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RESEARCH PAPER

# A mechanistic study of the influence of nitrogen and energy availability on the $NH_4^+$ sensitivity of nitrogen assimilation in *Synechococcus*

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## Abstract

**In most algae, $NO_3^-$ assimilation is tightly controlled and is often inhibited by the presence of $NH_4^+$. In the marine, non-colonial, non-diazotrophic cyanobacterium *Synechococcus* UTEX 2380, $NO_3^-$ assimilation is sensitive to $NH_4^+$ only when N does not limit growth. We sequenced the genome of *Synechococcus* UTEX 2380, studied the genetic organization of the nitrate assimilation related (NAR) genes, and investigated expression and kinetics of the main NAR enzymes, under N or light limitation. We found that *Synechococcus* UTEX 2380 is a β-cyanobacterium with a full complement of N uptake and assimilation genes and NAR regulatory elements. The nitrate reductase of our strain showed biphasic kinetics, previously observed only in freshwater or soil diazotrophic *Synechococcus* strains. Nitrite reductase and glutamine synthetase showed little response to our growth treatments, and their activity was usually much higher than that of nitrate reductase. $NH_4^+$ insensitivity of NAR genes may be associated with the stimulation of the binding of the regulator NtcA to NAR gene promoters by the high 2-oxoglutarate concentrations produced under N limitation. $NH_4^+$ sensitivity in energy-limited cells fits with the fact that, under these conditions, the use of $NH_4^+$ rather than $NO_3^-$ decreases N-assimilation cost, whereas it would exacerbate N shortage under N limitation.**

**Keywords:** Ammonium, cyanobacteria, glutamine synthetase, limitation, nitrate reductase, nitrite reductase, NtcA regulation, N metabolism.

Abbreviations: 2OG, 2-oxoglutarate; *glnA*, GSI gene; *glnB*, PII gene; Glu, glutamate; GOGAT, glutamate synthase; GSI, type I glutamine synthetase; NAR, Nitrate Assimilation Related; *narB*, NR gene; NiR, nitrite reductase; *nirA*, NiR gene; NR, nitrate reductase; NrtP, nitrate/nitrite permease; NrtS1 and NrtS2 (NrtS1/S2), nitrate/nitrite bispecific transporter; *nrtS1 and nrtS2*, NrtS1 and NrtS2 gene; NtcA, global nitrogen regulator; *ntcA*, NtcA gene; NtcB, activator of NAR genes; *ntcB*, NtcB gene; PII, nitrogen regulation protein; RbcL, Rubisco large subunit; PipX, PII interacting protein; *pipX*, PipX gene.

## Introduction

Nitrate and ammonium are the most common chemical forms of combined inorganic N in nature (Giordano and Raven, 2014); the energetic costs for their assimilation are very different due to the different requirement of reductants for their assimilation into organic matter. The assimilation of one $NO_3^-$ into glutamate requires the oxidation of 10 NADPH and the hydrolysis of 2 ATP. Assuming that the energy obtained for the oxidation of 1 NADPH is equal to that released by the hydrolysis of 3 ATP (Nitschmann and Peschek, 1986; Scherer *et al.*, 1988), this process has a cost of 32 ATP equivalents. The assimilation of 1 $NH_4^+$ requires 2 NADPH and 1 ATP, corresponding to about 7 ATP equivalents; i.e. 78% fewer ATP equivalents are needed for the assimilation of $NH_4^+$ than for that of $NO_3^-$ (Lee and Erickson, 1987; Noctor and Foyer, 1998), although ammonium assimilation may have additional costs associated with minimizing its potential toxicity (Dai *et al.*, 2008; Collos and Harrison, 2014). Assuming a cell C:N:P:S stoichiometry of 124:16:1:1.3 (Giordano, 2013), $NH_4^+$-grown cells, other things being equal, consume 13% less energy than $NO_3^-$-grown cells to produce the same amount of biomass (Ruan and Giordano, 2017). This becomes relevant when energy is in short supply (Ruan *et al.*, 2017).

In algae (including cyanobacteria; Giordano and Raven, 2014), the concomitant presence of $NO_3^-$ and $NH_4^+$ very often leads to the shutdown of $NO_3^-$ assimilation (Dortch, 1990; L'Helguen *et al.*, 2008). However, the underlying mechanisms of this regulation appear to be different in cyanobacteria and eukaryotic algae (Fernandez and Galvan, 2008; Ohashi *et al.*, 2011). In *Chlamydomonas reinhardtii*, $NH_4^+$ interferes with gene activation via an impediment on the activity of the transcription factor NIT2. Cyanobacteria—commonly divided into two group, α-cyanobacteria with Form 1A Rubisco and β-cyanobacteria with Form 1B Rubisco—contain a rather complex and not fully elucidated mechanism controlling the negative regulation of $NO_3^-$ assimilation by $NH_4^+$ (Ohashi *et al.*, 2011). Although β-cyanobacteria have the 2-oxoglutarate decarboxylase bypass (Zhang and Bryant, 2011; Zhang *et al.*, 2016; Wan *et al.*, 2017), the carbon flux from 2-oxoglutarate to glutamate, catalysed by the glutamine synthetase (GS)–glutamate synthase (GOGAT) cycle, is considered to be the dominant route (Forchhammer and Selim, 2020), especially since cyanobacteria seem to preferentially perform a non-cyclic tricarboxylic acid cycle during the light period (Esteves-Ferreira *et al.*, 2018). 2-Oxoglutarate is still accepted as the metabolic signal superintending the expression of nitrate assimilation related (NAR) genes in cyanobacteria: when 2-oxoglutarate is abundant, such as when the cell experiences a shortage of reduced N, it triggers the activation of NAR genes via the transcriptional regulator NtcA (Tanigawa *et al.*, 2002). Conversely, the presence of $NH_4^+$ leads to a depletion of the cell pool of 2-oxoglutarate and to the interruption of the activation of NAR genes via NtcA (Muro-Pastor *et al.*, 2005; Llácer *et al.*, 2010; Herrero and Flores, 2018). The NtcA-mediated regulation of NAR genes is favored by the interaction of NtcA with the PII interacting protein (PipX), a coactivator of NtcA, which appears to stabilize the active form of NtcA (Llácer *et al.*, 2010). When 2-oxoglutarate is low, the PipX protein preferentially binds to the PII protein; this prevents the formation of the NtcA–PipX complex and leads to a down-regulation of NAR gene expression (Espinosa *et al.*, 2014; Forcada-Nadal *et al.*, 2018). In β-cyanobacteria, when $NO_3^-$ is scarce, the activation of some of the NAR genes is controlled by both $NO_2^-$ and NtcA; the $NO_2^-$-dependent regulation of NAR gene is mediated by the transcription factor NtcB (Aichi *et al.*, 2004; Ohashi *et al.*, 2011).

