) under the same filter set. Images were captured with a digital camera (Spot; Diagnostics Instruments) under the same exposure times and exported to Adobe Photoshop (version 5.0).

Controls with strain CC9605 cultures included substitution of primary antiserum by blocking solution only, or preimmune antiserum only, in each case followed by secondary antibody treatment. Control incubations using only primary antiserum were tested as well. These controls did not yield any labeled cells. The strains listed in Table 1 were tested at the optimized conditions (1 : 50 primary and 1 : 150 secondary). For strains CC9701, SS9401, and SS10U5, which are phylogenetically closely related to CC9605, we also tested lower CC9605 antiserum dilutions (1 : 25 and 1 : 10). CC9701 and SS9401 were also tested at two growth stages (log- vs. stationary-phase cultures). We also grew strain CC9605 under conditions partially simulating the lower euphotic zone (blue light; see above) to evaluate whether antigenicity decreases with these culture conditions.

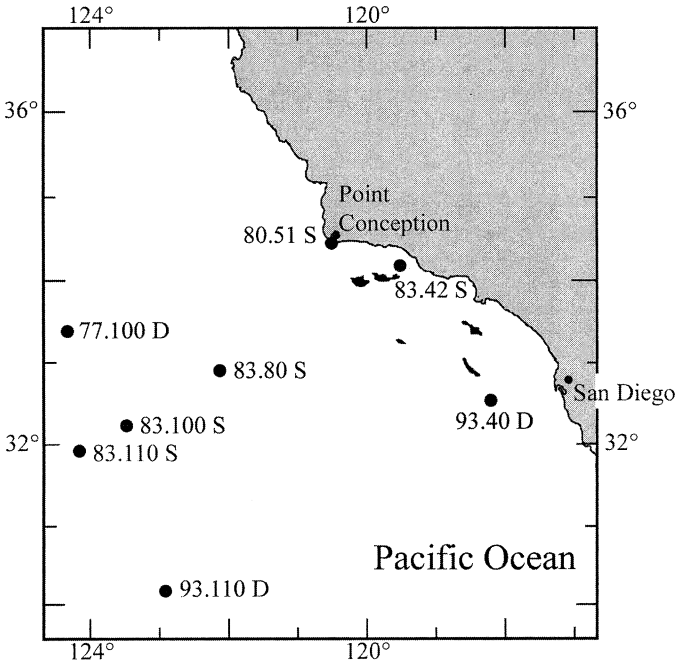

Fig. 1. California's coastline showing the sites in the CalCOFI grid, where samples were collected during September 1998 (cruise NH9809). Numbers represent the sites in the grid followed by S: stations where only surface samples (<2 m) were taken, or D: samples were collected at several depths. The geographical coordinates for each site can be obtained online (*see Methods*).

Sample collection—Field samples were obtained in September 1998 during the cruise CalCOFI 9809 of the R/V *New Horizon.* The station network and the sampled sites are shown in the Fig. 1. Hydrographic data for these sites are shown in Table 2. The geographical coordinates for each station and additional physical, chemical, and biological data can be accessed at http://www.calcofi.org/data/data.html. Vertical profiles were conducted at two offshore stations, 77.100 (20, 40, 60, 80, 110, 170, and 200 m) and 93.110 (25, 50, 70, 90, 110, and 170 m), and a near-shore station 93.40 (12, 20, 30, 40, 60, and 100 m). Surface samples were not obtained for these profiles. Surface samples (<2 m) were collected at four stations along a transect on line 83 (Sta. 83.110, 83.100, 83.80, and 83.42) and Sta. 80.51. For each sample, water was collected with 10-liter Niskin bottles from which 2 liters were drawn in a polypropylene (PP) dark bottle and transported to the onboard laboratory. The samples were then divided in two categories: 1.5 ml stored in 1.8-ml cryovials (Nalgene) and 45 ml stored in 50-ml disposable PP tubes (Corning). All samples were then fixed with 0.25% glutaraldehyde at room temperature for 15 min. The cryovials were stored in liquid nitrogen (−170°C) and the 50 ml tubes at −20°C for the rest of the cruise. On arrival at the laboratory, the samples in liquid nitrogen were transferred to −80°C, and the rest remained at −20°C until analyzed. The 1.5-ml samples were used to enumerate the total *Synechococcus,* picoeukaryotes, and *Prochlorococcus* popula-

Table 2. Hydrographic data and *Synechococcus* abundance for the shallow depths at Sta. 77.100, 93.110 and 93.40 and surface samples along the transect in line 83 and Sta. 80.51 during September 1998.

Station	Regime*	Depth (m)	Temp. (°C)	Salinity	PO_4 (μmol L^{-1})	NO_3 (μmol L^{-1})	NO_2 (μmol L^{-1})	Chl *a* (μg L^{-1})	Total % *Synechococcus*†	CC9605 positive
77.100	Offshore	20	17.97	33.123	0.27	0.1	0.00	0.14	4,384	29±(9.5)
93.110	Offshore	25	19.61	33.595	0.20	0.1	0.00	0.13	2,862	10±(6.5)
83.110	Offshore	1	19.15	33.572	0.23	0.0	0.00	0.08	1,814	8.4±(2.7)
83.100	Offshore	1	19.09	33.441	0.22	0.0	0.00	0.08	2,154	4.0±(2.1)
83.80	Offshore	1	17.37	33.119	0.34	0.0	0.00	0.13	8,923	3.7±(3.9)
83.42	N. coastal	1	19.17	33.396	0.17	0.1	0.00	1.4	27,648	7.1±(0.5)
80.51	Variable	1	17.05	33.380	0.24	0.1	0.00	0.78	13,912	1.4±(0.4)
93.40	S. coastal	10	21.10	33.468	0.19	0.0	0.00	0.15	16,556	3.0±(4.2)

