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# An Immunological Approach To Detect Phosphate Stress in Populations and Single Cells of Photosynthetic Picoplankton†

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**In the marine cyanobacterium *Synechococcus* sp. strain WH7803, PstS is a 32-kDa cell wall-associated phosphate-binding protein specifically synthesized under conditions of restricted inorganic phosphate ($P_i$) availability (D. J. Scanlan, N. H. Mann, and N. G. Carr, Mol. Microbiol. 10:181–191, 1993). We have assessed its use as a potential diagnostic marker for the P status of photosynthetic picoplankton. Expression of PstS in *Synechococcus* sp. strain WH7803 was observed when the $P_i$ concentration fell below 50 nM, demonstrating that the protein is induced at concentrations of $P_i$ typical of oligotrophic conditions. PstS expression could be specifically detected by use of standard Western blotting (immunoblotting) techniques in natural mesocosm samples under conditions in which the N/P ratio was artificially manipulated to force P depletion. In addition, we have developed an immunofluorescence assay that can detect PstS expression in single *Synechococcus* cells both in laboratory cultures and natural samples. We show that antibodies raised against PstS cross-react with P-depleted *Prochlorococcus* cells, extending the use of these antibodies to both major groups of prokaryotic photosynthetic picoplankton. Furthermore, DNA sequencing of a *Prochlorococcus pstS* homolog demonstrated high amino acid sequence identity (77%) with the marine *Synechococcus* sp. strain WH7803 protein, including those residues in *Escherichia coli* PstS known to be directly involved in phosphate binding.**

In oligotrophic oceans, the spatial and temporal distribution of photoautotrophic picoplankton is tightly controlled by the availability of nutrients. Although nitrogen is widely considered to be the major limiting nutrient in the marine environment (see, for example, references 19 and 20), there is now good evidence to support a role of iron in limiting phytoplankton biomass in high-nitrate, low-chlorophyll areas of the equatorial Pacific and the Antarctic (10, 40, 41). Phosphate is generally considered to be the limiting nutrient in the eastern Mediterranean Sea (32), but it has also been suggested to limit phytoplankton growth in the Sargasso Sea (11) as well as the Chesapeake Bay (17), which is subject to freshwater input. Over longer time scales, it has been proposed that P limits new and export production in the North Pacific subtropical gyre, alternating with nitrogen (N) limitation over 10-year periods (28, 29). Moreover, recent modelling data suggests that P-limited marine productivity plays a role in redox stabilization of the atmosphere and oceans over geological time scales (54).

The interpretation of the biological implications of restricted nutrient availability for the picoplankton must take into account the fact that the term nutrient limitation conventionally applies to the restriction of further increase in biomass of an organism by virtue of an inability to transport the nutrient in question when the environmental concentration falls below a certain critical threshold value. However, the transition from growth to nongrowth at the concentration threshold is preceded by a series of adaptive responses on the part of the organism, which may include the induction of a high-affinity transport system, utilization of reserves, and adaptation to utilization of alternative sources of the nutrient, etc. This period of nutrient stress, which may be referred to as nutrient-depleted growth, is clearly distinguished from genuine nutrient limitation but may exert a significant effect on competition with other organisms and on productivity, etc. Much recent effort has been directed towards the development of direct methods for assessing the nutrient status of phytoplankton, circumventing the previous indirect methods which could not yield information regarding specific groups of phytoplankton. Of great potential are the use of biophysical methods for measuring photochemical energy conversion efficiency and identifying general nutrient stress (22, 23, 31), cell cycle variables to monitor nutrient bioassays (55, 56), or diagnostic molecular markers, polypeptides expressed specifically during a particular nutrient limitation (21, 34–36, 45, 50, 51). The latter approach has led to the biochemical characterization of the phytoplankton response to nutrient limitation and identification of candidate proteins which might be useful as markers of the specific nutrient-depleted state. During iron depletion, for example, many organisms replace ferredoxin with flavodoxin, and it is possible that the presence or absence of flavodoxin in natural marine diatom assemblages can be used to infer iron limitation. Certainly, natural populations driven to iron depletion express flavodoxin (35), while along a 900-km east-west transect in an area of the northeastern subarctic Pacific, changes in the degree of iron stress have been demonstrated by use of flavodoxin as a biochemical marker (34).

We have described previously a molecular characterization of the inorganic phosphate ($P_i$) acquisition system in the marine *Synechococcus* sp. strain WH7803 (50). This study identified a phosphate-binding protein (PstS) homolog of the high-affinity (Pst) $P_i$ transport system of members of the family *Enterobacteriaceae* (61) present after growth in P-depleted medium. Immunological and nucleic acid probing showed the existence of PstS homologs in all the phycoerythrin-containing

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marine *Synechococcus* species examined and suggested that "immunological interrogation" of natural cyanobacterial populations with PstS antibodies could serve as an indicator of P depletion in these organisms. We introduce the term immunological interrogation to identify a specific strategy in which antibodies raised against a diagnostic protein are used to assay the physiological or nutritional state of natural populations or single cells of an organism. We report here data that extends the use of PstS as a potential diagnostic marker for the P status of marine picoplankton. In particular, we have developed a single-cell immunofluorescence assay which permits analysis of the P status of single picoplankton cells, allowing separation of an individual cell response within a species from those of the population and hence the whole phytoplankton-bacterium assemblage. Such an assay provides an ideal means for more accurately determining the spatial and temporal changes in the P-depleted physiology of these organisms by combining the specificity and sensitivity of immunological and fluorescence techniques.

## MATERIALS AND METHODS

**Organisms and culture conditions.** *Synechococcus* sp. strain WH7803, a member of marine cluster A (59), originally isolated from the North Atlantic (33°45′N, 67°30′W) (60), was routinely maintained in an axenic culture at 25°C, under continuous illumination (5 to 36 μmol of photons $m^{-2}$ $s^{-1}$) in an artificial seawater medium (ASW) (62). Cultures were checked for contamination at regular intervals by microscopy. P-depleted conditions (N/P ratio, 500:1) were obtained by removing late-exponential-phase cells from ASW medium, in which the $P_i$ concentration was 120 μM, and diluting 1 to 10 into ASW-P medium, which lacks added $P_i$; at least three generations were monitored until visible alterations in phycobilisome composition indicated nutrient stress (50). *Synechococcus* sp. strain WH7803 was also grown in ASW-P medium with various organic phosphates (cyclic AMP [cAMP], dCTP, *p*-nitrophenyl phosphate, glucose-6-phosphate, and glycerol phosphate) as the sole source of P; concentrations of 172 μM, which were equivalent to the $K_2HPO_4$ concentration in ASW medium, were routinely used. Growth, assessed as the change in optical density at 750 nm, and the $P_i$ concentration in the medium were determined at daily intervals. Samples for dissolved-$P_i$ analysis were taken immediately after harvesting of cells by centrifugation at 10,000 × *g* for 2 min at room temperature; analysis used a standard colorimetric method in which a phosphomolybdate complex is used to shift the absorption maximum of malachite green (25). Assays were performed daily on the supplemented ASW-P medium to determine the production of dissolved $P_i$ from organic phosphate hydrolysis. Control flasks were set up to measure the extent of hydrolysis of organic phosphates in ASW-P medium in the absence of *Synechococcus*; this was found to be negligible.