The negative effect of $NH_4^+$ on $NO_3^-$ assimilation can also be exerted at the level of $NO_3^-$ transport. Cyanobacteria are equipped with an ABC-NRT type bispecific nitrate/nitrite transporter, present in most freshwater β-cyanobacteria (*Nostoc punctiforme* ATCC29133 is the only known exception), an NRT2 type nitrate/nitrite permease (NrtP), present in α-cyanobacteria apart from *Prochlorococcus* (Scanlan *et al.*, 2009; Ohashi *et al.*, 2011), or a newly found transporter, NrtS, which has been found in both α- and β-cyanobacteria (Maeda *et al.*, 2019). A few strains of β-cyanobacteria of the genera *Cyanothece* and *Arthrospira* possess both ABC-NRT and NrtP (see Table 1 in Ohashi *et al.*, 2011). It has been shown that ABC-NRT are post-translationally regulated by $NH_4^+$, via the ATP binding subunits NrtC and NrtD (Watzer *et al.*, 2019), whose long C-terminal extension is the site to which an $NH_4^+$-induced negative effector binds (Kobayashi *et al.*, 1997, 2005). On the contrary, NrtP is probably $NH_4^+$-insensitive, as observed for *Nostoc punctiforme* ATCC29133 (Aichi *et al.*, 2006). In this strain, nitrate reductase (NR) is $NH_4^+$-sensitive and thus the $NH_4^+$-inhibitory effect on $NO_3^-$ assimilation is retained. It is not clear to what extent this fact can be generalized. No information is available for the regulation of NrtS (Maeda *et al.*, 2019).

To elucidate the mechanisms behind the inhibitory effect of $NH_4^+$ on $NO_3^-$ assimilation (or the lack of it), and to attain a better understanding of the influence of energy availability on N assimilation, we cultured *Synechococcus* sp. UTEX 2380, originally isolated from coastal waters in Western Caroline Islands, Palau (see details of inorganic N, pH, etc. in Ruan *et al.*, 2017), under either N or energy (light) limitation, in the presence of either $NO_3^-$ or $NH_4^+$, and conducted a genomic, enzymological, and expression-based study on the main nitrate assimilation related (NAR) genes, with special attention to nitrate reductase.

## Materials and methods

### *Culture conditions*

The unicellular marine non-diazotrophic cyanobacterium *Synechococcus* UTEX LB 2380 was cultured semicontinuously as in Ruan and Giordano

(2017), under a 12 h light: 12 h dark photoperiod and an irradiance of 100 μmol photons $m^{-2}$ $s^{-1}$ (Ruan *et al.*, 2018). The Artificial Multipurpose Complement for the Nutrition of Algae (AMCONA) medium (Fanesi *et al.*, 2014) was modified to have two concentrations (22 and 550 μM) of either $NO_3^-$ or $NH_4^+$. At the lower N concentration, growth was limited by N, and cells grew faster if N concentration increased (up to 550 μM). At the higher N concentration, growth was limited by light (energy), and when cultures were moved to an irradiance of 200 μmol photons $m^{-2}$ $s^{-1}$, a higher growth rate was elicited regardless of N chemical form; however, this was not true for low N grown cultures (Ruan and Giordano, 2017; Ruan *et al.*, 2017). The semi-continuous cultures were diluted at 0.40 $d^{-1}$ (22 μM nitrate), 0.35 $d^{-1}$ (22 μM ammonium), 0.60 $d^{-1}$ (550 μM nitrate), and 0.67 $d^{-1}$ (550 μM ammonium) (Ruan and Giordano, 2017). These dilution rates corresponded to the maximum specific growth rates measured in batch cultures under the same culture conditions. The cultures were maintained under these conditions for at least 16 d (≥8 generations) prior to any measurement. Measurements were done on samples collected 3 h after the onset of the light period.

### *Growth, protein abundance, C and N cell quotas, and C and N net assimilation rates*

The methods used to determine growth, protein abundance, C and N cell quotas, and C and N net assimilation rates were reported in Ruan and Giordano (2017). In brief, cell numbers (from which specific growth rates were derived) and cell volume were measured with an automatic cell counter (Casy TT, Innovatis AG, Reutlingen, Germany). The protein cell content was determined according to Peterson (1977). C and N cell quotas were assessed with an elemental analyser (EA1108, Carlo Erba Instruments, Milan, Italy). C and N assimilation rates were calculated by multiplying the cell quotas by the specific growth rates.

### *Enzyme activities and kinetics*

Cells were collected by centrifugation at 16 000 *g* for 20 min with a Beckman J2-MC centrifuge (Beckman Coulter, Brea, CA, USA); they were then washed twice in a solution containing 25 mM HEPES–NaOH (pH 7.0) and 1 mM KCl, and resuspended in the same solution (Herrero *et al.*, 1981; Sakamoto and Bryant, 1999).