* Hayward and Venrick (1998).
† *Synechococcus* numbers estimated by flow cytometry ''fresh counts.''

tions by flow cytometry (FACSort; Becton Dickinson) 3 weeks after the cruise, and we refer to these numbers as ''fresh counts.'' The 45-ml samples were used for the IF labeling experiments because *Synechococcus* cell densities are usually low, especially at the offshore stations, and larger volumes are required to have statistically significant numbers. These samples were stored for ~7 months before analysis. Alternatively, samples of 4.5 ml were collected at coastal Sta. 80.51, stored in 5-ml cryovials, and treated similarly to the samples for fresh counts. The comparison between flow-cytometer fresh counts and counts on each individual sample for the complete data set showed similar trends; however, numbers were generally twofold higher for fresh counts. The 45-ml volume samples were as well preserved as the 4.5-ml samples (data not shown).

IF labeling of environmental samples—On the day of IF labeling, an aliquot of 995 μl (from 45-ml samples) or 100 μl plus 895 μl PBS (from 4.5-ml samples) were taken, combined with 5 μl of green fluorescent beads (diameter, 0.9 μm; Duke Scientific), and analyzed by flow cytometry. This allowed us to evaluate the preservation of the larger volume samples by comparing with the fresh counts from the 1.5-ml samples and to have a total *Synechococcus* count independent of the fluorescence microscope counting of each individual sample. The IF procedure was similar to that of the laboratory cultures, except that a wash with 100 ml of PBS instead of 20 ml was done, to eliminate excess glutaraldehyde from the sample.

Controls for the environmental samples included substitution of CC9605 antiserum with preimmune serum and incubations with secondary antibody only. The former resulted in a small proportion of cells with a ring of green fluorescence similar to cells scored as positive in incubations with CC9605 antiserum. We refer to these cells in preimmune treatments as ''false positives.'' The incubations using only secondary antibody did not yield any labeling.

Cell counts and statistical analyses—Total *Synechococcus* counts were obtained by fluorescence microscopy and flow cytometry. Cell counts by fluorescence microscopy can be determined using either blue or green excitation. The blue excitation can underestimate total *Synechococcus* if cells have low total PE or if cells have relatively low PUB : PEB ratios, because these two cell types display dim fluorescence and are difficult to visualize. These types are relatively more abundant in surface samples. Green excitation can overestimate *Synechococcus* abundance, because other small cell types (picoeukaryotes or prochlorophytes) with chlorophyll fluorescence, cell size, and shape similar to *Synechococcus* can be mistakenly scored as *Synechococcus.* Therefore, neither blue nor green excitation is ideal. Because we had several surface samples, it was decided, for most work, to use green excitation to estimate total *Synechococcus.* We used a green excitation filter (Zeiss 487914) to visualize Chl *a*–containing cells that fluoresce red and counted cells per field of view at 1,000× magnification. Then we switched the filter to blue excitation, to count the antibody-positive cells in the same field of view. Total *Synechococcus* counts using the blue excitation filter set were done only on one sample per depth at the vertical profiles and one sample per station in the transect. We counted at least 100 total *Synechococcus* per filter or 45 fields of view, when cell densities were very low, and estimated the abundance similar to Waterbury et al. (1986). The proportion of positive cells was calculated by dividing the antibody-positive count by the total *Synechococcus* microscope counts (green excitation) or the flow cytometry counts (from the aliquot analyzed for each sample). At depths ≤110 m in the vertical profiles or at each location in the transect, three replicate filters were treated with CC9605 antiserum, and two were treated with preimmune antiserum. For deeper depths, we analyzed only one sample and one control without replicates. The proportion of positive cells from each of the three replicate filters was corrected by subtracting the average from the two treatments with preimmune serum (false positives) at the same depth. The values were then transformed to the corresponding arcsine with the formula $p' = \text{arcsine}(\sqrt{p})$ (Zar 1984). This transformation was done to correct the data set from a binomial distribution typical of data expressed as percentage to a normal distribution for further analysis of variance (ANOVA). One-way ANOVA was done using the SYSTAT package (version 5.2.1.; SYSTAT) on the corrected data set to evaluate statistically significant differences of the proportion of CC9605 among depths in the vertical profiles and stations in the transect. Post hoc tests using Fisher's least-

squares difference allowed pairwise comparisons between depths for vertical profiles and sites for the surface transect.

Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis (PAGE) and Western blot analyses—Cultures of strains CC9605 and CC9311 were harvested by centrifugation at 13,000 × *g* for 6 min. The cell pellets containing 2.1 × 10^9 and 1 × 10^9 cells, respectively, were washed with 700 μl of sterile SN medium and spun again at the same speed and time. The resulting pellet was resuspended in 100 μl of sterile SN and used for further protein analysis. A volume of 10 μl or a 10-fold dilution (1 μl of cell suspension in 9 μl of sterile SN) was then boiled in electrophoresis loading buffer (Laemmli 1970) at 100°C for 5 min. The cell slurry was centrifuged at 13,000 × *g* for 5 min and 10 μl were loaded in a NuPAGE 10% Bis-Tris precast polyacrylamide minigels (Novex). The protein separation was conducted at 170 V for 60 min with 3-[N-Morpholino]propanesulfonic acid (MOPS) as the running buffer, as recommended by the manufacturer. The gels were then stained with Coomasie brilliant blue (Bio Rad) in a solution of 46% methanol and 0.1% acetic acid and destained, typically for 4 h, in a 9.5% ethanol and 7.5% acetic acid solution. Alternatively, the gels were electrophoretically blotted onto PVDF membranes (Millipore). The transfer buffer was Bicine (25 mmol L^{-1}), Bis-Tris (25 mmol L^{-1}), ethylene diaminetetraacetic acid (EDTA; 1 mmol L^{-1}), and methanol (20% v/v). After 1 h transfer at 24 V, the membranes were blocked overnight in 5% nonfat dry milk on PBS and 0.05% Tween-20. CC9605 antiserum was applied at a dilution of 1 : 20,000 and incubated for 1 h, followed by four washes of 15 min each with 100 ml of PBS-Tween. We used, as secondary antiserum, horseradish peroxidase conjugated goat anti-rabbit IgG (Sigma) diluted at 1 : 10,000 in 50 ml of a buffer that contained Tris (50 mmol L^{-1}), EDTA (1 mmol L^{-1}), NaCl (150 mmol L^{-1}), and 0.25% bovine serum albumin for 1 h, followed by four washes of 15 min each. Cross-reacting bands were detected using the ECL chemiluminiscence Western blotting detection kit (Amersham Pharmacia) and X-ray film (Kodak), according to the manufacturer suggestions. To assess the specificity of the cross-reaction, we included a blot to which CC9605 antiserum was substituted by preimmune antiserum at a dilution of 1 : 20,000.

Results

IF labeling of laboratory cultures—*Synechococcus* strain CC9605 showed a clear cross-reaction with CC9605 antiserum. The labeling intensity observed by fluorescence microscopy allowed the rapid scoring of cross-reacting cells (Fig. 2F). Typically, we observed in the same stock of fixed cells used on different days an efficiency (± standard deviation) of 86% ± 4.3 (n = 7) of labeled cells at the optimal titer of 1 : 50. The substitution of anti-CC9605 with preimmune serum did not yield any false-positive cells with laboratory cultures (Fig. 2B). No significant changes in label intensity or the percentage of positive-labeled cells were observed when CC9605 cultures were grown under blue light at 20°C (85% positive), compared with those grown under white light at 21°C (92% positive). Little difference was found when comparing cells in the logarithmic (88% positive) and stationary (78% positive) growth phases.

The CC9605 antiserum was very specific for strain CC9605. For example, strain CC9702 did not cross-react (Fig. 2E). From the 23 strains tested (Table 1), only CC9705 cross-reacted with similar intensity as strain CC9605. Both strains are almost identical in the gene sequence of a 612-bp fragment of the RNA polymerase *rpoC1* (1-nt difference) and were isolated in two consecutive years from offshore sites using a similar isolation strategy. The strains CC9701 and SS9401, isolated from offshore in the CalCOFI sampling grid and from the Sargasso Sea, respectively, and included in the same phylogenetic cluster as CC9605, did not show labeling with CC9605 antiserum, even when low antibody dilutions (1 : 10) were used (data not shown).

SDS-PAGE analysis and Western blotting—To understand the specificity of the antiserum, we analyzed total protein extracts using SDS-PAGE and Western blotting. When cell densities of 10^8 were used, the CC9605 antiserum cross-reacted with many proteins of strain CC9605 and also with three abundant proteins in strain CC9311 (Fig. 3B). A 10-fold dilution of the cells resulted in cross-reaction of two bands of 73 and 68 kDa in CC9605. They both seem relatively abundant compared with most cell proteins (Fig. 3A). CC9311 showed cross-reaction only in the most abundant protein band of 116 kDa at this cell density. It appears that the cross-reactivity of the antiserum with whole cells of CC9605, but not CC9311, must be due to the inaccessibility of cross-reacting proteins inside glutaraldehyde-fixed cells. The incubation with preimmune antiserum showed only a few very faint bands for CC9605 (2 × 10^8 cells), and these were different from those observed when immune antiserum was used (Fig. 3C).

IF labeling of environmental samples—The CC9605 antiserum clearly detected positive cells in field samples. The antibody label intensity per cell on positive cells was lower (Fig. 2H) than that found with laboratory-grown CC9605 cells (Fig. 2F), and the green fluorescence was observed as a ring around the cells *Synechococcus* natural populations at shallow depths were also dimmer in their PE and chlorophyll autofluorescence than laboratory cultures (Fig. 2C,G compared with 2A,E), possibly because of the different light and nutrient regimes of natural populations.

When CC9605 antiserum was substituted by preimmune antiserum on field samples, we observed a small fraction of cells (1.43% ± 1.3, n = 36; Fig. 2D) that yielded a similar reaction to cells scored as positive in the CC9605 antiserum incubations. The red autofluorescence under green excitation showed that these false positives were not ''ghosts'' (structures resembling *Synechococcus* cells but lacking autofluorescence). These false-positive cells are due to the existence of a natural population with chlorophyll fluorescence and some affinity to our rabbit antiserum or cells that nonspecifically absorb antiserum because incomplete blocking by the 30-min incubation with blocking solution.