*Prochlorococcus* strains from two different geographic regions were used in this study. Both are clonal isolates obtained by serial dilution (8). CCMP1378 is a MED strain derivative isolated from a 5-m depth in the Mediterranean Sea by D. Vaulot and F. Partensky, and CCMP1375 is a SARG strain derivative isolated from a 120-m depth in the Sargasso Sea by B. Palenik. Cultures were grown routinely at 19 ± 1°C in polycarbonate tissue culture bottles (Nalgene Brand Products, Rochester, N.Y.) under continuous blue light at 7 μmol of photons $m^{-2}$ $s^{-1}$, by using white fluorescent lights wrapped with a moonlight blue LEE filter (Panavision) in nutrient-poor seawater modified as described previously (46). Strains were grown under P-depleted conditions in which the P concentration was 0.3 μM $Na_2HPO_4$ and the N/P ratio was 500:1 as described elsewhere (46).

Heterotrophic marine bacterial strains were isolated off Plymouth Sound, Plymouth, United Kingdom, and from station A (29°28′N, 34°55′E) in the Gulf of Aqaba, Red Sea. They are all gram-negative rods but otherwise uncharacterized. Strain designations are as follows: 040 (probably a *Cytophaga* sp.), PL01, PL02, PL03, PL04, PL07, and PL08 were from Plymouth, and RS-1, RS-2, and RS-4 were from the Red Sea. We also obtained two isolates from the Baltic Sea, H5 and H54 (a gift of K. Moebus). Cultures were grown in ASW or ASW-P medium containing 0.2% (wt/vol) glucose or in 75% ASW or ASW-P medium containing 5 g of peptone per liter, 1 g of yeast extract per liter, and 0.05% (wt/vol) Casamino Acids at 25°C.

**Induction of the *Synechococcus* PstS polypeptide as a function of $P_i$ concentration in the medium.** *Synechococcus* sp. strain WH7803, grown previously in ASW medium, was inoculated into ASW-P medium at a dilution of 1/10, and initial $P_i$ concentrations were determined colorimetrically (see above). At zero time, approximately 100 μCi of carrier-free $^{32}P_i$ (specific activity, 9,000 Ci $mmol^{-1}$) was added and the radioactivity was measured in 100 μl of supernatant following removal of cells by centrifugation at 10,000 × *g* for 5 min at room temperature. $P_i$ concentrations in the medium at each time point (~24-h intervals) were correlated from $^{32}P_i$ measurements of the supernatant, with reference to the chemically determined $P_i$ concentration and counts per minute at time zero. Correction was made for the decay of $^{32}P_i$ specific activity over time. At each time point, cell samples (2 ml) were harvested by centrifugation and the cell pellet was resuspended in 200 μl of 0.5 M Tris-HCl (pH 6.8) and stored frozen at −20°C before sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (immunoblot) analysis (see below). SDS-PAGE gels were loaded on an equal cell number basis.

**Southern blotting and DNA hybridization.** *Prochlorococcus* and *Synechococcus* chromosomal DNAs, extracted as described previously (49), were digested with various restriction enzymes under conditions recommended by the manufacturer (Gibco BRL). Southern blotting of the DNA onto nylon or nitrocellulose membranes (Amersham) was performed as described by Maniatis et al. (37). A 571-bp *Eco*RI-*Bgl*II *pstS* gene fragment, containing only the PstS coding sequence, was used as a hybridization probe and labelled to high specific activity with [α-$^{32}$P]dCTP by the random priming method (16). Prehybridization and hybridization of nitrocellulose filters was carried out at low stringency in 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaH_2PO_4$, and 1 mM EDTA [pH 7.4]), 5× Denhardt's solution (37), 0.1% (wt/vol) SDS, and 100 μg of herring sperm DNA (Sigma) per ml at 55°C. Blots were washed in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% SDS at room temperature for 10 min, 50°C for 10 min, and 55°C for 30 min, followed by 65°C for 20 min.

**DNA sequencing and computer analysis.** The *Prochlorococcus pstS* gene was cloned from a 5- to 11-kb *Bgl*II partial genomic library of the TATL1 strain on an approximately 7-kb *Bgl*II fragment. A 0.855-kb *Hin*dIII subclone was subsequently identified, and the nucleotide sequence was determined by the dideoxy chain termination method with a Sequenase kit (U.S. Biochemicals). The sequence was completed by using specific primers to sequence parts of the entire fragment cloned into M13mp18. Computer analyses of nucleic acid and protein sequences were carried out via the Seqnet computer facility at the Daresbury Laboratory with the University of Wisconsin Genetics Computer Group software package (13).

**SDS-PAGE analysis.** Picoplankton grown to the exponential phase were harvested by centrifugation at 10,000 × *g* and resuspended in 50 μl of 0.5 M Tris-HCl (pH 6.8), and cells were disrupted by boiling in SDS sample buffer (33) (1% SDS [wt/vol] final concentration) for 5 min. Protein concentrations were determined by use of the Bio-Rad DC protein assay with bovine serum albumin (BSA) as the standard. Extracts were loaded on an equal protein basis (25 to 100 μg per lane) or by using equal cell numbers ($5 \times 10^7$ cells total). Proteins were separated on either 12.5% slab or 10 to 30% gradient polyacrylamide gels and transferred electrophoretically to nitrocellulose before Western blotting with antiserum raised against PstS as described previously (50).

**Mesocosm experiments.** Natural *Synechococcus* populations were sampled during June and July 1995 in large seawater enclosures, with volumes of 11 $m^3$ each (14), located in a fjord 20 km south of Bergen, Norway. At 1400 h on 7 June (defined as day 0 of the experiment), nutrient additions were made to selected enclosures. Nitrate was added as sodium nitrate ($NaNO_3$; Analar), phosphate was added as potassium phosphate ($KH_2PO_4$; Analar), and silicate was added as sodium metasilicate ($NaSiO_3 \cdot 5H_2O$). Enclosures 1 and 2 were used as controls and had no additions; enclosure 3 was fertilized with N and P at concentrations of 15 and 1 μM, respectively, plus silicate at 10 μM; enclosure 5 was fertilized with N and P alone at concentrations of 15 and 1 μM; respectively; and enclosures 7 and 8 were manipulated to simulate conditions of P depletion, i.e., for enclosure 7, 15 μM N was added, and for enclosure 8, 15 μM N and 0.2 μM P were added. At 1200 h on day 18 (25 June; enclosure 7) or day 21 (28 June; enclosure 8), additions were made to produce nutrient concentrations of 1 μM P. On addition of the nutrients, the air uplifts were switched on to begin the mixing process and create a homogeneous water column. Ten percent of the enclosure volume was renewed daily with seawater from the fjord, and to compensate for the loss of nutrients by this renewal of water, 10% of the initial experimental concentrations of nitrate and phosphate were also added daily. Nutrients were added at 1200 h, after completion of the daily sampling program. Water samples for nutrient analysis were taken from the mesocosms between 0730 and 0830 h daily and analyzed with a Skalar autoanalyzer. Phytoplankton were concentrated from water samples of 50- to 100-liter volumes by tangential flow filtration; three 0.3-μm-pore-size screen channel Omega membrane filters gave a total surface area of 0.675 $m^2$ and were used in conjunction with minisette hardware supplied by Flowgen Instruments Ltd., Kent, United Kingdom. The samples were concentrated to a volume of 150 ml, centrifuged at 7,500 × *g* for 10 min at room temperature, and frozen at −20°C. Proteins were extracted as described above. *Synechococcus* counts were made by filtering 10-ml seawater samples onto 0.2-μm-pore-size Nuclepore filters. Filters were mounted in immersion oil on a microscope slide, and orange-fluorescing *Synechococcus* cells were enumerated with a Nikon Labophot 2A fluorescence microscope fitted with a G-2A filter block (excitation, 510 to 560 nm; 590-nm barrier filter). Chlorophyll concentrations were measured daily in 100- or 250-ml water samples, which were filtered in triplicate onto GF/F glass fiber filters. Filters were placed in glass scintillation vials and extracted at 4°C overnight with 10 ml of 90% acetone, and samples were read with a Turner design fluorometer.