### *Nitrate reductase activity assay*

Prior to NR activity measurements, 1 ml of cell suspension containing 5–8 × $10^9$ cells was whirl-mixed in the presence of 20 μl of toluene for 2 min to permeabilize the cells (Herrero *et al.*, 1981). The cells were assayed within 60 min after this procedure and kept on ice until used. To avoid temperature variation in the course of the assay, a 60 μl aliquot of permeabilized-cell suspension was added to a 2 ml reaction mixture pre-incubated at the assay temperature (30 °C), in a thermostated bath. The temperature of this mix was allowed to equilibrate for 5 min. The reaction mixture contained: 0.1 M $Na_2CO_3$–$NaHCO_3$ buffer (pH 10.5), 1 mM KCl, 4 mM methyl viologen, and $NaNO_3$ at a concentration varying from 0 to 80 mM (see Fig. 3). The reaction was initiated by the addition of 20 μl of a 1.0 M $Na_2S_2O_4$ solution. The $Na_2S_2O_4$ solution was freshly prepared in $N_2$-sparged 0.3 M $NaHCO_3$. The addition of this solution caused the reduction of methyl viologen, which acted as electron provider to NR. Aliquots of 0.7 ml of the reaction mix were sampled after 1 and 4 min (linearity in this time range was preliminarily checked) and shaken vigorously until the blue color associated to the reduced form of methyl viologen disappeared. Samples were then centrifuged for 5 min at 13 700 *g*, and 0.5 ml of the supernatant was transferred to a new Eppendorf tube. To this solution, 0.5 ml of 1% (w/v) sulfanilamide in 3 M HCl was added, followed by 0.5 ml of 0.02% (w/v) *N*-(1-naphthyl)ethylenediamine (Nicholas and Nason, 1957). The samples were incubated in the presence of these reagents for 15 min. Then the absorbance at 540 nm, due to interaction of the reagents with $NO_2^-$, was determined with a Beckman DU 640 spectrophotometer (Beckman Coulter). The $NO_2^-$ concentration was calculated by interpolating the absorbance values into a standard curve generated using 0–50 μM $NaNO_2$.

### *Nitrite reductase (NiR) activity assay*

Nitrite reductase (NiR) activity was assayed on toluene-permeabilized cells, as for the NR assay. An aliquot of 0.15 ml of cell suspension containing 0.5–1.3 × $10^9$ permeabilized cells was incubated for 5 min at 30 °C in 5 ml of reaction mix, before initiating the assay. The reaction mixture contained 25 mM HEPES buffer (pH 7.0), 1 mM KCl, 20 mM $NaNO_2$ and 5 mM methyl viologen. The reaction was started by the addition of 100 μl of a 2 M $NaNO_2$ stock. The $NaNO_2$ concentration in our reaction mixture was much higher than that used by other authors, e.g. 100 μM (Sakamoto and Bryant, 1999), because in preliminary experiments we noticed that the NiR of our *Synechococcus* strain was saturated above 30 mM $NaNO_2$. One hundred micromoles of sodium dithionite solution in 0.1 ml $N_2$-sparged 0.3 M $NaHCO_3$ was added to start the reaction. Aliquots of 0.4 ml were taken from the reaction mixture at time 0 and after 10 min (linearity was checked preliminarily; the enzyme activity was verified to be stable for at least 15 min). The reaction was terminated by vigorous shaking to oxidize the reduced methyl viologen. The samples were then centrifuged (13 700 *g* for 5 min) and the supernatant was diluted 1 in 400 (v: v). Aliquots (0.5 ml) of the diluted supernatant were used for the quantification of nitrite concentration (see above, NR activity assay).

### *Glutamine synthetase activity assay*

For the GS assay, alkyltrimethylammonium bromide (MTA, Chemical Abstract Service registry no. 8044-71-1) at a final concentration of 0.025% (w/v) was used as the permeabilizing agent, since toluene interferes with the GS assay (Marqués *et al.*, 1989).

The assay was carried out according to Marqués *et al.* (1989), except for the killing solution and the substrate: the killing solution contained 2.5% $FeCl_3$ (w/v), 5% trichloroacetic acid (w/v) and 1.5 M HCl. $NH_2OH$ was used as the inorganic substrate, according to Kaffes *et al.* (2010) and Oaks *et al.* (1980), substituting for the $NH_4Cl$ used by Marqués *et al.* (1989). This was done because $NH_2OH$, being less volatile than the $NH_3$ in equilibrium with $NH_4^+$, ensures a greater control of substrate concentration. The absorbance of the supernatant at 540 nm due to the reaction product (L-glutamic acid γ-monohydroxymate) was determined with a Beckman DU-64 spectrophotometer (Beckman Coulter).

An aliquot of 50 μl of permeabilized cell suspension (containing 2.5–4 × $10^8$ cells) was added to a reaction mixture containing 50 mM HEPES (pH 7.5), 50 mM $MgCl_2$, 100 mM monosodium glutamate (pH 7.5), 2 mM $NH_2OH$–HCl (pH 7.5), 7.5 mM ATP (pH ~7.4), in a total volume of 1 ml. The reaction mixture was carefully prepared to make sure that the pH was always 7.5, at which ATP is stable and the enzyme activity is high (close to optimum pH) (Gawronski and Benson, 2004). The reaction took place in a water bath at 30 °C and was initiated by ATP addition. After 15 min, the reaction was stopped by adding 200 μl of killing solution to the assay mixture. The control assays were stopped at time 0. After the termination of the reaction, cells were pelleted by centrifugation at 13 700 *g* for 5 min, in a Heraeus *fresco* microcentrifuge (Thermo Fisher Scientific, Langenselbold, Germany). The absorbance of the supernatant at 540 nm was determined with a Beckman DU-64 spectrophotometer. The amount of L-glutamic acid-γ-monohydroxymate generated over the 15 min of the assay was determined by interpolation of absorbance in a standard curve constructed with γ-monohydroxamate (0–250 μM).

GS kinetics was studied by varying both the inorganic ($NH_2OH$) and the organic (monosodium glutamate) substrates. One of the substrates was added to the reaction mix at saturating concentration; the other substrate

was added in incremental amounts to obtain a substrate concentration response. When glutamate was the 'variable' substrate, its concentration went from 2 to 200 mM. For the $NH_2OH$ response curve, the substrate concentration range was between 60 and 3000 μM.