Relative abundance of CC9605 serotype in the California Current—Two independent data sets were used to test two

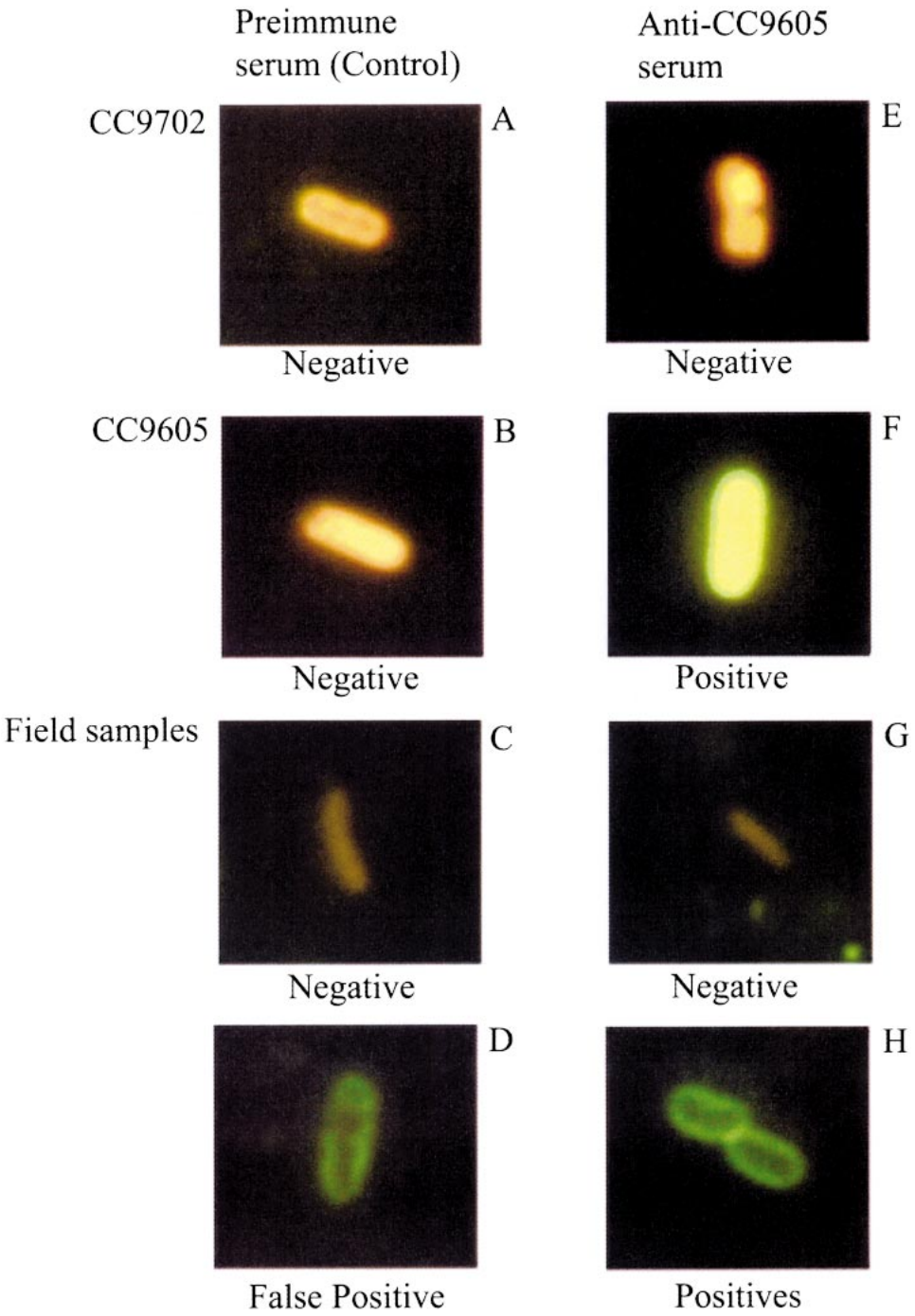

Fig. 2. Blue excitation fluorescence micrographs of laboratory cultures (CC9702 and CC9605) and field samples. The samples were treated with preimmune antiserum (A–D) or immune antiserum (E–H). Strain CC9605, treated with anti-CC9605 serum (F), rendered an evident change in fluorescence compared with preimmune treatment of the same strain (B), whereas for non–cross-reacting strains there was no difference in fluorescence (A,E). Some cells in the field samples treated with CC9605-antiserum showed a ring of green fluorescence, scored as a positive reaction (H), compared with a cell in the same sample lacking the ring and thus scored negative (G). When field samples were treated with preimmune antiserum, few false-positive cells resembled features of cells scored positive (D), and negative cells appear similar in both treatments (C).