**PstS immunofluorescence.** Whole-cell immunofluorescence assays were performed on glass slides with *Synechococcus* cells from both natural mesocosm samples and laboratory cultures. Cells were harvested by centrifugation (3 min, 10,000 × *g*, room temperature), resuspended in 100 μl of 1× phosphate-buffered

saline (PBS; 3× PBS solution was made by mixing 390 mM NaCl–30 mM $Na_2HPO_4$ and 390 mM NaCl–30 mM $NaH_2PO_4$ to a final pH of 7.2) at a final concentration of $10^9$ cells $ml^{-1}$, and decolorized by heating at 65°C for 60 min to reduce endogenous phycoerythrin fluorescence. Cells were harvested by centrifugation as described above, washed once in PBS, resuspended in 1/10 volume (10 μl) of PBS, and then permeabilized by the addition of SDS and sodium hydroxide to final concentrations of 0.1% (wt/vol) and 0.1 M, respectively, and incubation at 37°C for 10 min. Cells were washed once in PBS and resuspended in 100 μl of the same buffer. Three volumes of freshly prepared 4% paraformaldehyde in PBS were added, and the cells were fixed on ice for 60 min. Cells were pelleted by centrifugation, washed once in 100 μl of PBS, and resuspended in 100 μl of the same buffer. One microliter of this solution was spotted onto a 12-well (5-mm-diameter) immunofluorescence slide (Scientific Laboratory Supplies, Nottingham, United Kingdom) and air dried. Slides were dehydrated by immersion in 100% methanol for 5 min followed by a 30-s immersion in 100% acetone, both at 4°C. Air-dried slides were incubated in 10 μl of blocking solution (0.1% [vol/vol] Triton X-100, 2% BSA in PBS) for 15 min at room temperature. Blocking solution was then aspirated off, PstS affinity-purified primary antibody (diluted 1:50 in blocking solution) was added, and the slides were incubated for 1 h at room temperature. Each well was then washed three times in 10 μl of PBS before being reblocked for 15 min as described above. Secondary antibody, i.e., anti-rabbit immunoglobulin fluorescein-linked whole antibody from donkey (Amersham), was added and diluted 1:50 in blocking solution, and the slides were incubated in the dark for 60 min at room temperature. Each well was again washed three times in PBS, before mounting in 50% (vol/vol) PBS-glycerol and examination with a Nikon Labophot 2A fluorescence microscope fitted with a B-2A filter block (excitation, 450 to 490 nm; 520-nm barrier filter). Photomicrography involved the use of a 35-mm camera attachment (Nikon FX-35DX) and Ektachrome 160 ASA transparency film. Control slides were examined in parallel; these slides contained either fixed cells without incubation in primary antisera or fixed cells incubated with preimmune rabbit antisera.

Several agents were initially assessed for their ability to adequately permeabilize *Synechococcus* cells. These included SDS (0.01 to 1% [wt/vol]) plus NaOH (1 mM to 1 M), SDS (1% [wt/vol]), Triton X-114 (1% [vol/vol]), dodecyl maltoside (0.84% [vol/vol]), lysozyme (2 mg $ml^{-1}$) (12), saponin (0.1 to 1% [wt/vol]), and toluene (5% [vol/vol]). To examine the effectiveness of permeabilization during optimization of the immunofluorescence protocol, which was particularly important for time course experiments for determining cell permeabilization with the various agents, cells were treated with DNase (final concentration, 0.1 mg $ml^{-1}$) at 37°C for 1 h prior to staining with DAPI (4′,6-diamidino-2-phenylindole; 5 μg $ml^{-1}$) for 10 min at room temperature. Stained (nonpermeabilized) and unstained (permeabilized) cells were examined by fluorescence microscopy as described above with a UV-2A filter block (excitation, 330 to 380 nm; 420-nm barrier filter).

**Affinity purification of PstS antibodies.** A simple procedure was developed for rapid purification of PstS antibodies by use of a Western blotting technique, based on a published method (24). Since the PstS polypeptide was known to be localized to the cell wall, a preparative SDS-polyacrylamide gel was run with a *Synechococcus* sp. strain WH7803 cell wall fraction, isolated from total membranes by differential solubility in Triton X-100 (50, 51), from cells grown under P-depleted conditions and ~15 μg of protein per lane. Polypeptides were transferred electrophoretically to nitrocellulose, and the filter was stained with Ponceau S (0.5% [wt/vol] in 5% trichloroacetic acid) to reveal all polypeptides. A region of the filter containing the PstS polypeptide was cut out, washed in PBS to remove the stain, and blocked and treated with primary antibody in the normal way (50). After being washed three times for 10 min in 0.1% (vol/vol) Triton X-100–PBS, the filter was cut into small pieces with a scalpel and 0.9 ml of 100 mM glycine (pH 2.5) was added. After incubation for 10 min at room temperature, 0.1 ml of 1 M Tris-HCl (pH 8.0) was added to neutralize the solution, which was stored at either 4 or −20°C in 100-μl aliquots. In addition, the crude primary antiserum was also subjected to a preabsorption scheme by preabsorption at 4°C overnight against a concentrated extract of cells grown under P-replete conditions. Both antisera (affinity purified and preabsorbed) were assayed for their cross-reactivity by Western blotting and by in situ labelling with fluorescein isothiocyanate (FITC). Western analysis showed that the affinity-purified antibody gave a specific cross-reaction against the PstS polypeptide only, whereas the preabsorbed antiserum still cross-reacted against a potential GroEL homolog as well as PstS. Affinity-purified antibody was subsequently used in immunofluorescence assays.

**Nucleotide sequence accession number.** The nucleotide sequence of the *Prochlorococcus pstS* gene has been deposited in the GenBank database under accession no. U75514.

