

# *Prochlorococcus* can use the Pro1404 transporter to take up glucose at nanomolar concentrations in the Atlantic Ocean

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*Prochlorococcus* is responsible for a significant part of CO<sub>2</sub> fixation in the ocean. Although it was long considered an autotrophic cyanobacterium, the uptake of organic compounds has been reported, assuming they were sources of limited biogenic elements. We have shown in laboratory experiments that *Prochlorococcus* can take up glucose. However, the mechanisms of glucose uptake and its occurrence in the ocean have not been shown. Here, we report that the gene Pro1404 confers capability for glucose uptake in *Prochlorococcus marinus* SS120. We used a cyanobacterium unable to take up glucose to engineer strains that express the Pro1404 gene. These recombinant strains were capable of specific glucose uptake over a wide range of glucose concentrations, showing multiphasic transport kinetics. The *K<sub>s</sub>* constant of the high affinity phase was in the nanomolar range, consistent with the average concentration of glucose in the ocean. Furthermore, we were able to observe glucose uptake by *Prochlorococcus* in the central Atlantic Ocean, where glucose concentrations were 0.5–2.7 nM. Our results suggest that *Prochlorococcus* are primary producers capable of tuning their metabolism to energetically benefit from environmental conditions, taking up not only organic compounds with key limiting elements in the ocean, but also molecules devoid of such elements, like glucose.

high-affinity glucose transport | marine cyanobacteria | multiphasic uptake kinetics

Cyanobacteria is a phylum of the bacterial domain distinguishable by their unique capacity to perform oxygenic photosynthesis. This process, which relies on the existence of two photosystems and a chain of electron transporters, enables these organisms to use light energy and electrons from water to produce reductant molecules and fix atmospheric CO<sub>2</sub> to synthesize carbon compounds. Cyanobacteria probably arose on Earth billions of years ago (1), and prolonged evolutionary divergence has made them very diverse in terms of morphology, metabolism, and lifestyle.

A number of cyanobacterial strains can use glucose (2), using it as a source of reduced carbon and energy, although the majority of isolated strains lack this ability. The inability of many species to use carbon sources that are widely used by many prokaryotes is in some cases due to toxic effects (3) or lack of metabolic capabilities, whereas in other cases it is due to the lack of transport systems for the intake of such compounds (4, 5). Unicellular marine cyanobacteria were included in the group of cyanobacteria unable to use glucose (6), long being considered to be photoautotrophic organisms (7). *Prochlorococcus* is the most abundant marine cyanobacterium (8), being one of the main primary producers on Earth (9, 10), and has hitherto been considered to be photoautotrophic. This fact notwithstanding, different studies have shown that *Prochlorococcus* strains can take up organic compounds, whose interest was usually considered to come from their containing limited elements, such as nitrogen in amino acids (11), or sulfur in dimethylsulfoniopropionate (12). Initial studies on *Prochlorococcus* sp. strain PCC 9511 showed no evidence for glucose utilization

(13). Nevertheless, we have since shown that *Prochlorococcus* cultures of different strains do take up glucose (14), showing clear changes in gene expression after glucose addition. The current annotations of *Prochlorococcus* genomes (15–17) show no genes involved in glucose transport. Nevertheless, we observed that the expression of the gene Pro1404, annotated as *melB* (a putative melibiose/sodium symporter) in the genomes of *Prochlorococcus*, showed a clear increase in *Prochlorococcus marinus* strain SS120, upon glucose addition (14). In this study, our goal was to determine the mechanism of glucose uptake by *Prochlorococcus*, to investigate the possible involvement of the Pro1404 gene, and to examine whether glucose could be taken up by *Prochlorococcus* populations in the Atlantic Ocean.