### *Expression of nitrate reductase gene (narB)*

Thirty-four amino acid sequences of NR from marine *Synechococcus* strains were downloaded from NCBI's publicly available database and were aligned using DNAMAN V6 (Lynnon Biosoft, San Raman, CA, USA). Two highly conserved regions were identified: NNFDANSRL-CMSSA and GPFSLTGQPNAMGGREAGGL. An alignment of 27 *Synechococcus* nitrate reductase gene (*narB*) sequences obtained with DNAMAN was examined for codon preference. Five pairs of degenerate primers were designed according to CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) (Rose *et al.*, 2003). CODEHOP primers consists of a short 3′ degenerate core region with three to four highly conserved amino acid residues and a longer 5′ consensus clamp region that permits stable annealing during PCR amplification. Primer efficiencies were verified on serial dilutions of cDNA. LightCycler 480 Software (F. Hoffmann-La Roche Ltd, Basel, Switzerland) was used to analyse the data. Analyses of the denaturation curves for the reactions showed a single peak in all cases and no amplification products of primer-dimer formations were observed. The primer pair shown in Supplementary Table S1 was used for template amplification in a Block Incubator (ASTEC BI-525A, Fukuoka, Japan), according to the protocol described in Supplementary Table S2. The target segment was retrieved and purified with Midi Purification Kit (DP209, Tiangen, Beijing, China) following the manufacturer's specifications and amplified again to increase the amount of product. Two microliters of the final PCR product was electrophoresed on 1.0% agarose gel to verify fragment size and quality. An additional 2 μl aliquot of PCR product was used to determine the product concentration in a Microvolume Nanodrop Spectrophotometer (NanoDrop 2000, Thermo Fisher scientific, Waltham, MA, USA); the concentration was >30 ng $\mu l^{-1}$. The target product was sequenced with Illumina Hiseq (Illumina, San Diego, CA, USA). Based on this sequence, a new pair of 'confirmation' primers (Supplementary Table S1) was designed with Primer Premier V5.00 (Premier Biosoft International, Palo Alto, USA). PCR products obtained with the confirmation primers were sequenced to ensure no replication error had occurred during amplification. A partial sequence of UTEX 2380 *narB* was submitted to NCBI Genebank (accession no. MH684745). This sequence was used in PrimerQuest (Integrated DAN Technology, Skokie, IL, USA) to design RT-qPCR primers.

Total RNA was extracted from $8 \times 10^7$ cells ground in a mortar with TRNZol Universal reagent (cat. no. DP424, Tiangen) following the manufacturer's instructions. Total RNA was resuspended in 20 μl of diethylpyrocarbonate-treated water and quantified in a Nanodrop Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA); protein and carbohydrate contamination was assessed by determining the $OD_{260}/OD_{280}$ and $OD_{260}/OD_{230}$ ratio respectively; a value in the range of 1.8~2.0 for the former and a value >2.0 for the latter were considered acceptable for further processing. Samples of the extracted RNA (5 μl) were mixed with 1 μl of 6× Loading Buffer containing 0.05% bromophenol blue and 0.05% xylene cyanol FF (Tiangen) and subject to electrophoresis on 1.0% agarose gels. After completion of the electrophoretic run, the gel was inspected with a Gel-Imaging System (Bio-Rad Laboratories EOBR/01438, Hercules, CA, USA) to check for RNA quality. The template RNA was mixed with 2 μl of 5× gDNA buffer and RNase-free $ddH_2O$ at 42 °C to remove residual genomic DNA, then stored on ice. Reverse transcription was carried out using the FastQuant RT Kit (Cat. No. KR106, Tiangen, Beijing, China). The reverse transcription mix was composed of 10× Fast RT Buffer (2 μl), RT Enzyme Mix (1 μl), FQ-RT Primer Mix (2 μl), and RNase-free $ddH_2O$ (5 μl). This mix was added to the template RNA mix, at 42 °C for 15 min, and then incubated at 95 °C for 3 min. For RT-qPCR, the SuperReal PreMix Plus (SYBR green) kit (cat. no. FP205, Tiangen) was used. The RT-qPCR was conducted in a Roche LightCycler 480II (Hoffmann-La Roche Ltd), in a reaction mix composed of 12.5 μl of 2× SuperReal PreMix Plus, 0.75 μl of forward and reverse primers (20 μM), 5.0 μl of cDNA (30 ng/μl) and 6.75 μl of RNAse-free $ddH_2O$. Samples were subject to 15 min pre-denaturation at 95 °C, followed by forty 10-s cycles of denaturation at 95 °C, annealing at 55 °C for 32 s, and elongation at 72 °C for 20 s. Fluorescence was measured after the elongation step. The RNase P gene (*rnpB*) (Paerl *et al.*, 2012) was used as the internal control gene, after verifying that its expression was stable under all our experimental conditions (Supplementary Table S3).

### *Genome sequencing and annotation*

Observing that NR activity in UTEX 2380 was insensitive to $NH_4^+$ inhibition while energy was sufficient for growth, and that the UTEX 2380 enzyme showed biphasic kinetics prompted us to look for peculiarities in the sequences of N transport and assimilation genes and in their regulatory elements. We thus sequenced genomic DNA from *Synechococcus* UTEX 2380 using Illumina Hiseq XTen and Miseq platforms (Illumina). Sequencing, assembly, and annotation were carried out with the assistance of Sangon Biotech Shanghai Co. Ltd. (Shanghai, China; contract no.: GEN1815830SZ).

DNA for sequencing was prepared using a Rapid Bacterial Genomic DNA Isolation Kit (cat. no.: B518225, Sangon Biotech) according to the manufacturer's instructions. DNA integrity was checked by agarose electrophoresis (0.7%, 20 V for 15 h), and concentration was determined by fluorometric assay using a Qubit 4.0 Fluorometer (Thermo Fisher Scientific). DNA was then fragmentated into 300–500 bp segments with a S220 Focused-Ultrasonicator (Covaris, Woburn, MA, USA). A genomic paired-end library was prepared with a NEB Next Ultra DNA Library Prep Kit for Illumina (cat. no.: NEB#E6177, New England Biolabs, Ipswich, MA, USA). Adaptor-ligated fragments were then purified and enriched for PCR with Agencourt AMPure XP Clean Beads. Library volume was checked by 2% agarose gel electrophoresis, and concentration was determined by Qubit 4.0 fluorometric assay. The range of DNA fragments size was determined using a 2100 DNA 1000 Kit (recorder no.: 5067-1504, Agilent Technologies, Waldbronn, Germany).