## RESULTS

**Induction of the *Synechococcus* PstS polypeptide as a function of $P_i$ concentration in the medium.** The level of $P_i$ causing induction of the PstS polypeptide was assessed by using $^{32}P_i$ as a tracer. Following an initial colorimetric measurement of the $P_i$ concentration in the medium, the radioactivity remaining in the medium was measured as growth progressed. Western analysis of cell extracts taken at approximately 25-h intervals indicated that *Synechococcus* sp. strain WH7803 induced the synthesis of the PstS polypeptide only when $P_i$ concentrations declined below 50 nM (Fig. 1a). In comparison, the PstS polypeptide could be detected throughout the 30 days of growth following transfer of P-depleted *Synechococcus* sp. strain WH7803 to ASW-P medium; however, there was no further induction of the polypeptide (Fig. 1b). To assess whether PstS induction was a response to $P_i$ concentrations in the medium per se rather than to changes in the growth rate, *Synechococcus* sp. strain WH7803 was grown in ASW medium, i.e., under P-replete conditions, under irradiance with 70, 10, and 1.5 μmol of photons $m^{-2}$ $s^{-1}$, which resulted in specific growth rates of 0.037, 0.016, and 0.0007, respectively. Such conditions did not significantly induce PstS expression (data not shown).

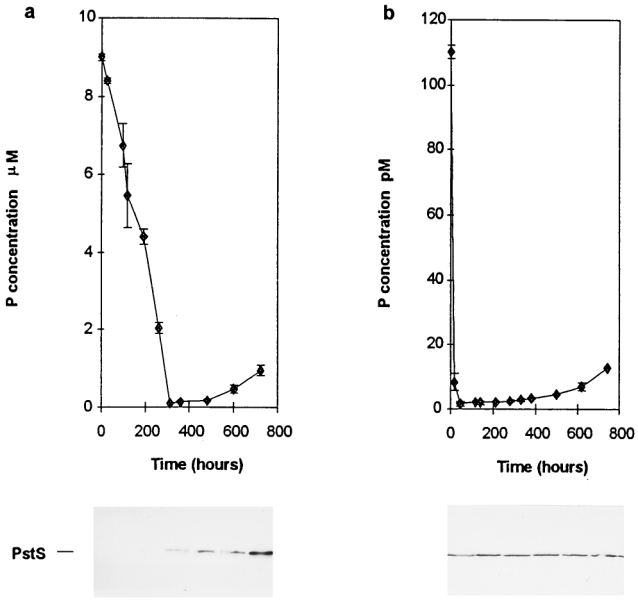


FIG. 1. Time course of $P_i$ concentration in the medium and PstS induction during growth of *Synechococcus* sp. strain WH7803 transferred from P-replete to P-depleted medium (a) and P-depleted to P-depleted medium (b). Induction of the *Synechococcus* sp. strain WH7803 PstS polypeptide as a function of the external $P_i$ concentration was determined by Western blotting with PstS antibodies of total cell extracts taken at each time point and subsequently separated by SDS-PAGE on a 10% gel. Standard deviations of the means ($n$ = 2) are indicated by the solid vertical lines.

**Organic phosphate repression of PstS synthesis in *Synechococcus* sp. strain WH7803.** PstS produced by *Synechococcus* sp. strain WH7803 under P-depleted conditions was repressed when the cells were grown in ASW-P medium supplemented with 172 μM dCTP, *p*-nitrophenyl phosphate, glucose-6-phosphate, or glycerol phosphate (Fig. 2); typically, the growth rate (μ) was 0.011 $h^{-1}$ under these growth conditions. *Synechococcus* sp. strain WH7803 displayed little or no growth in medium containing cAMP as the sole source of phosphorus; the growth rate was 0.004 $h^{-1}$ in this medium as compared to 0.032 $h^{-1}$ obtained for growth in P-replete medium. Under this condition, the PstS polypeptide was induced or derepressed in a fashion similar to that observed with cells grown under P-depleted conditions (Fig. 2, lane c), presumably reflecting the inability of *Synechococcus* sp. strain WH7803 to utilize cAMP as a sole source of phosphorus.

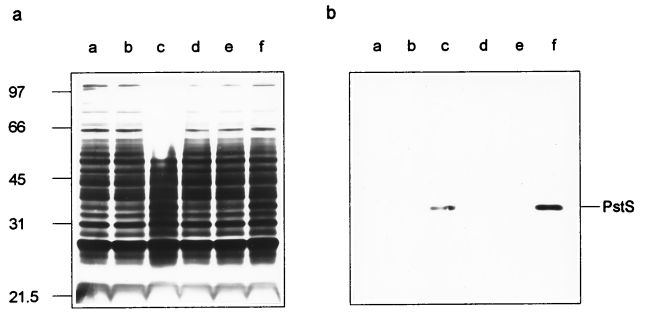


FIG. 2. (a) A silver-stained SDS-polyacrylamide gel of whole-cell extracts of *Synechococcus* sp. strain WH7803 grown in ASW medium in which $P_i$ was replaced by various organic phosphates. Phosphorus sources were dCTP (lane a), *p*-nitrophenyl phosphate (lane b), cAMP (lane c), glucose-6-phosphate (lane d), and glycerol phosphate (lane e). P-depleted medium was used as a control (lane f). The sizes of the molecular weight markers are indicated (in kilodaltons). (b) A Western blot probed with antibody raised against the PstS polypeptide of the SDS-polyacrylamide gel shown in panel a.

**Immunological cross-reaction of PstS in other picoplankton species.** Several isolates of heterotrophic marine bacteria were grown in batch culture under P-depleted or -replete growth conditions. Western analysis of cell extracts showed no cross-reaction with PstS antibodies for any of the heterotrophs examined under either growth condition (data not shown). Recent examination of the response of a marine diatom, *Thalassiosira weissflogii*, to P-depleted conditions also failed to detect a PstS homolog (13a). In contrast, Western analysis of cell extracts of two *Prochlorococcus* strains, both clonal isolates but genetically distinct and isolated from different geographical regions (49), showed strong cross-reactivity against the PstS antibody after growth in P-depleted medium (Fig. 3). Similarly, two *Synechococcus* isolates obtained from station A in the Red Sea showed strong and specific cross-reactivity with the PstS antibody only under P-depleted growth conditions (data not shown). Therefore, this antibody should have applicability for detecting the PstS antigen, and hence phosphate limitation, in prokaryotic picoplankton from several ocean provinces.

**Identification of the *pstS* gene in various *Synechococcus* and *Prochlorococcus* strains.** Cross-reaction of PstS antibodies with *Prochlorococcus* isolates contradicts earlier DNA hybridization work using the *pstS* gene as a probe (50), which failed to detect a *Prochlorococcus* homolog. More recent hybridization data resulting from the use of an internal *pstS* gene fragment to probe Southern blots of *Prochlorococcus* DNA, rather than a 1.43-kb *Eco*RI fragment used previously (which contained approximately 690 bp of upstream noncoding sequence), revealed homologous sequences at low stringency (Fig. 4, lanes q to s). A *Prochlorococcus pstS* homolog was subsequently cloned and sequenced from one of these strains, a tropical Atlantic isolate (TATL1). Comparison of this amino acid sequence with that obtained for *Synechococcus* sp. strain WH7803 showed significant homology (85% similarity and 77% identity), including identity at residues known to be directly involved in hydrogen bond formation between phosphate and the phosphate-binding protein in *Escherichia coli*. *PstS* gene homologs were also detected in marine *Synechococcus* strains isolated from the Mediterranean Sea as well as different isolates from the Sargasso Sea (Fig. 4).