## Results and Discussion

To investigate the putative role of the Pro1404 (*melB*) gene in the uptake of glucose by *Prochlorococcus*, we chose to express this gene ectopically in a cyanobacterium naturally incapable of glucose transport, *Synechococcus elongatus* PCC 7942. Two constructions were made where the promoterless Pro1404 gene was placed downstream of kanamycin resistance cassettes, C.K1 or C.K3, respectively. In these constructions, expression of Pro1404 is driven by the promoters of the C.K1 or C.K3 cassettes that show different activity in cyanobacteria, C.K1 (moderate) and C.K3 (strong) (18). Recombinant strains were selected where the C.K1::Pro1404 and C.K3::Pro1404 constructs were introduced by recombination in the *S. elongatus* chromosome splitting the dispensable *asnS* locus (coding for asparaginyl-tRNA synthetase).

The recombinant strains, named C.K1 and C.K3, were grown and used to study the uptake of 1 μM <sup>14</sup>C-glucose. *Synechocystis* sp. PCC 6803 was used as positive control, whereas an *S. elongatus* recombinant strain with a cassette insertion in the *asnS* gene (*AsnS*-1) was used as a negative control. *P. marinus* SS120 was also used as reference. The results are shown in Fig. 1: the ectopic expression of Pro1404 in *S. elongatus* conferred the capability for glucose uptake to the derived recombinant strains C.K1 and C.K3. Therefore, Pro1404 encodes a glucose transporter in *P. marinus* SS120, and we can infer this gene probably has the same function in the rest of *Prochlorococcus* and marine *Synechococcus* strains that have been sequenced and shown to have this gene.

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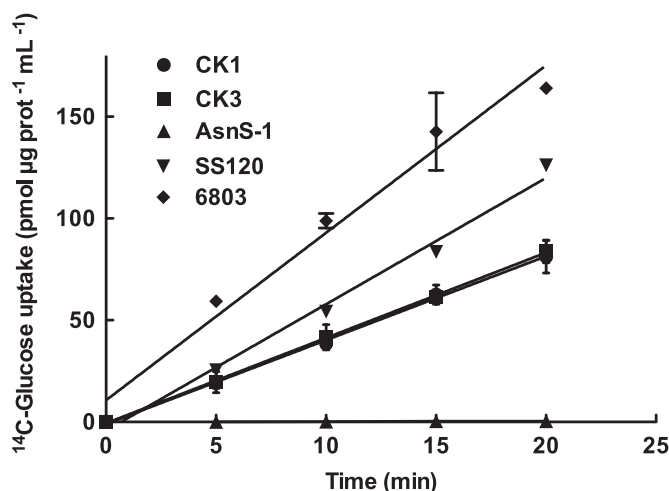
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**Fig. 1.**  $^{14}\text{C}$ -Glucose uptake in recombinant strains of *S. elongatus* sp. PCC 7942 expressing the gene Pro1404 from *P. marinus* SS120. The Pro1404 gene was inserted into *asnS* of the *S. elongatus* chromosome as described (Fig. S2), under the control of two constitutive promoters of moderate (C.K1) and high (C.K3) activity in cyanobacteria. A glucose-transporting species, *Synechocystis* sp. PCC 6803 was used as positive control, whereas the recombinant strain AsnS-1 of *S. elongatus* was used as negative control. *P. marinus* sp. SS120 was also used as reference. Data presented are the average of three independent biological replicates. Bars show SD for each sample. SD bars for *P. marinus* sp. SS120 are smaller than symbols.

Unexpectedly, both recombinant strains exhibited similar glucose transport rates, suggesting that the expression level in the C.K1 strain was sufficient to populate the plasma membrane of the host cell with saturating amounts of the transporter.

Kinetic properties of glucose uptake were studied in the recombinant *S. elongatus* C.K1 strain, by testing a range from 25 nM to 50  $\mu\text{M}$  glucose (Fig. 2A). We observed multiphasic kinetic behavior, showing a high affinity  $K_s$  constant in the nanomolar range (123.4 nM), in good agreement with the low glucose concentrations estimated in the oligotrophic Atlantic Ocean. Cultures of *P. marinus* SS120 were assayed for  $^{14}\text{C}$ -glucose transport, finding the same high affinity constant (124.6 nM); this value is not significantly different with respect to the  $K_s$  obtained in *S. elongatus* C.K1 (as confirmed by *t* test,  $P < 0.05$ ,  $P$  value 0.4601); furthermore, glucose transport by *Prochlorococcus* also shows multiphasic uptake kinetics (see Fig. 4A), thus reinforcing the physiological meaning of the results obtained in the recombinant strains.