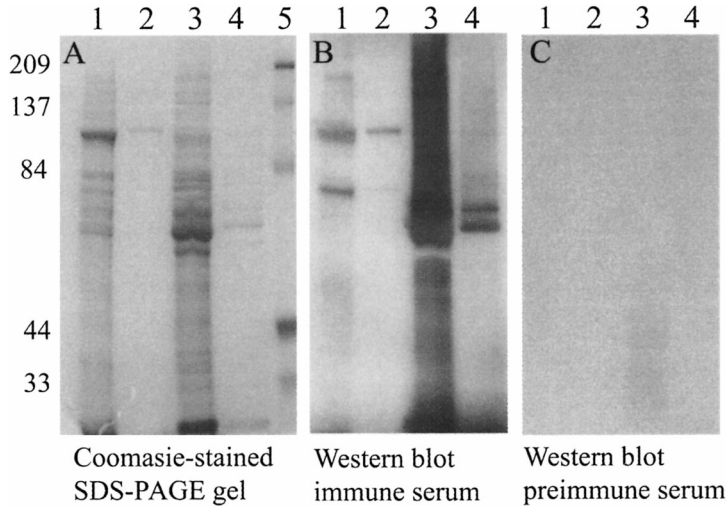

Fig. 3. Protein analyses of strains CC9605 and CC9311 cultures. (A) Coomasie-stained SDS-PAGE gel. (Lanes 1 and 2) Strain CC9311 at 1×10^8 and 1×10^7 cells, respectively. (Lanes 3 and 4) Strain CC9605 at 2.1×10^8 and 2.1×10^7 cells, respectively. (Lane 5) Molecular weight marker Kaleidoscope; sizes (in kDa) are indicated at left. (B) Western blot of an equivalent gel incubated with CC9605 serum at 1 : 20,000 dilution. (C) Western blot using preimmune antiserum at 1 : 20,000 dilution.

hypotheses: (1) that the CC9605 serotype is relatively more abundant in the surface mixed layer than below it and (2) that the CC9605 serotype is relatively more abundant offshore than near shore. To test (1), we analyzed the data for the vertical profiles at oligotrophic Sta. 77.100 and 93.110 in the California Current. The number of positive cells was determined and divided by the total number of *Synechococcus* cells. The total number of cells can be determined using blue or green excitation on a fluorescence microscope, neither of which is ideal, as discussed in *Methods* and below.

At Sta. 77.100, we observed good correspondence between total counts done by flow cytometry and fluorescence microscopy (blue excitation showing orange cells and green excitation showing red cells) for depths <100 m (Fig. 4A, top). At depths >100 m, there was a discrepancy between orange and red cell counts, whereas flow cytometry counts were very close to orange cell counts. This difference in the estimation of total *Synechococcus* may stem from the presence of chlorophyll-containing cells that resemble *Synechococcus* in size and morphology but lack PE. In the flow cytometer counts, we observed an abundance of cells scored as picoeukaryotes, similar to red fluorescent cells at this depth, that may account for the difference in numbers (data not shown), or the red fluorescent cells could also be *Prochlorococcus.*

Figure 4B (top) shows the proportion of positive and false-positive cells for each individual filter, normalized to microscope red cell counts. At depths <100 m, we observed that the proportion of false-positive cells was relatively low and constant, whereas the CC9605 serotype was clearly detectable at 20 and 40 m. For depths >100 m, we observed a very high proportion of positive AND false-positive cells. This population(s) is (are) clearly not the CC9605 serotype, because there was cross-reaction with both immune and preimmune antiserum. When the proportions of positive and false-positive cells were normalized to flow cytometry counts (or orange cell counts; data not shown) rather than red fluorescent counts, the samples from depths >100 m resulted in ≥1,000% positive and false positives, whereas there was no significant change for samples from depths <100 m. This suggests that the positive and false-positive populations at depths >100 m are different from what we score as *Synechococcus.* Of interest, at depths <100 m, the picoeukaryotes were more abundant than *Synechococcus,* and the size of the false positives appeared to be larger than expected for *Prochlorococcus.* Thus, we hypothesize that the false positives are due to picoeukaryotes, but we cannot rule out a subpopulation of *Prochlorococcus.*

For the statistical analysis, we considered only the samples from <100 m with the corrected values (subtraction of controls at each depth). For Sta. 77.100, there was a significant difference between depths when proportions were normalized to red fluorescence counts (Fig. 4C, top) or flow cytometry counts (not shown). Samples from 20 m had a significantly higher relative abundance of CC9605 serotype than other depths.

Sta. 93.110 was very similar overall to Sta. 77.100. Total counts estimated by microscopy and flow cytometry were congruent. However, at depths >100 m, there was again a discrepancy between red cell counts and orange cell or flow cytometry counts (Fig. 4A, bottom). Again, there was an increase in cells scored as picoeukaryotes in flow cytometry (data not shown). The proportion of positive cells at shallow depths at Sta. 93.110 was lower than at Sta. 77.100 (Fig. 4B). The corrected values for the proportion of positive cells showed statistically significant differences between depths, with the highest values at 25 m, and the same result was obtained when the positive cells were normalized to total counts by red fluorescence (Fig. 4C, bottom) or flow cytometry (not shown). Analyses of the depth profiles at the two

