

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

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Edited by David M. Karl, University of Hawaii, Honolulu, HI, and approved March 15, 2013 (received for review December 14, 2012)

***Prochlorococcus* is responsible for a significant part of CO₂ 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_s 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 CO₂ 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 ¹⁴C-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.

Author contributions: M.d.C.M.-M., I.L., M.V.Z., P.G.H., J.D., and J.M.G.-F. designed research; M.d.C.M.-M., I.L., and P.G.H. performed research; M.d.C.M.-M., I.L., M.V.Z., P.G.H., J.D., and J.M.G.-F. analyzed data; and M.d.C.M.-M., I.L., M.V.Z., P.G.H., J.D., and J.M.G.-F. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

See Commentary on page 8323.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1221775110/-DCSupplemental.

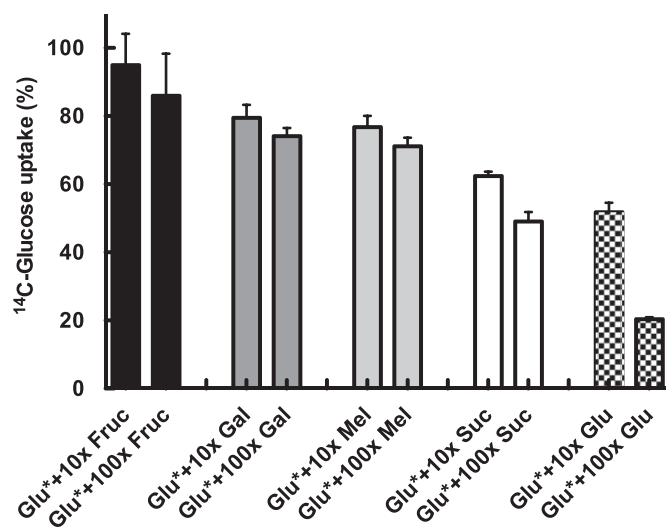


Fig. 3. ^{14}C -Glucose uptake in *S. elongatus* C.K1 in the presence of additional concentrations of different sugars. Fruc, fructose; Gal, galactose; Glu, glucose; Mel, melibiose; Suc, sucrose. Data presented are the average of three independent biological replicates. Bars show SD for each sample.

(Fig. 3). ^{14}C -glucose ($0.5\ \mu\text{M}$) was added to all samples, and, additionally, 5 or $50\ \mu\text{M}$ of the competing, unlabeled sugar—fructose, galactose, melibiose, or sucrose—were added. Glucose uptake was determined, assigning 100% to the results obtained with $0.5\ \mu\text{M}$ ^{14}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 \times and 100 \times 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_s 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_s values of 21.55 nM and 2.86 μM . Interestingly, the high affinity K_s 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 $\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg}\ \text{prot}^{-1}\cdot\text{mL}^{-1}$,

respectively). If we take both parameters into account to calculate the uptake efficiency (maximum uptake rate/ K_s) 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 ^3H -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 ^3H -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

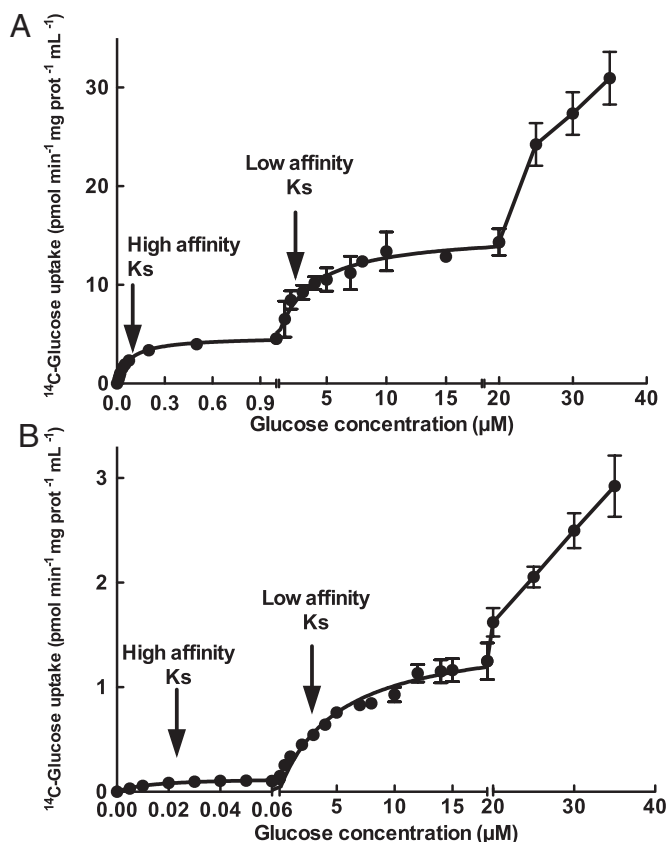


Fig. 4. ^{14}C -Glucose concentration versus its uptake in cultures of *P. marinus* sp. SS120 (A) and *Synechococcus* sp. WH7803 (B). The scale used in the y axis is different to allow a proper representation of results. Data presented are the average of three independent biological replicates. Bars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