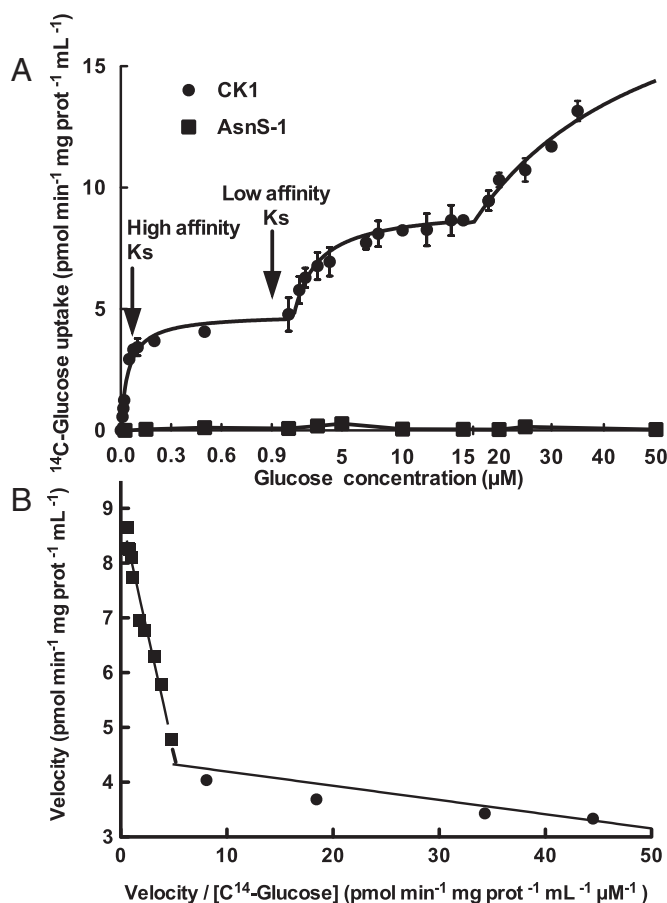
It is worth noting that the high-affinity  $K_s$  constant of Pro1404 for glucose is 3,000-fold lower than the one described for the glucose transporters of model freshwater cyanobacteria, with  $K_s$  constants in the millimolar range (3, 19, 20).

An interesting aspect of our results is the fact that a single gene product shows multiphasic uptake kinetics, indicating that the same transporter is enabled to behave differently depending on the glucose concentration. Whereas the third phase could not be fully characterized due to the elevated concentrations of glucose required to reach saturation (Fig. 2A), two phases showing Michaelis–Menten kinetics with  $K_s$  affinity constants of 123.4 nM and 0.9  $\mu\text{M}$  could be determined. These two phases are reflected in the two lines with clearly different slopes shown in the representation of Eadie–Hofstee (Fig. 2B). Thus, we will refer to Pro1404 as a dual affinity transporter. To our knowledge, only two other transporters, a potassium and a nitrate transporter from plants, have been shown to be dual-affinity transporters with a biphasic kinetics by the use of an ectopic expression approach similar to the one shown here (21–23). In the case of the nitrate transporter, the alternation from the high-affinity to the low-affinity mode and vice-versa is controlled by phosphorylation

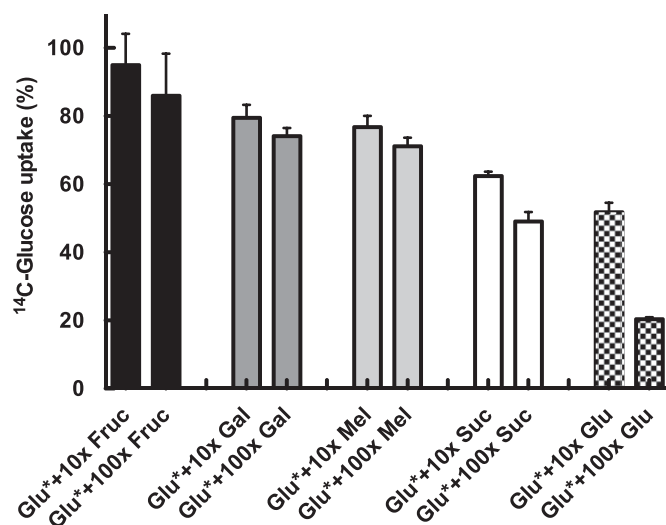
(24). Whether this transition in Pro1404 is regulated by post-translational modification remains to be investigated.