Sequencing was completed using both Illumina Hiseq Xten and Illumina Miseq platforms. Data quality was assessed with FastQC 0.11.2 (Babraham Bioinformatics, Cambridge, UK), and artifact primer sequence and read pairs below quality threshold (where $Q<20$ and/or length <35 nt) were trimmed with Trimmomatic 0.36 (The Usadel Lab of RWTH Aachen University, Aachen, Germany) (Bolger *et al.*, 2014). Filtered reads were assembled with SPAdes 3.5.0 (Center for Algorithmic Biotechnology, St Petersburg State University, Russia). Contig gaps were filled using GapFiller 1.11 (Boetzer and Pirovano, 2012), and then PrInSeS-G 1.0.0 (Massouras *et al.*, 2010) was utilized to detect and correct structural variants incurred during assembly. Putative gene annotation was accomplished with the bacterial annotation pipeline Prokka 1.10 (Torsten Seemann, The University of Melbourne, Australia).

Specific genes involved in N acquisition and metabolism (*narB*, *nrtP*, *nrtS1*, *nrtS2*, *nirA*, *focA*, *glnA*, *glnN*, *glsF*, *glnB*, *ntcA*, *ntcB*, *glnB*, *pipX*) were identified by BLASTing known genes from *Synechococcus* sp. PCC 7002 (phylogenetically close to UTEX 2380, Fig. 1) against our assembly of *Synechococcus* UTEX 2380 (Altschul *et al.*, 1990). Multiple sequence alignment was used to visually assess BLAST hit similarities. Putative matches were verified though BLASTing the NCBI Conserved Domain Database, where the genes of interest (*narB*, *nrtP*, *nrtS1*, *nrtS2*, *nirA*, *focA*, *glnA*, *glnN*, *glsF*, *glnB*, *ntcA*, *ntcB*, *glnB*, *pipX*) were found to contain the conserved domains common to other cyanobacteria. The $NH_4^+$-sensitive


**Fig. 1.** Phylogenetic tree based on the sequences of Rubisco large subunit.

regulatory motifs in the genome were identified by blasting the NtcA or NtcB motif against the upstream of identified N-related gene sequences. The query sequences used were GTA-$N_8$-TAC-$N_{22/23}$-TA-$N_3$-T (the consensus binding sites for NtcA-motif) and ATGC-$N_7$-GCAT-$N_7$-ATGC-$N_7$-GCAT (consensus binding sites for NtcB-motif) (Jiang *et al.*, 2000; Ohashi *et al.*, 2011).

For *nrtS* and *pipX*, a simple BLAST failed to determine matching sequence. In these cases, we obtained 67 and 42 homologous sequences (Supplementary Tables S4, S5), respectively. The 67 homologs of *nrtS* were derived from Maeda *et al.* (2019, supplementary data). The 42 homologs of *pipX* were obtained by BLASTing a known *pipX*, UniProtKB entry Q7X386_SYNE7 (PipX, *Synechococcus elongatus* strain PCC 7942), against NCBI's Conserved Domain Database to identify Conserved Protein Domain Family DUF3539. All family members were obtained as a multiple sequence alignment. For each set, hidden Markov models were built using HMMER's hmmbuild function with standard parameters (http://hmmer.org). We then used HMMER's hmmsearch function to identify putative matches in our translated dataset. The *nrtS* search returned exactly two matches, with scores of 108.8 and 101.9, with E-values <1e-30. The *pipX* search returned exactly one match, with a score of 100.2, E-value <1e-29.

### *Classification of Synechococcus UTEX 2380*

We sought to determine whether *Synechococcus* UTEX 2380 should be classified as an α-cyanobacterium or a β-cyanobacterium. Typically, the attribution to α- or β-cyanobacteria is based on the type of Rubisco and/or carboxysome proteins (Badger and Bek, 2008). Given the comparatively larger availability of Rubisco sequence data relative to those of carboxysome protein, we proceeded to compare the sequence of Rubisco large subunits (RbcL) in our genome with orthologs from a large number of strains of both α- and β-cyanobacteria. Seventy-six non-redundant cyanobacterial and two terrestrial bacterial RbcL peptide sequences were obtained from NCBI's publicly available sequence database (Supplementary Table S6). Sequence orientation was manually confirmed, and sequences were subsequently aligned using Clustal Omega, with standard parameters (Sievers *et al.*, 2011). The resulting fasta-format multiple sequence alignment was converted to phylip format via Fasta2Phylip (https://github.com/jvollme/fasta2phylip). The final maximum likelihood tree was generated using RAxML version 8 (Stamatakis, 2014), employing the GTR gamma substitution model with 100 bootstrap replicates.

### *Statistical methods*

The programs and statistical packages used for the analysis of the genome are given above. For all other measurements, data were expressed as means and standard deviation of three biological replicates (i.e. three distinct cultures). The significance of variance was assessed by two-tailed *t*-test using the Origin 7.0 SR0 software (OriginLab Corp., Northampton, MA, USA) or by two-way ANOVA followed by Student–Newman–Keuls multiple comparison test using GMAV 5.0 for Windows XP (Underwood and Chapman, Institute of Marine Ecology, University of Sydney, Australia). In the two-way ANOVA, the significance of homogeneity of variance was always checked by Cochran's test, before analysis; if it was significant, the original data were transformed until the homogeneity of variance was not significant. The level of significance was always set at 95%.

## Results

### *Growth, protein abundance, C and N cell quotas, and C and N net assimilation rates*

The information on basic cell parameters is summarized in Supplementary Table S7. These data were already published in (Ruan and Giordano, 2017), but are essential for the comprehension of this dataset and were thus deemed necessary to include herein. In brief, the cells cultured under energy limitation (550 μM N) grew faster, were larger, contained more protein, and had higher net rate of C and N assimilation than the N-limited cells. The C cell quota was instead similar under the two limitations. Overall, a greater allocation of C to protein was observed in the cells grown under energy limitation (which also had more N available). The N chemical form had an impact on the various parameters in Supplementary Table S7 (growth, protein abundance, C and N cell quotas, and C and N net assimilation) only when energy was limiting, with higher values for all parameters in the $NH_4^+$ grown cells.