**Expression of PstS in natural *Synechococcus* populations.** To bridge the gap between laboratory cultures and the natural environment, we conducted large-scale experiments in mesocosm enclosures at the Marine Biology Field Station, Bergen, Norway. These enclosures have been used previously to study

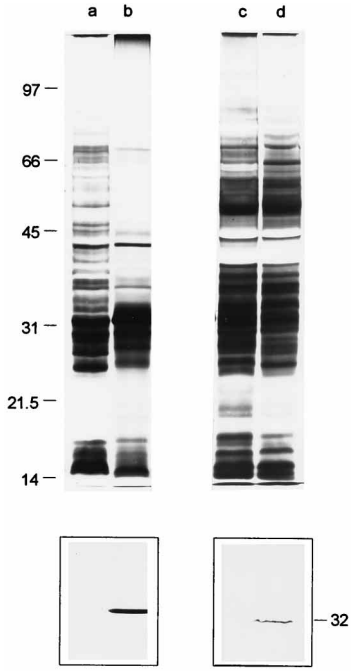


FIG. 3. (Upper panels) A silver-stained gel of whole-cell extracts of *Prochlorococcus* sp. strain CCMP1378 (MED [lanes a and b] and SARG [original strain; lanes c and d]) grown in P-replete (lanes a and c) and P-depleted (lanes b and d) medium. (Lower panels) Western blots corresponding to the gels in the upper panels showing cross-reaction of *Synechococcus* PstS antibodies with P-depleted *Prochlorococcus* SARG and MED strains. The sizes of the molecular weight markers are indicated on the left, and that of the cross-reacting polypeptide is indicated on the right (both in kilodaltons).

the effects of nutrient fertilization on phytoplankton physiology (15, 53) and appear both to be reproducible and to effectively simulate the phytoplankton community structure obtaining in the surrounding water. *Synechococcus* counts, phosphate and nitrate concentrations (Fig. 5 and 6), and chlorophyll concentrations were measured daily. The highest chlorophyll concentration occurred in enclosure 3 (day 4; 36.1 μg of

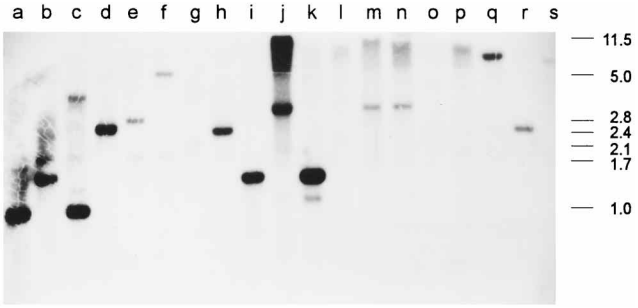


FIG. 4. An autoradiograph of a Southern blot of restriction enzyme digestions of DNA from various *Synechococcus* and *Prochlorococcus* strains after hybridization with a 0.571-kb *Eco*RI-*Bgl*II *pstS* gene fragment (the sizes of the λ *Pst*I markers are indicated in kilobases on the right). The DNA samples that were restricted with *Pst*I (lanes a to h), *Eco*RI (lanes i to p), and *Bgl*II (lanes q to s) are as follows: *Synechococcus* sp. strain WH7803 (lanes a and i), *Synechococcus* sp. strain WH8103 (lanes b and j), *Synechococcus* sp. strain WH8018 (lanes c and k), *Synechococcus* sp. strain WH8012 (lanes d and l), *Synechococcus* sp. strain Max01 (lanes e and m), *Synechococcus* sp. strain Max42 (lanes f and n), *Synechococcus* sp. strain Ros (lanes g and o), *Synechococcus* sp. strain MED (lanes h and p), *Prochlorococcus* sp. strain TATL1 (lane q), *Prochlorococcus* sp. strain SARG (lane r), and *Prochlorococcus* sp. strain CCMP1426 (lane s).

chlorophyll liter$^{-1}$), which was dominated by the diatoms *Skeletonema costatum* and *Leptocylindrus minimus* and the coccolithophore *Emiliania huxleyi* (13b). *Synechococcus* numbers fluctuated throughout the experimental period in enclosures 1, 3, 5, and 7; initial cell densities of approximately 100,000 cells ml$^{-1}$ (day 1) dropped to 20,000 cells ml$^{-1}$ around day 16 and increased gradually thereafter. The exception was enclosure 8, which showed a steady increase in *Synechococcus* numbers to 500,000 cells ml$^{-1}$ 3 days subsequent to $P_i$ addition at day 21, before declining rapidly (Fig. 5). Flow cytometric analysis of fixed samples failed to detect any prochlorophytes in the enclosures. P concentrations in the two P-depleted enclosures (7 and 8) remained low almost throughout the study; an exception was day 19 in enclosure 7 just after the addition of 1 μM $P_i$, although this P concentration was depleted rapidly. In contrast, nitrate concentrations in enclosures 7 and 8 remained high prior to $P_i$ addition on days 18 and 21, respectively. In comparing enclosures 7 and 8, the former retained significantly higher nitrate concentrations throughout the period prior to $P_i$ addition (Fig. 6). This correlates well with the absence of any added $P_i$ in this enclosure.

Expression of PstS was readily detected by Western blotting samples from enclosure 7 on day 17 and enclosure 8 on day 21, where the N/P ratio was specifically manipulated to simulate a P-depleted environment (Fig. 7). Subsequent $P_i$ fertilization of these enclosures resulted in strong repression of PstS synthesis (Fig. 7). Similar results were obtained when protein gels were loaded with equal cell numbers (data not shown). Interestingly, on day 19, low detectable PstS expression was observed in enclosure 1, which did not have any nutrient manipulation and hence represented in situ water conditions. At this time, $P_i$ concentrations in the enclosure were barely measurable.

**Single-cell detection of PstS expression in *Synechococcus*, in both laboratory and mesocosm samples, by use of immunofluorescence.** Detection of PstS expression in single *Synechococcus* sp. strain WH7803 cells, grown under P-depleted conditions, was obtained by use of an immunofluorescence method combining an affinity-purified PstS primary antibody and an FITC-labelled secondary antibody. It included a 1-h period of incubation at 65°C to reduce inherent phycoerythrin fluorescence of cells, which masked FITC labelling, especially in laboratory cultures, and an SDS-NaOH permeabilization step. Optimization of the permeabilization protocol was aided by the use of DNase and DAPI staining to monitor the effectiveness of the various permeabilizing agents (Fig. 8a and b). Efficient permeabilization conditions were assumed to exist if, after DNase treatment, cells were not subsequently stained by DAPI. Specific and uniform labelling of cells around the cell periphery, which correlates well with the proposed cell wall localization of the PstS polypeptide, was observed only in those cells grown under P-depleted conditions (Fig. 8d). Cells of *Synechococcus* sp. strain WH7803 grown under P-replete conditions (Fig. 8c) or control cells incubated in secondary antibody alone or incubated in preimmune rabbit serum failed to show any FITC fluorescence. The autofluorescence observed here derives from the phycoerythrin remaining after heat treatment. Initial permeabilization experiments using a variety of agents had shown that the SDS-NaOH combination provided the most efficient way of mediating the accessibility of the antibody to the PstS antigen while leaving cells structurally intact. Preliminary experiments had confirmed that the antibody, which was raised against denatured protein, could recognize native PstS. Dodecyl maltoside (0.84% [vol/vol]) was used to solubilize *Synechococcus* sp. strain WH7803 cell walls prior to native PAGE and Western blotting. Similarly, preservation of the antigenicity of PstS was demonstrated by examination of electrophoretic protein blots of P-depleted cell extracts treated with 4% paraformaldehyde prior to antibody addition (data not shown).

