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 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.

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, Synechocystis 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.


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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 μM 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 $^{14}$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 μM 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.
(Muñoz-Marín et al. PNAS). When we compared the decrease in radioactive glucose uptake by addition of cold glucose as control, the observed values were 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* CK1 (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 CK1, as compared by the Student’s t test ($P < 0.05$). In the case of *Synechococcus* WH7803, again a multiphasic kinetics was observed, with measurable $K_c$ values of 21.55 nM and 2.86 μM. Interestingly, the high affinity $K_c$ value of *Synechococcus* WH7803 is roughly sixfold 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$^{-1}$ mg prot$^{-1}$ mL$^{-1}$, respectively). If we take both parameters into account to calculate the uptake efficiency (maximum uptake rate/$K_c$) 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 $^3$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 $^3$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 take up glucose at low concentrations.
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 ± 0.56%; *Prochlorococcus* cellular glucose uptake relative to the average bacterioplankton cell uptake was 13 ± 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 × 10⁻⁸, 1.597 × 10⁻⁸, and 0.321 × 10⁻¹⁰ μCi-cell⁻¹-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 × 10⁻⁸ μCi-cell⁻¹-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. 2A and 4A. 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).

![Flow cytometric scatter plot showing a signature of SYBR Green I DNA stained picoevents 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.](image)

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 CO₂ (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 CO₂ fixation at a rate of 1.2 fg C-cell⁻¹-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 0.16–0.31 fg C-cell⁻¹-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 CO₂ 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

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

<table>
<thead>
<tr>
<th>Geographical coordinates</th>
<th>Station</th>
<th>Depth, m</th>
<th>Total Prochlorococcus uptake, μCi/mL</th>
<th>Total bacterial uptake, μCi/mL</th>
<th>% total uptake by Prochlorococcus</th>
<th>[Glucose], nM</th>
</tr>
</thead>
<tbody>
<tr>
<td>33° 30.11’ N 28° 45.21’ W</td>
<td>15</td>
<td>80</td>
<td>4.24 × 10⁻⁵</td>
<td>128 × 10⁻⁵</td>
<td>3.3</td>
<td>0.50</td>
</tr>
<tr>
<td>10° 45.43’ N 31° 52.40’ W</td>
<td>35</td>
<td>47</td>
<td>17.7 × 10⁻⁵</td>
<td>480 × 10⁻⁵</td>
<td>3.7</td>
<td>0.52</td>
</tr>
<tr>
<td>06° 31.34’ N 29° 07.96’ W</td>
<td>38</td>
<td>55</td>
<td>4.18 × 10⁻⁵</td>
<td>160 × 10⁻⁵</td>
<td>2.6</td>
<td>2.7</td>
</tr>
</tbody>
</table>

![Red fluorescence](image)
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 NaGene 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 μE m⁻² 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 μE m⁻² 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 CO₂ and air (1%, vol/vol). For recombinant strains, kanamycin was used at 7 μg 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′ GGAGGTCATGTTCCATGGAAT 3′; and
MELB-1R: 5′ TGCCAGGTTTTAAGCCAACGG 3′.

The amplified fragment was cloned between the Ncol 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. [U-¹⁴C] 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 filters (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. 51). At each of 38 stations, surface seawater was collected in Niskin bottles and decanted onto HCl-washed vacuum flasks. Samples were analyzed within 1 h of sampling. 

- samples were added to 1.6 mL of seawater samples, in a 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 20 mL of scintillation mixture, using a liquid scintillation counter (Tri-Carb 3100; Perkin-Elmer).

To determine cell abundance, 1 mL of untreated seawater was added to 1.6 mL of seawater samples, in a liquid scintillation counter (Tri-Carb 3100; Perkin-Elmer). Glucose turnover times were plotted against the concentrations of added glucose; in this presentation, 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 nuclear acid stain (Sigma) in the presence of 30 mM potassium citrate (38) for 1 h. Samples were analyzed in a flow cytometer (FACS-Calibur; Beckton Dickinson Biosciences) at a flow rate of 15–60 mL min⁻¹, depending on the cell concentration, for 1 min. Multifluorescent 0.5-µm beads (Fluoresbe 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 mM [5,6-3H]glucose for 4 h. Samples were fixed with 1% PFA, and DNA was stained as described above. Prochlorococcus cells were sorted from 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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