### *Phylogeny of* Synechococcus *UTEX 2380*

Our phylogenetic analysis of the sequence of the Rubisco large subunit gene of *Synechococcus* UTEX 2380 (GenBank accession no.: MN373284) conclusively places this organism among the β-cyanobacteria (Tabita *et al.*, 2008) and within a single clade containing all other unicellular β-cyanobacteria examined herein (Fig. 1), with the branch dividing α- and β-cyanobacteria showing 100% bootstrap support. The α-cyanobacteria can be considered a monophyletic group, which previously reported multi-gene phylogenies have indicated as being nested within the paraphyletic β-cyanobacteria, with *Gloeobacter* placed as sister to all other β-cyanobacteria—though a consensus has not been reached for finer details of the topology (Rae *et al.*, 2013; Sánchez-Baracaldo *et al.*, 2014; Moore *et al.*, 2019). Within the β-cyanobacteria, the unicellular and the multicellular (filamentous or colonial) species generally group separately; however, the relationships between these β-subsets are not sufficiently resolved in the present analysis to be described as strictly distinct clades. Four outlier species within the unicellular β-subset preclude this observation: *Chroococcidiopsis thermalis* and *Dactylococcopsis salina* are both rooted outside the greater clade of unicellular β-cyanobacteria, with bootstrap support of 22 and 51, respectively. These values are too low to be certain of these species' placement in the presented topology. More deeply within the unicellular β-cyanobacteria, we observed *Bacillus subtilis* (Ashida *et al.*, 2003), a terrestrial non-autotrophic bacterium, placed with 100% support. *Bacillus* Rubisco-like proteins (RLP) have been reported to be involved in methionine metabolism (Sekowska *et al.*, 2019 and references therein), and it has been long recognized that Rubisco phylogenies can topologically differ from those based on other loci due to differential retention of duplicated gene copies, or as may be the case with *Bacillus*, a horizontal gene transfer (Delwiche and Palmer, 1996). Perhaps surprising is the highly supported presence of a filamentous *Leptolyngbya* within the unicellular β-cyanobacteria.

Among the included filamentous and colonial β-cyanobacteria, and excepting the aforementioned *Leptolyngbya* sp., both *Prochlororothix hollandica* and *Arthrospira* sp. are

rooted outside the greater clade, with respective bootstrap values of 75 and 22. We consider these values inconclusive, and cannot be certain of their true placement at this time.

### *Nitrate assimilation genes in* Synechococcus *UTEX 2380*

The Illumina HiSeq platform sequencing effort provided 2 482 535 paired-end reads, totaling 360 847 093 base pairs (bp), with an average read length of 143.1 bp. The MiSeq platform effort provided 982 685 paired-end reads totaling 237 484 177 bp in length, with reads averaging at 241.7 bp. Our *de novo* assembly was resolved to a total concatenated length of 3 478 287 bp across 95 contigs, with a mean GC content of 49%. Contigs averaged 36 613.3 bp in length, and the current draft genome has a N50 of 80 905 base pairs. A total of 3360 protein coding genes were identified across all available databases and ranged from 37 bp to 8163 bp in length. At the date of submission, 97.52% and 97.03% of identified protein coding genes have maintained accession records in the NCBI NR and TrEMBL databases, respectively. Total coding bases in the draft genome amounted to 3 035 878 bp, for a coding ratio of 87.28%. We identified 130 repeated regions, totaling 8672 bp, with an overall ratio of 0.25% repeated sequence.

Among our genes of interest, two pairs of genes were found to co-occur in their given contigs of the current assembly. The first pair included *narB* (GenBank accession no.: MN373283) and *narP* (MN373275); the second pair was constituted by *nrtS1* (MN603758) and *nrtS2* (MN603759). The remaining nitrate assimilation genes, *focA* (MN603757), *nirA* (MN373276), *glnA* (MN373277), *glnN* (MN373278), *glsF* (MN373279), *ntcA* (MN373280), *ntcB* (MN373281), *glnB* (MN373282), and *pipX* (MN793312), were distributed in independent contigs. Further sequencing and analysis are required to resolve sequence gaps and determine operon structure and organization in UTEX2380.

### *Nitrate transporters*

In the genome of *Synechococcus* UTEX 2380, we found one *nrtP* gene; no ABC type transporter was found. Upstream of *nrtP*, one NtcA and one NtcB binding motif are present (Fig. 2). Two nitrate/nitrite bispecific transporter genes, *nrtS1* and *nrtS2* (Supplementary Table S4), possibly forming heteromeric transporter complexes (Maeda *et al.*, 2019), were closely related (96–100%) to the *nrtS1* and *nrtS2* of *Synechococcus* strains PCC7117, PCC7002, PCC8807, and PCC7003. In UTEX 2380, these were found to occur in tandem, sharing a reading frame and separated by only 14 amino acid residues. No typical NtcA or NtcB motif was found upstream of these two genes. In *Synechococcus* PCC7003 and NIES-970, we found only a single *nrtS* gene; the similarity of this gene to the *nrtS* of our experimental strain is much lower than that of the aforementioned *Synechococcus* strains and appreciably higher with *nrtS1* than with *nrtS2* (Supplementary Table S8).

### *Nitrate reductase*

Immediately downstream to the *nrtP* gene, a gene coding for NR was identified (*narB*), with 81–97% similarity to *narB* genes of other *Synechococcus* strains (Supplementary Table S8). No other NR encoding gene was identified in the genome. Both *nrtP* and *narB* have the same orientation, but they are separated by a 208 nt intergenic region, suggesting they occupy different reading frames. A putative NtcA-binding motif (GTA-$N_9$-TAC) was found between these two genes, which contained one base more than normal (NtcA-binding motifs are GTA-$N_8$-TAC). Furthermore, a −10 promoter box is usually located 22 or 23 bases after the NtcA-binding motif, but is not present in *Synechococcus* UTEX 2380 (Fig. 2). This could indicate the presence of a pseudogenic or non-functional NtcA-binding motif. We were unable to identify any other putative promoter sequence in the intergenic region: thus, *narB* and *nrtP* could potentially share an operon (Fig. 2).