The same immunofluorescence protocol was used to monitor PstS expression in single *Synechococcus* cells from the various mesocosm samples. Positively labelled cells were detected in enclosures 7 and 8 (Fig. 8e). The low cell density observed here resulted from repeated freeze-thawing of samples during Western analysis prior to immunofluorescence staining.

## DISCUSSION

Microbial growth in the marine environment is frequently restricted by nutrient availability. The progress towards nutrient limitation involves a series of steps by which the organism attempts to maintain growth through the utilization of stored reserves or by the induction of high-affinity uptake systems, allowing trace amounts of the limiting nutrient to be scavenged (38). The identification of proteins specifically induced or derepressed under these nutrient-depleted conditions hence gives an indication of the physiological response of the organism to a specific environmental stimuli, in this case low nutrient concentrations, rather than identification of a growth rate-limited state per se.

Such information is extremely valuable, especially if the nutrient concentration causing specific protein synthesis is known and expression of the protein is nutrient specific. In the case of PstS, we have previously shown that nitrate or sulfate depletion does not induce synthesis of the protein (50). In this study, *Synechococcus* sp. strain WH7803 induced, or derepressed, PstS synthesis when the external $P_i$ concentration fell below 50 nM during growth in medium with an initial N/P ratio of 500:1 (Fig. 1a). Changes in growth rate, as a function of light intensity, did not significantly induce PstS synthesis. Similar control of the Pho regulon, of which the PstS protein is a component, by external $P_i$ but not the cytoplasmic $P_i$ concentration has been shown in *E. coli* (47).

If induction of PstS synthesis is to be useful as a general marker of P stress, it is also necessary that synthesis be readily repressed by dissolved organic phosphorus (DOP) sources, since these molecules may comprise a high proportion of the available phosphorus in the marine environment (9, 30, 44). Although both bacteria and phytoplankton produce extracellular enzymes capable of hydrolyzing phosphate monoesters and nucleotides (for examples, see references 3, 7 and 18), suggesting competition only for the released $P_i$ (52), it is also possible that picoplankton possess sugar-P transport systems which might allow direct uptake of a part of the DOP, as in enteric bacteria (26). Marine *Synechococcus* spp., in natural systems, may indeed utilize the $P_i$ released through the action of external hydrolytic enzymes produced by other organisms. However, that a laboratory strain was capable of utilizing a variety of organic phosphates, with direct repression of PstS synthesis (Fig. 2), suggests the capacity for some DOP hydrolysis; further work is needed to assess specific production of alkaline phosphatases and 5′-nucleotidases, etc. Recent examination of the bioavailability of organic phosphorus compounds in Hawaiian coastal waters showed that nucleotides were the most readily utilizable of the combined phosphorus sources investigated, with consistently lower bioavailability of phosphate monoesters. However, $P_i$ appeared to be the preferred, and apparently universal, substrate in these natural assemblages of microorganisms (5). The exception to the DOP substrates tested in this study was cAMP, which could not be hydrolyzed by *Synechococcus* sp. strain WH7803. The ability to utilize cAMP may be confined to a fraction of the marine

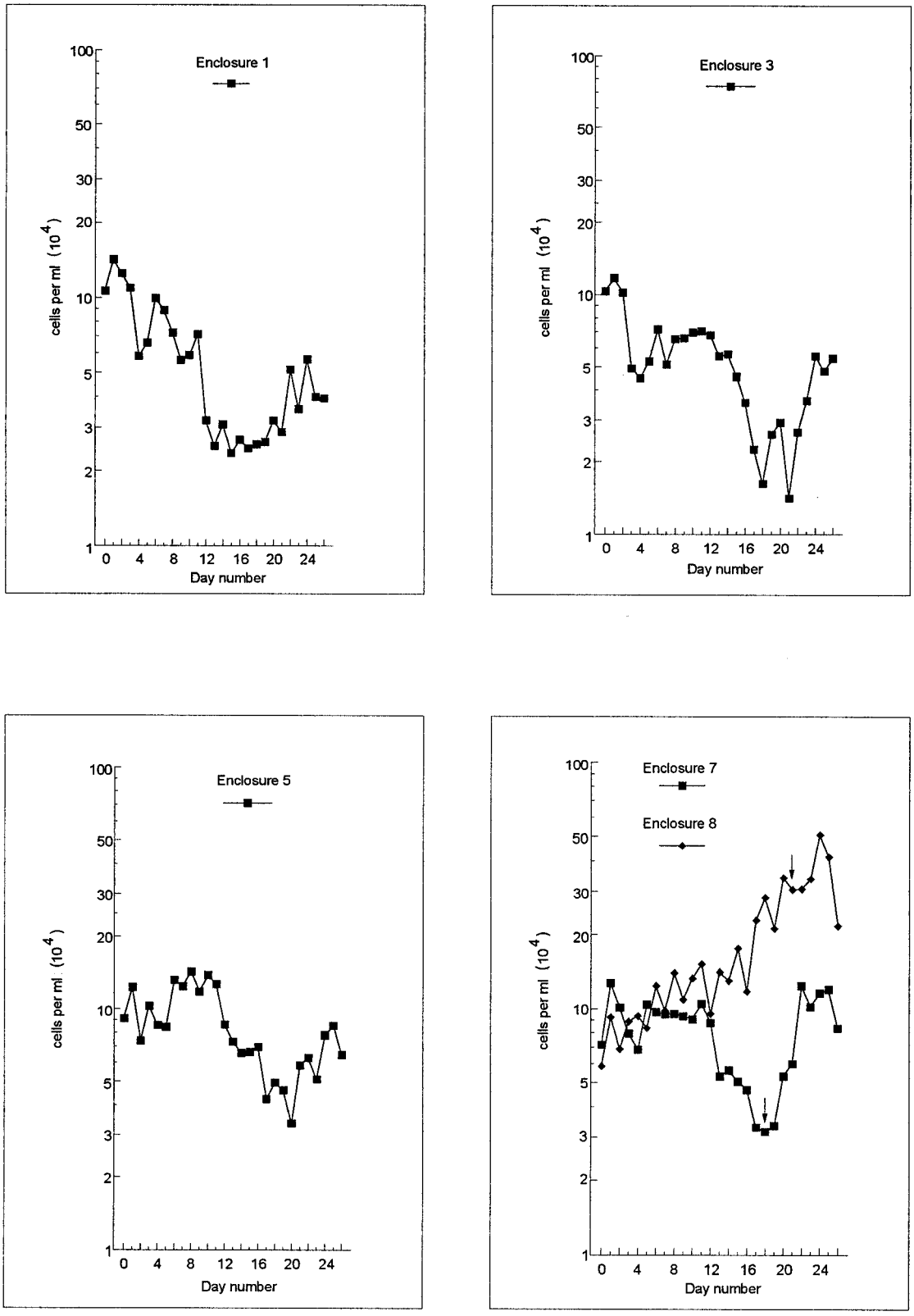


FIG. 5. Changes in *Synechococcus* numbers in enclosures 1, 3, 5, 7, and 8 during a mesocosm experiment from 6 June (day 0) to 3 July (day 26) 1995. The arrows indicate when enclosures 7 and 8 were enriched with $P_i$ (final concentration, 1 μM). Additions of 0.1 μM phosphate were made each day to compensate for the 10% of the water column replaced each day.