Table 1. ^3H -Glucose uptake determined onboard during the AMT-21 cruise, showing the results obtained with sorted *Prochlorococcus* populations vs. total bacterial uptake

Geographical coordinates	Station	Depth, m	Total <i>Prochlorococcus</i> uptake, $\mu\text{Ci}/\text{mL}$	Total bacterial uptake, $\mu\text{Ci}/\text{mL}$	% total uptake by <i>Prochlorococcus</i>	[Glucose], nM
33° 30.11' N 28° 45.21' W	15	80	4.24×10^{-5}	128×10^{-5}	3.3	0.50
10° 45.43' N 31° 52.40' W	35	47	17.7×10^{-5}	480×10^{-5}	3.7	0.52
06° 31.34' N 29° 07.96' W	38	55	4.18×10^{-5}	160×10^{-5}	2.6	2.7

take up glucose in its natural habitat. Furthermore, we analyzed the relative contribution of populations to total glucose uptake, finding that *Prochlorococcus* was responsible for 2.6–3.7% of total bacterioplankton glucose uptake observed at those stations (Table 1). On average, the *Prochlorococcus* population contribution to total bacterioplankton glucose uptake was $3.2 \pm 0.56\%$; *Prochlorococcus* cellular glucose uptake relative to the average bacterioplankton cell uptake was $13 \pm 2\%$ (Fig. 6).

The uptake values shown in Fig. 6 are rather low, compared with previous results from our team obtained with *Prochlorococcus* cultures (Fig. 6 vs. figure 1a in ref. 14 by Gómez-Baena et al.). When we calculated the specific uptake values per cell in natural populations, we obtained 0.642×10^{-10} , 1.597×10^{-10} , and $0.321 \times 10^{-10} \mu\text{Ci}\cdot\text{cell}^{-1}\cdot\text{h}^{-1}$ for samples obtained at stations 15, 35, and 38 (Table 1), respectively. By contrast, our results with flow sorted cells from cultures of *P. marinus* strain PCC 9511 were $1.02 \times 10^{-8} \mu\text{Ci}\cdot\text{cell}^{-1}\cdot\text{h}^{-1}$ (14). This fact might be due to the big environmental differences between natural *Prochlorococcus* populations from the open ocean vs. *Prochlorococcus* cells growing in the laboratory under optimized conditions of light, nutrients, temperature, etc. Furthermore, it has to be taken into account that glucose uptake of natural populations was determined after incubations with 2 nM glucose (Table 1) whereas glucose uptake in cultures of *P. marinus* PCC 9511 was determined after incubations with 1 μM glucose (14), which correspond to two different

phases of the uptake kinetics, as shown in Figs. 24 and 44. Finally, it is worth noting that, because glucose uptake was found to be higher in the light (14), laboratory cultures might have higher glucose uptake rates due to an increased light availability, compared with natural samples obtained at 47, 55, and 80 m depth (Table 1).

Pro1404 belongs to the Major Facilitator Superfamily of transporters (31), which couple solute transport to the downgradient flow of an ion. Although the free energy for the transport of a glucose molecule by Pro1404 would need to be calculated, it is predicted to be much lower than the metabolic effort required for the synthesis of a molecule of glucose from CO_2 (18 molecules of ATP and 12 of NADPH). Therefore, taking up glucose is energetically advantageous for *Prochlorococcus* rather than to synthesize it de novo.

Previous works have shown that *Prochlorococcus* carried out CO_2 fixation at a rate of $1.2 \text{ fg C}\cdot\text{cell}^{-1}\cdot\text{h}^{-1}$ (32) in the same sampling area used in the present study. According to our estimations, based on the results obtained during this work (Table 1 and Fig. 6), *Prochlorococcus* was taking up carbon from glucose at rates of $0.16\text{--}0.31 \text{ fg C}\cdot\text{cell}^{-1}\cdot\text{h}^{-1}$. Therefore, our data suggest that, if carbon from glucose is incorporated to metabolism by *Prochlorococcus*, its ratio with respect to that incorporated from CO_2 would be roughly 1/6. Although this is an initial estimation that needs confirmation in larger field studies, it indicates that the importance of glucose uptake cannot be considered as negligible.