### *Nitrite reductase*

A single *nirA* gene was identified. The similarity of this gene to those of other *Synechococcus* is 91–99% (Supplementary Table S8). The *nirA* sequence is preceded by NtcB and NtcA binding sites (Fig. 2). Only one of the two NtcB-binding sites described by Ohashi *et al.* (2011) is present upstream of the *nirA* gene.


**Fig. 2.** Putative promoters of nitrate assimilation genes and their regulatory genes.

### *Glutamine synthetase*

Genes encoding type I (*glnA*) and III (*glnN*) GS were identified in the genome of *Synechococcus* UTEX 2380. Their similarity with cyanobacterial orthologs was 84–99% (Supplementary Table S8). A typical NtcA-binding site is present upstream of GSI, but not of GSIII (Fig. 2).

### *Glutamate synthase*

A *glsF* gene, encoding a ferredoxin-dependent glutamate synthase (Fd-GOGAT), was identified in *Synechococcus* UTEX 2380 genome; its identity to other Fd-GOGAT of cyanobacteria was 77–98% (Supplementary Table S8). No NADP-dependent GOGAT was found in the genome. No canonical NtcA and NtcB binding motifs were found upstream of the gene encoding GOGAT.

### *Nitrogenase*

The genome analysis confirmed that this strain of *Synechococcus* does not contain genes encoding nitrogenases and is thus unable to fix $N_2$.

### *N metabolism regulators*

In *Synechococcus* UTEX 2380, the sequence of *glnB*, encoding the PII protein, was almost identical to that of *Synechococcus* PCC 7117, PCC 73109, PCC 7002, and PCC 8807 (99%). The NtcA and NtcB sequences were also highly similar (99% and 98%, respectively) to those of the above-mentioned *Synechococcus* strains (Supplementary Table S8). A putative NtcA binding motif was found upstream of the *ntcA* and *pipX* gene (Fig. 2; Supplementary Table S5). Upstream of the *ntcB* gene, an NtcA and two NtcB binding sites were found, and the −10 promoter box overlapped with one of the NtcB binding sites.

## *Enzyme activities and kinetics*

### *Nitrate reductase*

The kinetics of NR activity was studied for $NO_3^-$-grown cells (Fig. 3). NR activity showed biphasic kinetics. The first phase encompassed nitrate concentration between 0 and 1 mM; the second phase occurred between 1 and 80 mM $NO_3^-$ (Fig. 3). The NR $V_{max}$ (Fig. 3; Supplementary Table S9), expressed on a per cell basis, was independent of the nitrate growth concentration in phase I, but was 2.7-fold higher at 550 μM nitrate (energy limited cells) in phase II. If $V_{max}$ was expressed on a per protein basis, however, the value for phase II was about 3-fold higher in N-limited cells than that in their energy-limited counterparts. The $K_m(NO_3^-)$ was lower at low N (22 μM $NO_3^-$, nitrogen-limited), with

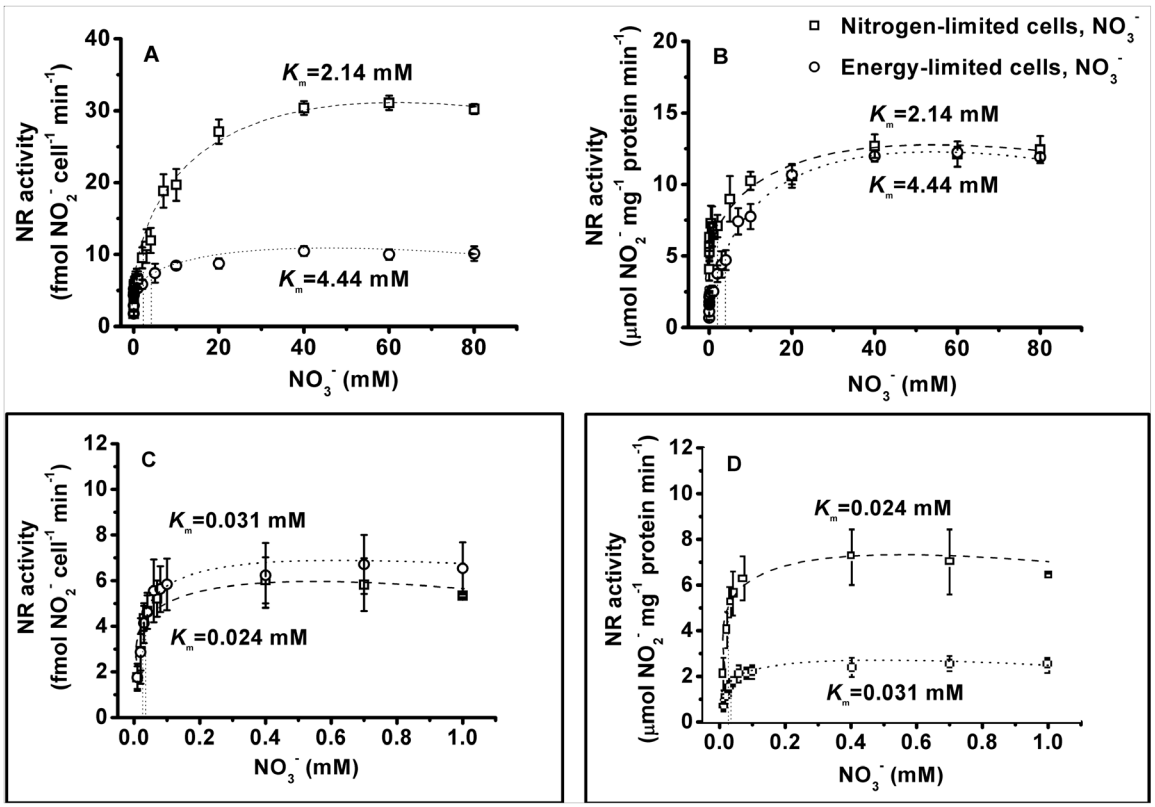


**Fig. 3.** Nitrate reductase (NR) kinetics for *Synechococcus* UTEX 2380 cells cultured under either N (□) or energy (○) limitation. The NR activity is shown as a function of $NO_3^-$ concentration on a per cell (A, C) and per mg of protein (B, D) basis. (C, D) enlarged views of the first phase of the curves in (A, B). The error bars indicate the standard deviations (*n*=3).

values that were about 75% and 48% those of 550 μM $NO_3^-$-grown cells, in phase I and phase II, respectively (Fig 3; Supplementary Table S9). Due to resource constraints, we were unable to study the kinetics of NR from N-limited $NH_4^+$-grown cells.