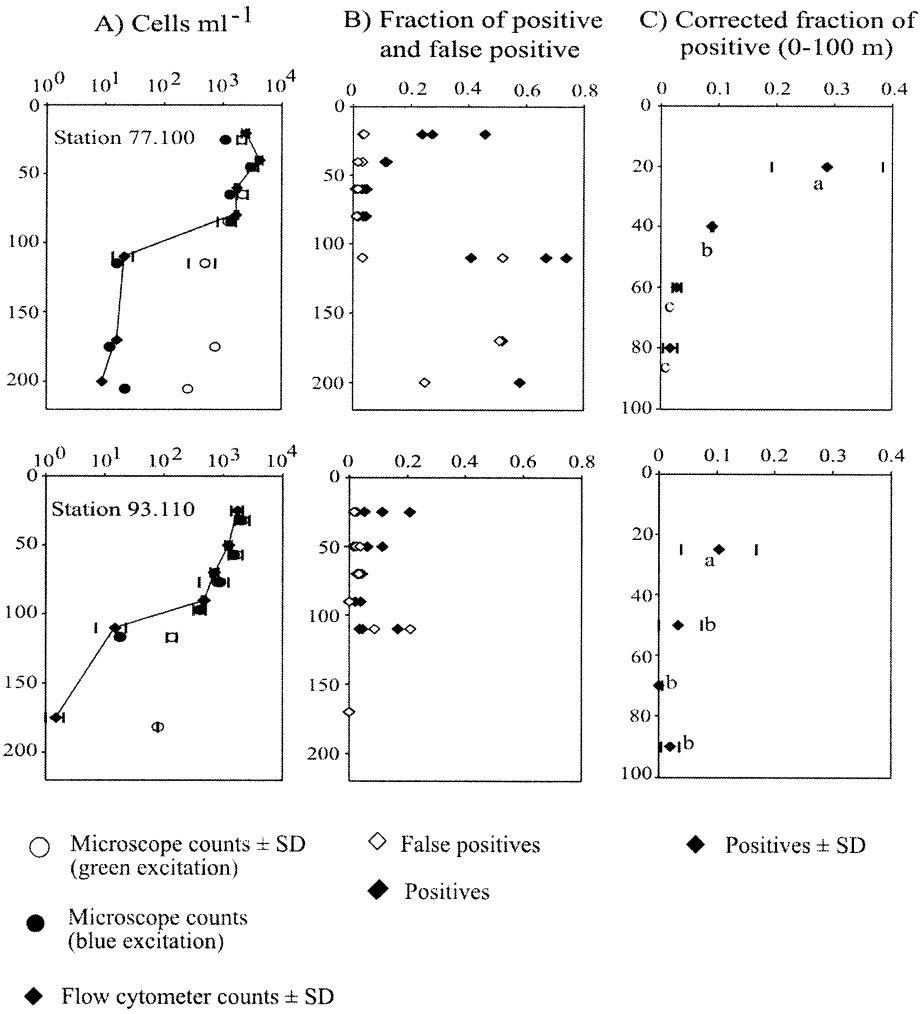

Fig. 4. Total *Synechococcus* and relative abundance of CC9605 serotype in a vertical profile from cruise NH9809 at Sta. 77.100 (top) and Sta. 93.110 (bottom). (A) Flow cytometry and microscope counts (± standard deviation). Diamonds: total *Synechococcus* counts obtained by flow cytometry, open circles: microscope counts under green excitation (± standard deviation), filled circles: counts under blue excitation. Note that some circles overlap. (B) Raw data representing the fraction of positive cells per filter (filled diamonds) and false-positive controls (open diamonds). Each symbol represents an independent filter replicate. (C) Corrected data (subtraction of false positives per depth, ± standard deviation). Different letters represent statistical differences by one-way ANOVA.

oligotrophic stations in the California Current indicated that CC9605 serotype was more abundant at shallow depths during September 1998, when the water column was strongly stratified.

Samples collected along an offshore-inshore transect in CalCOFI line 83 were used to assess (2), that the CC9605 serotype is relatively more abundant offshore than near shore. The controls with preimmune serum showed a low proportion of false positives along the line 83 transect (1.3% ± 1.26, $n = 10$). The CC9605 serotype was relatively more abundant at Sta. 83.110, located offshore. Sta. 83.100, also considered offshore, had a lower relative abundance of CC9605 serotype, and at Sta. 83.80 the abundance was similar to preimmune controls (Fig. 5B). Sta. 80.51 showed an average of 1.4% of positives, but the preimmune controls did not show false-positive cells. Because of the relatively higher total *Synechococcus* abundance at this site compared with the offshore stations, the 1.4% of positive cells may be significant, considering the CC9605 serotype population size. However, considering a global value for the false-positive cells among the five stations, 1.4% is not different from preimmune controls. Unexpectedly, at Sta. 83.42 we encountered a relatively high abundance of the CC9605 serotype with a low abundance of false positives. When the corrected proportion of positive cells at each station was analyzed by ANOVA, it was found that there were statistically significant differences between offshore Sta. 83.110 and inshore Sta. 80.51 and 83.80, whereas Sta. 83.42 was similar to Sta. 83.110 when cells were normalized to red fluorescent counts (Fig. 5C). However, when normalization