Dual affinity transport of solutes has been observed in many organisms, but in most cases this mode of uptake is achieved by the alternate use of distinct high-affinity and low-affinity transporters. The existence of multiple glucose transporters in *Prochlorococcus* cannot be ruled out by our data. However, we here demonstrate the existence of at least one glucose transporter with biphasic (or multiphasic) kinetic behavior. Glucose concentration in the ocean is generally low, but fleeting glucose enrichments may occur locally by transient fluxes of the sugar (3, 25). Interchanging multiple transporters of varying affinity in response to changes in glucose concentration may take time. By using a single transporter, *Prochlorococcus* can rapidly adapt the intake of glucose to sudden fluxes, changing from high-affinity, low capacity mode to low-affinity, high-capacity mode and back to the high-affinity, low-capacity mode when the concentration drops and *Prochlorococcus* needs to scavenge traces of the sugar. Interestingly, similar multiphasic glucose transport has been previously described in marine oligotrophic bacteria (26), with a high affinity  $K_s$  constant also in the nanomolar range. However, in these cases, it has not been addressed how many transporters are involved.

To study the specificity of the Pro1404-encoded transporter, we performed competition experiments where increased concentrations of other sugars were added, in addition to glucose



**Fig. 2.** (A)  $^{14}\text{C}$ -Glucose concentration versus its uptake by recombinant *S. elongatus* C.K1 strain and the control strain AsnS-1. The scale in the x axis is not linear, to allow a clearer representation of the determined glucose uptake values. Data presented are the average of three independent biological replicates. Bars show SD for each sample. (B) Eadie–Hofstee representation of the kinetics of glucose uptake shown in A for the recombinant *S. elongatus* C.K1 strain.



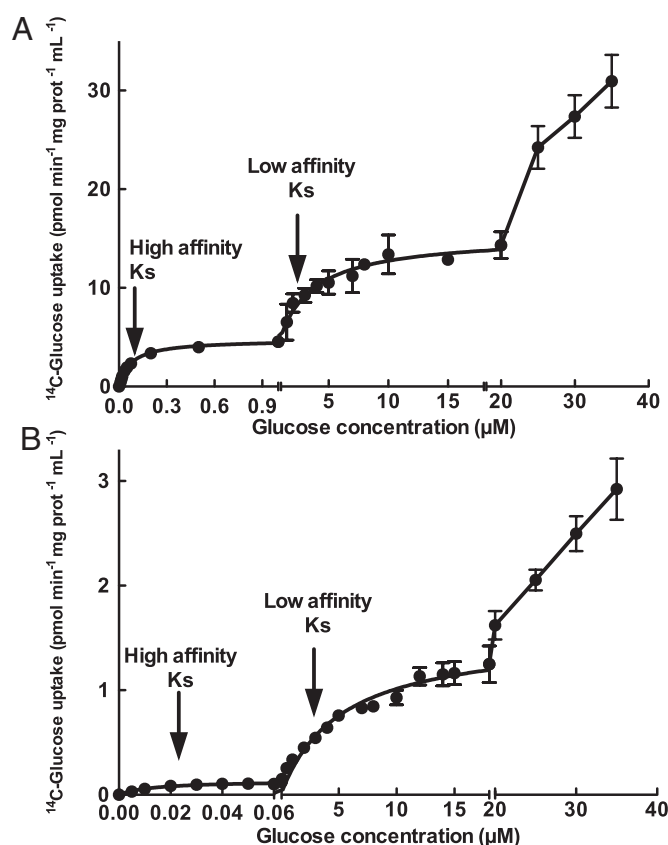
**Fig. 3.** <sup>14</sup>C-glucose uptake in *S. elongatus* C.K1 in the presence of additional concentrations of different sugars. Fruc, fructose; Gal, galactose; Glu, glucose; Mel, melibiose; Suc, sucrose. Data presented are the average of three independent biological replicates. Bars show SD for each sample.