The ecological meaning of the results described above is very significant. We provide conclusive evidence that *Prochlorococcus* does take up glucose in the open Atlantic Ocean. Laboratory experiments demonstrate that the gene Pro1404 is coding for a high affinity glucose importer in *Prochlorococcus*. This means that, besides being possibly the most abundant autotrophic organism on Earth, *Prochlorococcus* can use organic molecules devoid of other essential elements (such as nitrogen or sulfur) except carbon. Moreover, initial estimates indicate that the ratio of carbon obtained from glucose is noteworthy and might constitute a significant part of total carbon incorporated by *Prochlorococcus* cells when glucose is available in the environment. This estimate is consistent with the bioenergetic advantage of glucose uptake vs. glucose synthesis de novo, summarized above in this paper. Although glucose is usually detected at very low concentrations in the open ocean, there might be events of sudden increase in its availability [such as excretions from other organisms, or detrital particles (26)], which might be exploited by *Prochlorococcus* to save energy for other metabolic uses.

Different studies suggest that the metabolism of *Prochlorococcus* is optimized to be extremely efficient in growing autotrophically at very oligotrophic environments. In our view, *Prochlorococcus* is essentially a phototroph but is also capable of taking up glucose when available as a way to save energy for other purposes.

If we take into account the abundance and wide distribution of *Prochlorococcus*, it seems reasonable to expect it to be an important player in the global carbon cycle for an unexpected reason: in addition to its important and widely recognized contribution to primary production, one should consider its role in the consumption of sugars. The implications of this conclusion are far reaching because they could significantly alter the

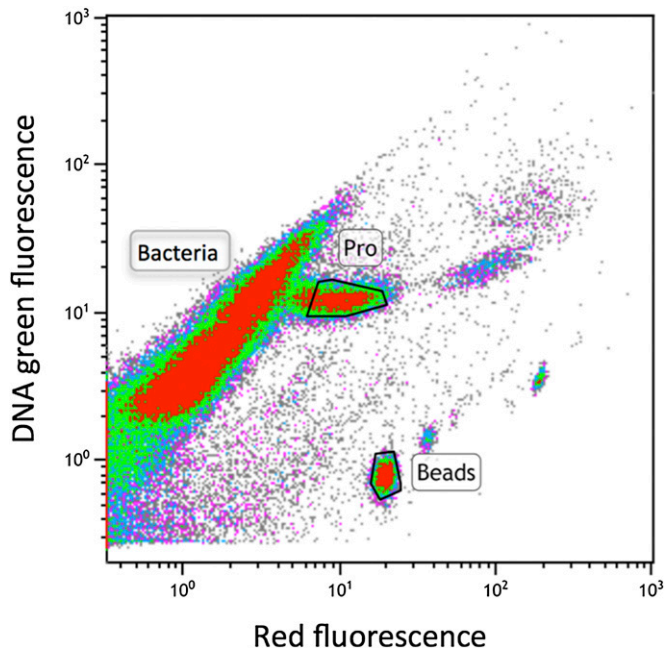


Fig. 5. Flow cytometric scatter plot showing a signature of SYBR Green I DNA stained picoplankton from station 35 of cruise AMT-21 (47 m depth). *Prochlorococcus* cells were identified by their extra red chlorophyll fluorescence, gated (area marked "Pro") and sorted to determine their ^3H -glucose uptake.