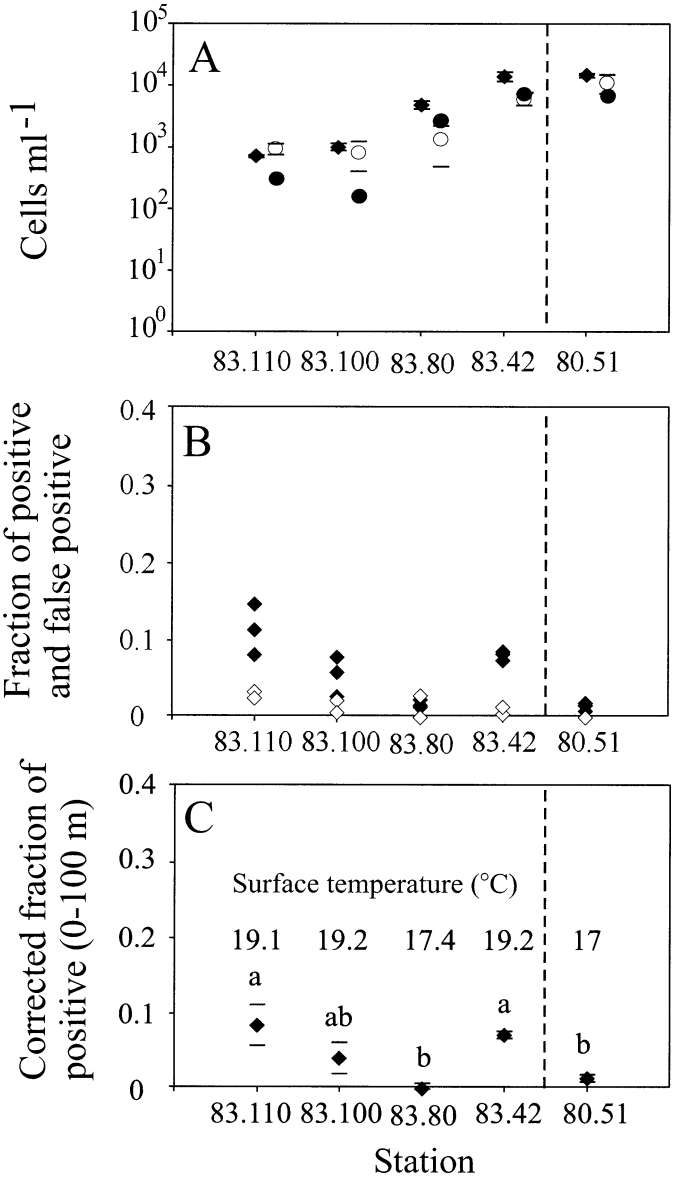

Fig. 5. Total *Synechococcus* and relative abundance of CC9605 serotype in surface samples in an offshore-inshore transect along line 83 and Sta. 80.51 (separated by a dashed line) during cruise NH9809. (A) Flow cytometry and microscope counts (± standard deviation). Diamonds: total *Synechococcus* counts obtained by flow cytometry, open circles: microscope counts under green excitation (± standard deviation), and filled circles: counts under blue excitation. Note that some circles overlap. (B) Raw data representing the fraction of positive cells per filter (filled diamonds) and false-positive controls (open diamonds). Each symbol represents an independent filter replicate. (C) Corrected data (subtraction of false positives per depth, ± standard deviation). Different letters represent statistical differences by one-way ANOVA. In situ surface temperatures are indicated for each site.

was done to flow cytometry counts, Sta. 83.42 was similar to Sta. 83.100 (not shown). The presence of the CC9605 serotype along line 83 was associated with in situ temperatures of 19°C, whereas at stations with low relative abundance the temperature was 17°C (Fig. 5C). These in situ surface temperatures corresponded to isothermal contour lines drawn for 10-m depth temperatures measured during the cruise (Scripps Institution of Oceanography 1999), but other hydrographic characteristics such as nitrate, phosphate, and chlorophyll were different between the coastal Sta. 83.42 and most of the offshore sites (Table 2).

We also analyzed a vertical profile at the more inshore Sta. 93.40 to evaluate the possibility of finding an inshore subsurface sample with a high relative abundance of CC9605 serotype. At this site, overall *Synechococcus* abundance was higher than the offshore stations (Table 2). The proportion of positive and false-positive cells were low along the vertical profile (≤3%), and there were no statistically significant differences between depths (data not shown), which again supports the hypothesis that CC9605 serotype is typically more abundant at more oligotrophic offshore sites.

Discussion

The IF technique has proved to be helpful for the identification and enumeration of cells with similar antigenic characteristics in aquatic environments, but the data must be interpreted cautiously. Using whole-cell polyclonal antibodies, IF has been used to identify bacterial groups with similar physiological adaptations (i.e., ammonia- and nitrite-oxidizing bacteria; Ward and Perry 1980; Ward and Carlucci 1985); however, morphological diversity has been observed among strains with similar antigenicity (Ward and Carlucci 1985). Therefore, the relationship between the taxonomic and serological groups is not always clear. Also, the cell-surface antigenicity of natural populations of the same morphospecies can be different in two geographic regions. For example, Lopez-Rodas and Costas (1997) found that similar *Microcystis aeruginosa* morphospecies from Denmark and Spain were antigenically different. In contrast, different morphospecies from the same locale showed similar antigenicity.

For marine *Synechococcus* polyclonal antisera to whole cells have been applied successfully to characterize isolates and identify serotypes in natural samples (Campbell et al. 1983; Campbell and Carpenter 1987; Campbell 1988; Campbell and Iturriaga 1988). However, for *Synechococcus*, phylogenetic data to correlate genotype with serotypes has been scarce; therefore, it has been difficult to determine whether strains from the same serogroup are monophyletic or they are polyphyletic and share common antigens. Recently, however, sequence data have supported the former. For example, some strains found in what has been referred to as the WH7803 serogroup (Campbell 1988; Campbell and Iturriaga 1988) (i.e., WH7803 and WH7805) are closely related as based on their *rpoC1* gene sequences (Palenik 1994; Ferris and Palenik 1998). However, some genetic diversity has been observed between strains of this serogroup (Wood and Townsend 1990). Therefore, this serogroup may be composed of monophyletic but highly divergent genetic lineages rather than a monophyletic group of closely related strains adapted to a specific niche.

A closer phylogenetic relatedness among strains in a serogroup can be observed with the WH8113 serogroup.