(Fig. 3). <sup>14</sup>C-glucose (0.5 μM) was added to all samples, and, additionally, 5 or 50 μM of the competing, unlabeled sugar—fructose, galactose, melibiose, or sucrose—were added. Glucose uptake was determined, assigning 100% to the results obtained with 0.5 μM <sup>14</sup>C-glucose alone. The results indicate that, besides being highly efficient, this transporter is more, but not exclusively, specific for glucose. When we compared the decrease in radioactive glucose uptake by addition of cold glucose as control, the observed values (both at 10× and 100× concentrations; Fig. 3) were significantly lower than those observed for all other sugars, according to the Student's *t* test. Sucrose was the most competitive of the sugars studied, which induced a 50% decrease in glucose uptake when added at 100-fold higher concentration than glucose. Furthermore, our data suggest that, despite its annotation as a melibiose transporter, the main function of this gene product is the uptake of glucose, and its name should be changed to sugar transporter.

Genes homologous to Pro1404 exist in the genomes of all marine cyanobacteria. Consequently, we decided to perform comparative experiments addressing the glucose uptake capabilities of *P. marinus* SS120 and *Synechococcus* sp. strain WH7803, which belongs to clade V in the marine *Synechococcus* phylogenetic tree (27), and is representative of one of the open ocean *Synechococcus* strains inhabiting oligotrophic environments, therefore being potential competitors of *Prochlorococcus*. Fig. 4 shows glucose uptake by cultures of both strains along the same range of concentrations studied in experiments with recombinant strains. It is worth mentioning that the glucose uptake observed cannot be explained on the basis of the minimal contamination observed in cultures, as described in detail in a previous manuscript (14). As stated above, a multiphasic kinetics was observed in cultures of *P. marinus* SS120 (Fig. 4A), remarkably similar to that observed in recombinant *S. elongatus* CK.1 (Fig. 2A), with measurable *K<sub>s</sub>* values of 124.6 nM and 2.37 μM. Both values were not significantly different from those found in the recombinant CK.1, as compared by the *t* test (*P* < 0.05). In the case of *Synechococcus* WH7803, again a multiphasic kinetics was observed, with measurable *K<sub>s</sub>* values of 21.55 nM and 2.86 μM. Interestingly, the high affinity *K<sub>s</sub>* value of *Synechococcus* WH7803 is roughly six-fold lower than *P. marinus* SS120 (124.6 nM), but the maximum glucose uptake rate is much higher in *Prochlorococcus* than in *Synechococcus* (6.4 vs. 0.16 pmol·min<sup>-1</sup>·mg prot<sup>-1</sup>·mL<sup>-1</sup>,

respectively). If we take both parameters into account to calculate the uptake efficiency (maximum uptake rate/*K<sub>s</sub>*) for each strain, the result is that *Prochlorococcus* is 6.7 times more efficient in glucose uptake than *Synechococcus*. Although these results have been obtained with laboratory cultures and require further comparative studies in the field, they point to a potentially important difference between the two main genera of marine cyanobacteria living in oligotrophic environments, which might help to explain their clearly different distributions and abundances (27, 28).