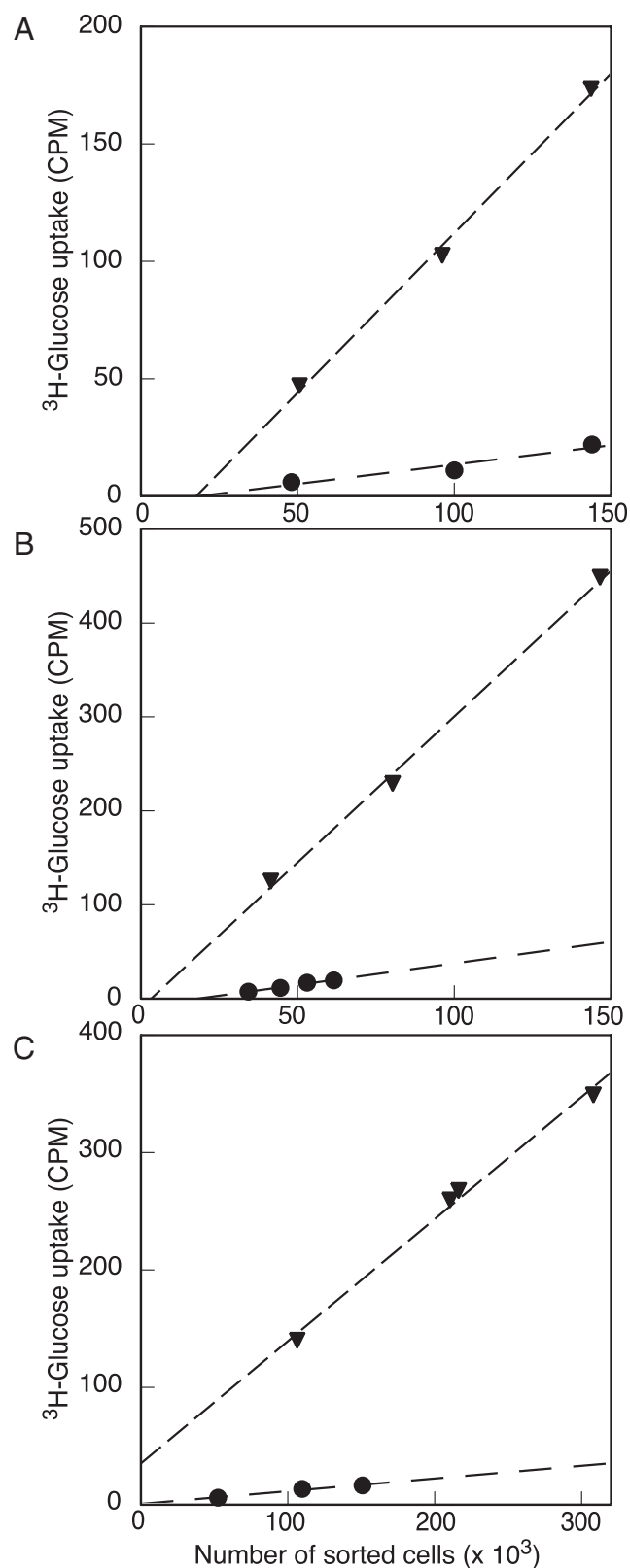


Fig. 6. ^3H -Glucose uptake vs. number of sorted *Prochlorococcus* (circles) or total bacterioplankton cells (triangles) at stations 15 (A), 35 (B), and 38 (C). Further details on the results obtained at those stations are shown in Table 1.

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 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' TGCCAAGCTTTTTAAGCCAACGG 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 *rnnB* 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 *rnnB* 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. [^3H] 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 μM to 20 μM , 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- μm (for *Prochlorococcus* sp. SS120 and *Synechococcus* sp. WH7803) and 0.45- μm (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-[5,6-³H]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- μ m 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 \cdot min⁻¹, depending on the cell concentration, for 1 min. Multifluorescence 0.5- μ m 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-[5,6-³H]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- μ m 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).

ACKNOWLEDGMENTS. We thank Prof. W. R. Hess (University of Freiburg) for providing cultures of *Synechococcus* WH7803, Mr. R. Holland (National Oceanography Centre, Southampton, United Kingdom) for help with flow cytometry analyses, and Prof. E. Flores (Instituto de Bioquímica Vegetal y Fotosíntesis) for insightful discussions. We acknowledge kind support from Dr. G. Tarran (principal scientist of the cruise D371-AMT21), as well as the crew of the *R.R.S. Discovery*, and thank Prof. S. García Navarro (Universidad de Córdoba) for kindly providing access to radioactivity facilities. We also thank the Roscoff Culture Collection (Station Biologique) for providing *Prochlorococcus* strains (ASSEMBLE project, Grant Agreement 227799, from the “Capacities” program, Seventh Framework Programme, European Union). This work was supported by Grants BFU-2009-08008/BMC and BFU2010-19544 (Spanish Ministerio de Educación y Ciencia cofunded by the European Union European Social Fund) and by Universidad de Córdoba (Programa Propio de Investigación). M.d.C.M.-M. received a grant from Projects BFU-2009-08008/BMC and P07-CVI-3055 (Proyectos de Excelencia). This study is a contribution to the international Integrated Marine Biogeochemistry and Ecosystem Research project and was supported by the UK Natural Environment Research Council National Capability funding to Plymouth Marine Laboratory and the National Oceanography Centre, Southampton. This is contribution number 232 of the Atlantic Meridional Transect (AMT) programme.

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