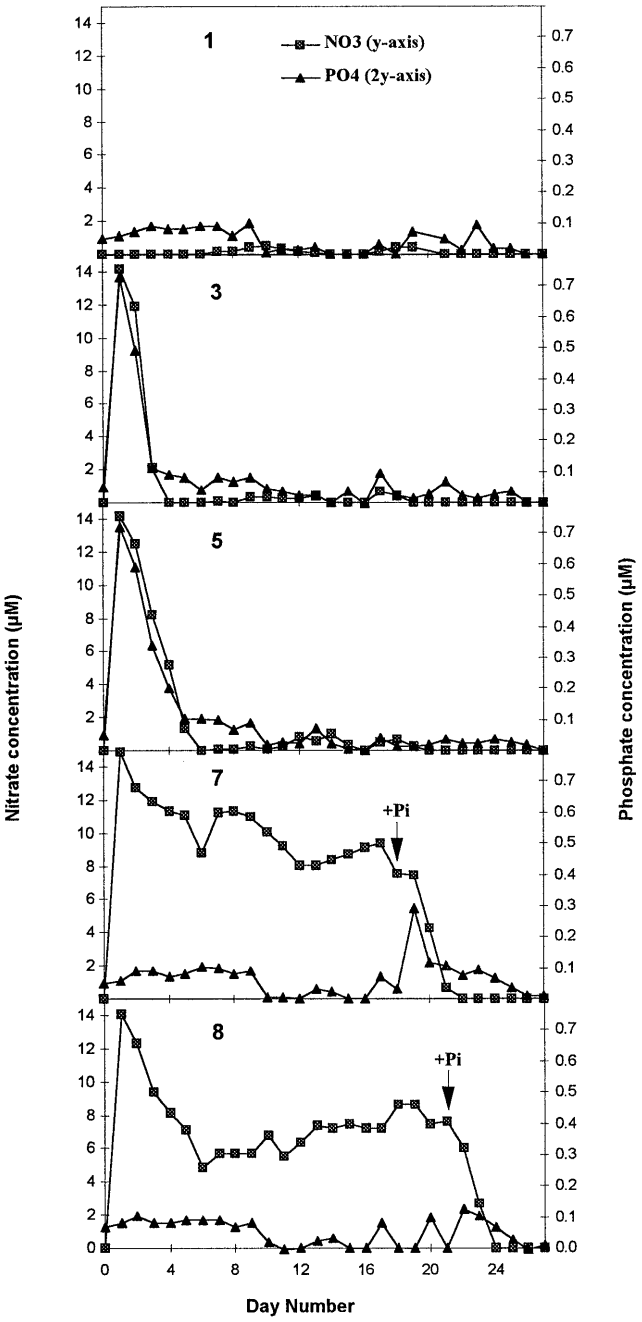


FIG. 6. Changes in phosphate and nitrate concentrations in enclosures 1, 3, 5, 7, and 8 from 6 June (day 0) to 3 July (day 26) 1995. The arrows labelled $+P_i$ indicate when enclosures 7 and 8 were enriched with $P_i$ (final concentration, 1 μM). Additions of phosphate equivalent to 0.1 μM were made daily thereafter. It is important to note the difference in scale of the *y*-axes (nitrate, 0 to 16 μM; phosphate, 0 to 0.8 μM).

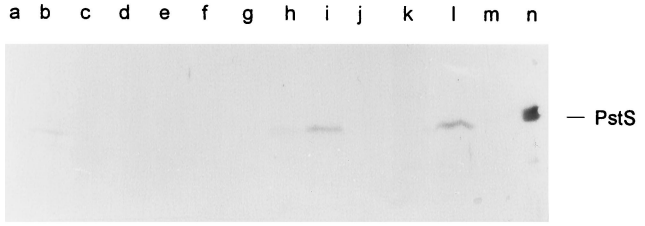


FIG. 7. Induction of PstS in natural *Synechococcus* assemblages from various mesocosm enclosures in a Norwegian fjord by Western blotting of total cell extracts from phytoplankton-bacterium populations (size, <20 μm) separated on a 10% SDS-polyacrylamide gel with PstS antiserum. Samples taken after the addition of $P_i$ to 1 μM in enclosures 7 and 8 are indicated in the following description of the lanes with +P in parentheses. Lanes: a, bag 1, day 8; b, bag 1, day 19; c, bag 3, day 1; d, bag 3, day 8; e, bag 3, day 19; f, bag 5, day 12; g, bag 7, day 1; h, bag 7, day 5; i, bag 7, day 17; j, bag 7, day 20 (+P); k, bag 8, day 1; l, bag 8, day 21; m, bag 8, day 23 (+P); n, positive control (cell wall fraction isolated from *Synechococcus* sp. strain WH7803 grown under P-depleted conditions with 1 μg of protein). Equal amounts of protein (100 μg) were loaded in lanes a to m.

microbial community since a high-affinity cAMP transport system is found in a minority of cells in mixed bacterial assemblages (4).

**Cross-reaction of anti-PstS polyclonal antibodies with other marine microorganisms.** The data presented here extends the use of the PstS antisera to include both of the dominant photosynthetic marine picoplankton genera, i.e., *Synechococcus* and *Prochlorococcus*. Synthesis of the PstS polypeptide is induced in all strains examined to date, including organisms isolated from diverse oceanic environments, e.g., the Sargasso Sea, the Mediterranean Sea, and the Red Sea, and only under P-depleted conditions. Cross-reaction has not been observed in any marine heterotroph. Indeed, the antisera do not appear to recognize the corresponding *E. coli* polypeptide with 35% amino acid identity, as deduced from nucleotide sequence data (50), since cell extracts derived from various *E. coli* strains grown under P depletion, with high alkaline phosphatase levels compared to that of P-replete cultures, showed no cross-reaction (unpublished results). Sequencing of the *Prochlorococcus pstS* gene reveals a much higher amino acid identity with the marine *Synechococcus* homolog (77%) than with the *E. coli* polypeptide (35% identity). A similar specificity of antibodies towards particular algal groups has been noted for both the large subunit of RuBisCO (43) and flavodoxin (36) polyclonal antisera.