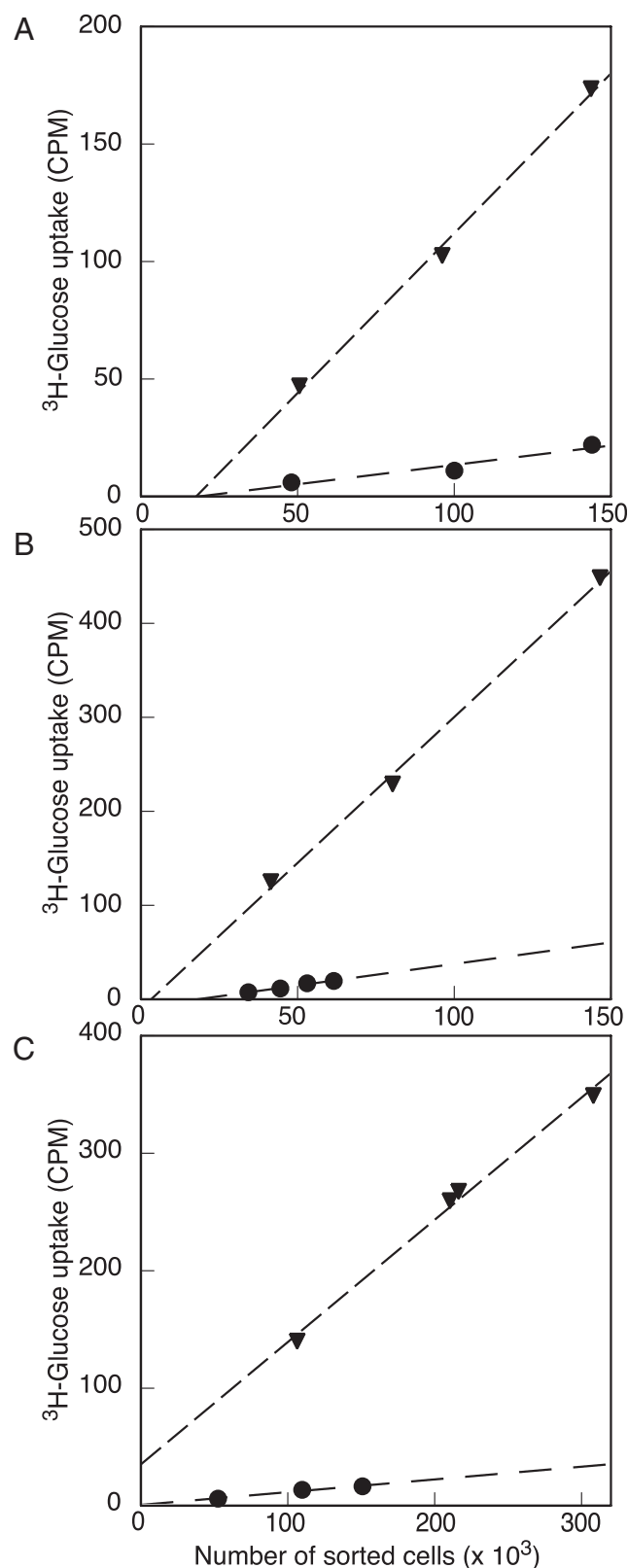
To confirm our laboratory results that *Prochlorococcus* takes up glucose at low concentrations, we assessed uptake of glucose by *Prochlorococcus* in the Atlantic Ocean on the Atlantic Meridional Transect 21 (AMT-21) research cruise, September–November 2011 (Fig. S1). Glucose bioavailability, determined using <sup>3</sup>H-glucose dilution bioassays (29), was in the nanomolar range (Table 1), with measured values ranging from 0.07 to 2.67 nM, in agreement with previous studies (29). To compare uptake rates of glucose by *Prochlorococcus* and average bacterioplankton cells, DNA-stained cells were flow sorted after 4 h labeling with 2 nM <sup>3</sup>H-glucose. The gated population (Fig. 5) corresponds to *Prochlorococcus*, showing its distinctive higher red chlorophyll fluorescence, compared with the total bacterioplankton population (11, 30). This type of measurement is technically challenging because of the low specific activity of the glucose tracer and relatively slow uptake of glucose by *Prochlorococcus* cells. Despite this technical limitation, at three stations, we could determine glucose uptake by *Prochlorococcus* (Table 1 and Fig. 6). We observed measurable rates of glucose import (Table 1), demonstrating that *Prochlorococcus* does indeed



**Fig. 4.** <sup>14</sup>C-glucose concentration versus its uptake in cultures of *P. marinus* sp. SS120 (A) and *Synechococcus* sp. WH7803 (B). The scale used in the y axis is different to allow a proper representation of results. Data presented are the average of three independent biological replicates. Bars show SD for each sample.