Campbell (1988) showed that most strains cross-reacting with this serum displayed a swimming phenotype. It has recently been reported that the marine motile *Synechococcus* form a tight phylogenetic cluster in their RNA polymerase gene sequence; thus, in this case there is an apparent correlation between serotype and genotype (Toledo et al. 1999). Of interest, this group of motile strains still shows a wide range of PUB : PEB ratios, demonstrating some phenotypic diversity (Toledo et al. 1999). Thus phylogenetic relationships, phenotypic diversity, and serotype are best considered together in providing an understanding of the microbiology of a microorganism.

In the present study, we prepared antiserum to whole cells of a strain of *Synechococcus* and characterized the cross-reactivity of that antiserum against strains whose phylogenetic relationships had been inferred from *rpoC1* sequence data. During the cross-reactivity tests, it was surprising to find the very high strain specificity of the antiserum, where only CC9705, a closely related strain based on *rpoC1* sequence data, cross-reacted with similar high intensity.

The relatively high specificity of CC9605 anti-serum may stem from multiple unique epitopes on different proteins on the cell surface of CC9605, given that Western blots of whole cell extracts showed cross-reaction with a large number of abundant protein bands. The localization of these proteins is unknown, however. The CC9605 antiserum likely only has access to the cell surface of fixed cells, and specificity may be due instead to a single highly abundant epitope on the cell surface. This epitope would likely be antigenic sugar moieties on an abundant glycoprotein. In marine *Synechococcus,* the abundant cell-surface protein SwmA, which is present in motile strains, provides an example of a highly abundant protein that is glycosylated (Brahamsha 1996). A polyclonal antiserum raised against this protein shows high specificity for motile strains in whole-cell immunofluorescence assays (Brahamsha and Ratliff, unpubl. data). It is possible that CC9605 similarly has an abundant cell surface protein, albeit for some function other than motility. If so, this protein was missing or highly divergent in related strains (clade 2; Ferris and Palenik 1998) that we tested.

CC9605 antiserum and IF was sensitive enough to detect variations in the relative abundance of this serotype along depth profiles and offshore-inshore transects. The offshore Sta. 77.100 and 93.110 were very similar hydrographically, and both showed a higher abundance of CC9605 at shallow depths within the surface isothermal layer. This finding supports laboratory evidence that suggested the preference of CC9605 for growth under conditions that simulate shallow depths such as high white-light and 21°C (compared with low blue-light and 19°C) (Palenik et al. unpubl. data). Additional evidence for a surface-offshore niche for strains CC9605 and CC9705 is that these were isolated from 51 and 30 m, respectively, in consecutive years, and clone libraries from similar shallow depths have shown closely related sequences (Palenik 1994; Ferris and Palenik 1998). Although IF, strain isolation, and clone libraries are subject to a number of well-known methodological biases, all of them consistently show the same pattern. There is, therefore, substantial evidence supporting the hypothesis that CC9605 is a surface-adapted serotype, although additional physiological evidence is clearly needed to prove this hypothesis.

In the CalCOFI sampling grid, off the coast of California, three marine regimes have been identified: southern coastal, northern coastal, and offshore-oligotrophic (Hayward and Venrick 1998). These regimes have usually a characteristic phytoplankton community, but some stations can be prone to changes in the flora composition as a result of local hydrodynamics, such as the formation of meanders and offshore tongues of warm waters inshore (Venrick 1998). Sta. 77.100, 83.110, 83.100, 83.80, and 93.110 are included in the offshore regime. Sta. 93.40 is southern coastal but sometimes can be influenced by the offshore regime, as seen in low nutrient and chlorophyll concentrations and phytoplankton species composition. Sta. 83.42 is northern coastal, whereas Sta. 80.51 does not fall consistently in the northern coastal because of fluctuations in the California Current jet. To test the hypothesis of an offshore-abundant strain, we sampled along a northern coastal-offshore transect. We observed a higher relative abundance in the offshore sites but also at coastal Sta. 83.42. There are several explanations for the presence of an offshore serotype at coastal sites: (1) there are coastal *Synechococcus* strains with antigenicity similar to CC9605 but from different genetic lineages that we do not have in our culture collection, (2) CC9605 serotype grows equally well at coastal and offshore sites, or (3) the presence of an offshore population in coastal sites can be explained by horizontal transport originated by local hydrographic conditions. In the Southern California Bight, a coastal northward flow of offshore waters from the central North Pacific can be originated by the merging of the latter with southward-flowing waters from the California Current generating the Ensenada Front (Venrick 2000). However, the dissimilar hydrographic characteristics between Sta. 83.42 and the offshore sites, the required time for advection of offshore water masses to inshore Sta. 83.42 and the absence of CC9605 serotype in surface samples at southern coastal Sta. 93.40 do not support the advection hypothesis. We cannot distinguish yet between (1) and (2) above. Thus, because of the results for Sta. 83.42, the data obtained at line 83 do not support the hypothesis that CC9605 serotype is found uniquely at offshore sites.

In the California Current, the CC9605 serotype was never >29% of the *Synechococcus* community, which indicates that there is significant in situ diversity and that other serogroups are present in shallow depths. The CC9605 antiserum does not cross-react with all members of clade 2 defined by *rpoC1* gene sequence (Ferris and Palenik 1998). Therefore, it might be that a number of strains of this clade together dominate the offshore surface environment or that other clades seen in *rpoC1* libraries (e.g., the motile *Synechococcus* seen in Ferris and Palenik 1998) are also important components of the community. The characterization of other *Synechococcus* strains and especially the development of specific probes for these strains will be needed to assess *Synechococcus* community structure and its response to environmental changes.

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