Previous DNA hybridization data revealed an absence of *pstS* gene homologs among freshwater cyanobacterial strains (50). Several components of the $P_i$ assimilation and regulatory machinery have now been identified in freshwater cyanobacterial strains (2, 27, 48, 58). Among these is a cytoplasmic membrane-located protein, SphX, present in P-limited *Synechococcus* sp. strain PCC7942 (1). This appears to belong to the phosphate-binding protein family, having identity at several residues implicated in interactions with P (39). Moreover, analysis of the complete *Synechocystis* sp. strain PCC6803 genome shows that homologs exist for both the *sphX* and *pstS* genes, suggesting an added complexity to P acquisition in these organisms (27).

**Detection of PstS in natural picoplankton populations.** Application of the use of the PstS antisera to the detection of PstS expression in natural *Synechococcus* assemblages was confirmed by use of large-scale enclosures in a Norwegian fjord. Organisms in enclosures forced into P depletion (N/P ratios of >75:1) specifically expressed the PstS polypeptide, while rapid repression of synthesis was observed upon P readdition, suggesting tight regulation of PstS expression by P in natural *Synechococcus* populations. It is not known whether PstS expression in these assemblages is a consequence of the very low ambient P concentrations or if it is a function of the high N/P ratio. That PstS was expressed in enclosures 7 and 8, when low but measurable $P_i$ was present, suggests that some of the phos-

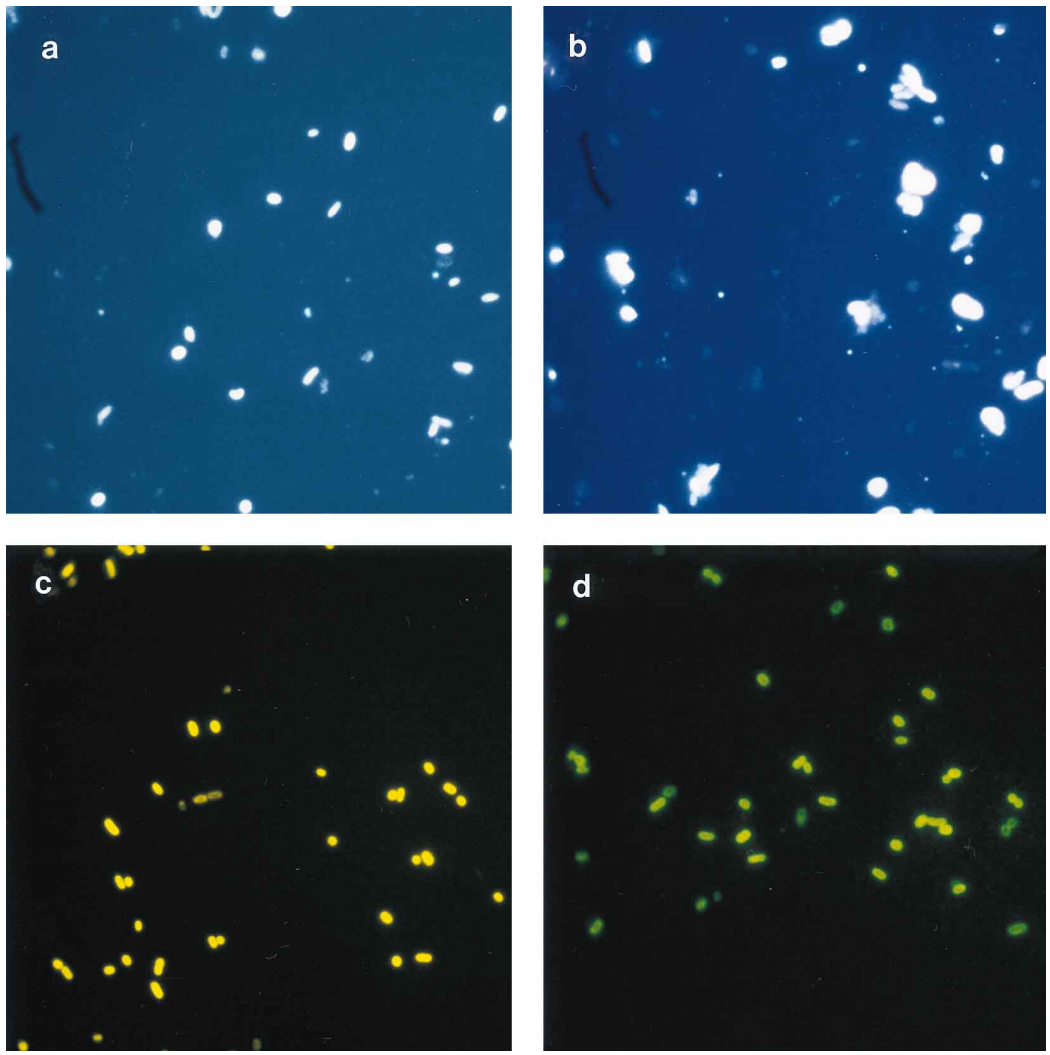


FIG. 8. Single-cell fluorescence microscope images of *Synechococcus* sp. strain WH7803 (a to d) and natural phytoplankton populations (e). (a and b) *Synechococcus* sp. strain WH7803 cells permeabilized with SDS-NaOH (a) or with no permeabilization (b), followed by DNase treatment and DAPI staining. (c to e) Immunofluorescence detection of PstS expression in *Synechococcus* cells with affinity-purified PstS primary antibody and FITC-labelled secondary antibody. Cells of *Synechococcus* sp. strain WH7803 grown under P-replete (c) or P-depleted (d) conditions and natural *Synechococcus* cells in a Norwegian fjord obtained from enclosure 7 on day 17 (e) were used. Each image represents a microscope field of 0.018 $mm^2$.

phate might have been biologically unavailable, at least over the time scales of the experiment.

**Single-cell immunofluorescence assay.** The use of immunofluorescence assays in the identification and enumeration of phytoplankton species, and the localization and quantification of cell components, has much potential in marine systems (for a review, see reference 57). Experimentation with detection of antigen molecules in single cells has led to the defining of permeabilization conditions useful for analyzing the expression of soluble antigens such as the RuBisCO large subunit and flavodoxin in larger eukaryotic phytoplankton cells (34, 42) as well as nitrogenase in filamentous cyanobacterial cells (12). Although polyclonal antisera raised against cell surface antigens of marine *Synechococcus* spp. have been used to identify and enumerate specific serogroups in natural populations (6), there is no published use of the technique to localize specific soluble or membrane-associated intracellular antigens in these organisms.

The method we describe here allows precise detection of a

membrane-associated antigen whose expression in single *Synechococcus* cells is dependent on ambient $P_i$ concentration. The assay has been optimized here, initially for use with cells that have been permeabilized, fixed, and dried onto glass slides. Much refinement of the permeabilization and fixation conditions was performed with *Synechococcus* sp. strain WH7803 cells prior to use with natural samples. Subsequent results with natural *Synechococcus* populations for detection of the PstS antigen confirm the applicability of this method (Fig. 8e). It is possible, however, that future refinements may be required to efficiently permeabilize some *Synechococcus* strains. Future use of this method will endeavor to apply the technique to cells suspended in aqueous solution. The solving of this technical problem would allow the application of flow cytometry to quantify immunofluorescence-stained PstS in natural samples, hence increasing the applicability of the method to the survey of large areas of the ocean for potential limitation of picophytoplankton growth.

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