**Fig. 6.**  $^3\text{H}$ -Glucose uptake vs. number of sorted *Prochlorococcus* (circles) or total bacterioplankton cells (triangles) at stations 15 (A), 35 (B), and 38 (C). Further details on the results obtained at those stations are shown in Table 1.

ecological role of these cyanobacteria as primary producers capable of tuning their metabolism to energetically benefit from environmental conditions.

## Materials and Methods

**Cyanobacterial Strains and Growth Conditions.** *P. marinus* strain SS120 (low-irradiance adapted) was routinely cultured in polycarbonate Nalgene flasks (10 L) using PCR-S11 medium as described (13). The sea water used as basis for this medium was kindly provided by the Instituto Español de Oceanografía (Madrid). *Synechococcus* sp. strain WH7803 was grown using artificial seawater medium, as described (33). Cells were grown in a culture room set at 24 °C under continuous blue irradiances ( $4 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ). The marine cyanobacterial cultures were clonal but not axenic although the contamination level was extremely low (< 3%), as assessed by flow cytometry.

*Synechococcus* sp. strain PCC 7942 was grown axenically at 30 °C in the light ( $75 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ ) in BG11 medium (34). Cultures of *S. elongatus* and derived strains were tested for axenicity in two ways, by microscopic observation and by plating samples of each culture on BG11 plates and on LB plates supplemented with 1% glucose that were cultured for at least 1 wk at 30 °C. The data presented in the manuscript correspond only to experiments where all strains involved were axenic. Liquid cultures were bubbled with a mixture of  $\text{CO}_2$  and air (1%, vol/vol). For recombinant strains, kanamycin was used at  $7 \mu\text{g}\cdot\text{ml}^{-1}$  final concentration. For plates, the medium was solidified with 1%, separately autoclaved agar (Difco).

**Construction of Recombinant *Synechococcus* sp. PCC 7942 Strains.** The Pro1404 gene from *P. marinus* SS120 (currently annotated as *melB*) was amplified by PCR using genomic DNA as a template and primers MELB-1F and MELB-1R:

MELB-1F: 5' GGAGGTCCATGGTTTCCTATGGAT 3'; and

MELB-1R: 5' TGCCAAGCTTTTAAGCCAACGG 3'.

The amplified fragment was cloned between the NcoI and HindIII sites of the pTrc99A plasmid vector (35) generating plasmid pcMM1. This molecule was used as a template for subsequent PCR amplification with phosphorylated primers PCMM1-1F and PCMM1-1R to generate a PCR fragment, including the *melB* gene and the downstream *rrnB* transcriptional terminator from pTrc99A, which was ligated with pCA12 plasmid (36) linearized by PCR with divergent primers 7942\_ASNS\_3F and 7942\_ASNS\_3R. The resulting plasmid, pcMM2, contained the *melB* gene from *Prochlorococcus* with a downstream *rrnB* terminator interrupting the *Synechococcus* sp. PCC 7942 *asnS* ORF, which has been shown to be dispensable for this organism (36). Kanamycin-resistance cassettes C.K1 and C.K3 (37) were cloned in the same orientation as the *melB* gene in the HpaI site upstream of it in pcMM2, generating plasmids pcMM3 and pcMM4, respectively. The construction of plasmids is outlined in Fig. S2.

Plasmids pcMM3 and pcMM4, which do not replicate in cyanobacteria, were introduced by transformation in *S. elongatus* sp. PCC 7942. Kanamycin-resistant transformants were selected, and the genomic structure of the *asnS* locus was analyzed. Those transformants that had integrated the constructions described above by double recombination in the *asnS* locus were selected for further analysis (Fig. S2).

**Determination of Glucose Uptake by Cultured Cyanobacterial Strains.** [ $^3\text{H}$ ] glucose (281 mCi/mmol; American Radiolabeled Chemicals, Inc) was added to 0.7–4.5 mL of culture sample to reach a final concentration of 0.01  $\mu\text{M}$  to 20  $\mu\text{M}$ , depending on the experiment. Mixtures of radiolabeled and unlabeled glucose were added to achieve the desired concentration; furthermore, unlabeled sugars were used in the competition experiments. Aliquots were taken at the indicated times, filtered through 0.22- $\mu\text{m}$  (for *Prochlorococcus* sp. SS120 and *Synechococcus* sp. WH7803) and 0.45- $\mu\text{m}$  (for *Synechocystis* sp. PCC 6803, *S. elongatus* sp. PCC 7942, and its recombinant strains) Millipore filters, washed with 25 mM Tris-HCl pH 7.5 and then placed into scintillation vials. In studies on cultured marine cyanobacterial strains, the aliquots were taken at 3, 6, and 9 min (for *Prochlorococcus* sp. strain SS120) and at 2, 5, and 10 min (for *Synechococcus* sp. strain WH7803), due to the different saturation times observed for the glucose uptake kinetics of each strain. Scintillation was started by the addition of 5 mL of Ready Protein Mixture (Beckman Coulter) and measured in an LS6000IC Scintillation Counter (Beckman Coulter).

**Determination of Protein Concentration.** Protein concentration was determined using the Bio-Rad Protein Assay kit, based on the method described by Bradford (38).

**Dilution Bioassay to Estimate Glucose Concentration in the Atlantic Ocean.** A series of dilution bioassays for the determination of glucose concentration, following the method described by Wright and Hobbie (39), were carried out at different sampling points, along the Atlantic Meridional

Transect 21 cruise (AMT-21), from September 29 to November 14, 2011 (Fig. S1). At each of 38 stations, surface seawater was collected in Niskin bottles and decanted into HCl-washed vacuum flasks. Samples were analyzed within 1 h of sampling.

D-[5,6-<sup>3</sup>H]glucose (specific activity 60 Ci/mmol; Hartmann Analytic) was added to 1.6 mL of seawater samples, in a final concentration range from 0.25 to 1.25 nM. The samples were incubated in sterile 2-mL capped polypropylene microcentrifuge tubes at in situ temperature and fixed with 1% paraformaldehyde (PFA) at 30, 50, 70, and 90 min. Following fixation, samples were filtered onto 0.2-μm polycarbonate filters (Nuclepore; Whatman) and washed twice with 4 mL of deionized water. The radioactivity retained on filters was measured as cpm, following the addition of 4 mL of scintillation mixture, in a liquid scintillation counter (Tri-Carb 3100; Perkin-Elmer). Glucose turnover times were plotted against the concentrations of added glucose; in this representation, the regression lines intercepts to the x axis are the ambient glucose concentration at each sampling point. Further details of the bioassay method have been described by Zubkov et al. (29).

**Determination of Glucose Uptake by Natural *Prochlorococcus* Populations Using Flow Cytometry.** To determine cell abundance, 1 mL of untreated seawater was fixed with 1% PFA for 1 h, then stained with SYBR Green I nucleic acid stain (Sigma) in the presence of 30 mM potassium citrate (38) for 1 h. Samples were analyzed by flow cytometry (FACSCalibur; Beckton Dickinson Biosciences) at a flow rate of 15–60 mL·min<sup>-1</sup>, depending on the cell concentration, for 1 min. Multifluorescent 0.5-μm beads (Fluoresbrite Microparticles; Polysciences) were used to check particle concentration and fluorescence.

To measure glucose uptake by natural *Prochlorococcus* populations, 1.6–8 mL of seawater was incubated with 2 nM D-[5,6-<sup>3</sup>H]glucose for 4 h. Samples were fixed with 1% PFA, and DNA was stained as described above. *Prochlorococcus* cells were sorted by flow cytometry, from a plot of red

chlorophyll fluorescence vs. green DNA fluorescence (Fig. 5) using CellQuest software (BD Biosciences). The total bacterioplankton population was sorted from a plot of side scatter against green DNA fluorescence, to compare the relative uptake of *Prochlorococcus* with the total community (Fig. 6, Table 1). Sorted cells were filtered onto 0.2-μm polycarbonate filters (Nuclepore; Whatman) and washed twice with 4 mL of deionized water. The radioactivity retained on filters was measured as cpm, following the addition of 20 mL of scintillation mixture, using a liquid scintillation counter (Tri-Carb 3100; Perkin-Elmer).

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