Interestingly, NR activity was present in $NH_4^+$-grown cells under N limitation (22 μM $NH_4^+$); the typical inhibitory effect of $NH_4^+$ on NR expression/activity was thus not observed in *Synechococcus* UTEX 2380 under N limitation. The NR $V_{max}$ of N-limited $NH_4^+$-grown cells was about half of that measured in the same conditions for $NO_3^-$-grown cells (Fig. 3; Supplementary Table S9). Under 550 μM $NH_4^+$ (and energy limitation), no NR activity was detected in the presence of $NH_4^+$.

### *Nitrite reductase*

NiR activity was two to three orders of magnitude higher than that of NR, under all conditions (Fig. 4), with very low affinity for $NO_2^-$ (in the mM range) and very high saturating concentration (>40 mM). This may imply that the intracellular $NO_2^-$ concentration could be very low to allow NiR activity to match that of NR. NiR $V_{max}$, whether expressed on a per cell or a per protein basis, had similar rates in all treatments, with only slight differences (Fig. 4; Supplementary Table S9).

### *Glutamine synthetase*

GS response to substrate concentration was studied for both glutamate (Fig. 5) and $NH_2OH$ (Supplementary Fig. S1). When glutamate concentration varied and $NH_2OH$ was saturating, the $V_{max}$ of GS responded to the N concentration, but not to the N chemical form. Only when $V_{max}$ was expressed on a protein basis, it was significantly higher in cells cultured in the presence of $NO_3^-$, under energy limitation and high N (Fig. 5; Supplementary Table S9). The $V_{max}$ of low N-grown cells was 2.0- ($NO_3^-$) and 1.7- ($NH_4^+$) fold higher than at high N on a per cell basis, and 2.4- ($NO_3^-$) and 3.7- ($NH_4^+$) fold on a protein basis (Supplementary Table S9). The $K_m$(Glu) of GS was not affected by either the N chemical form ($NO_3^-$ or $NH_4^+$) or the N concentration (22 μM or 550 μM). The same trends for both $V_{max}$ and $K_m$ were observed, except for $K_m$ between N-limited and energy-limited cultures grown on $NH_4^+$, when the $NH_2OH$ concentration was varied and glutamate was maintained constant at saturating concentrations (Supplementary Fig. S1; Supplementary Table S9).

### *Nitrate reductase expression*

The presence of NR activity on $NH_4^+$-grown cells prompted us to check if the expression of the *narB* gene, encoding nitrate reductase, followed the same trend (Fig. 6). At low N, no obvious difference in *narB* gene expression was shown between $NO_3^-$- and $NH_4^+$-grown cells. At high N, *narB* expression in $NH_4^+$-grown cells is indistinguishable from zero; in $NO_3^-$-grown cells, *narB* expression is much higher at high N than at low N.

## Discussion

The inhibitory role of even small amounts of $NH_4^+$ on $NO_3^-$ acquisition and assimilation in cyanobacteria has been known for many years (e.g. Dortch, 1990; Mérida *et al.*, 1991; Luque *et al.*, 1994). This is also the case for *Synechococcus* sp. UTEX 2380, under energy limitation. When, however, this strain is subject to N limitation (Supplementary Table S7), $NO_3^-$ is reduced even in the presence of $NH_4^+$ (Fig. 4). Our work was aimed at understanding the mechanisms that give rise to this phenotype.

### *Are transporters involved in the $NH_4^+$ insensitivity of nitrate reductase?*

From a metabolic point of view, the production and consumption of 2-oxoglutarate are key control points in N assimilation (Fig. 7): when reduced N is low within the cell, the 2-oxoglutarate pool is large and it favors the binding of NtcA to the promoters of most NAR operons, inducing their expression. When reduced N is generated in high amount, 2-oxoglutarate is aminated to glutamate, and the 2-oxoglutarate pool size decreases and NtcA binding to NAR gene regulatory sites is down-regulated (Herrero *et al.*, 2001; Forchhammer, 2008; Herrero and Flores, 2018; Forchhammer and Selim, 2020). In some organisms, the 2-oxoglutarate pool size is also at the base of post-translational modification of ABC-type NRT transport systems, whose activity is blocked in the presence of $NH_4^+$ (Ohashi *et al.*, 2011). Some NRT2 transporters (NrtP) found in cyanobacteria do not appear to be sensitive to $NH_4^+$. Our biochemical analysis did not allow us to directly test whether transport was affected by the presence of $NH_4^+$, since our activity assays were performed on permeabilized cells. The analysis of the genome showed that one *nrtP* gene and no ABC-type NRT genes encoding nitrate transporters are present in *Synechococcus* UTEX 2380. This is in agreement with previous reports for marine cyanobacteria (Ohashi *et al.*, 2011). If the *Synechococcus* UTEX 2380 NrtP transporter behaves like a typical cyanobacterial NRT2, then $NH_4^+$ should not have any effect on transport. It is worth noticing, however, that Sakamoto *et al.* (1999) reported on the $NH_4^+$ sensitivity of *nrtP* gene expression in *Synechococcus* PCC 7002, a strain that our Rubisco phylogeny places close to *Synechococcus* UTEX 2380. In *Synechococcus* UTEX 2380, *nrtP* is likely to be in the same operon with *narB*; the promoter region upstream of *nrtP* has typical NtcA and NtcB binding motifs, while the intergenic region separating it from *narB* contains only an atypical NtcA-binding motif that lacks a −10 box; we thus might expect *narB* and *nrtP* to show a similar expression pattern controlled primarily by the NtcA and NtcB systems. However, in *Nostoc punctiforme* ATCC29133, in which *nrtP* and *narB* share an operon with *nirA* (Ohashi *et al.*, 2011), *nrtP* is $NH_4^+$ insensitive, whereas *narB* appears to be down-regulated or inhibited in the presence of 3.75 mM $NH_4^+$; unfortunately, no data are available

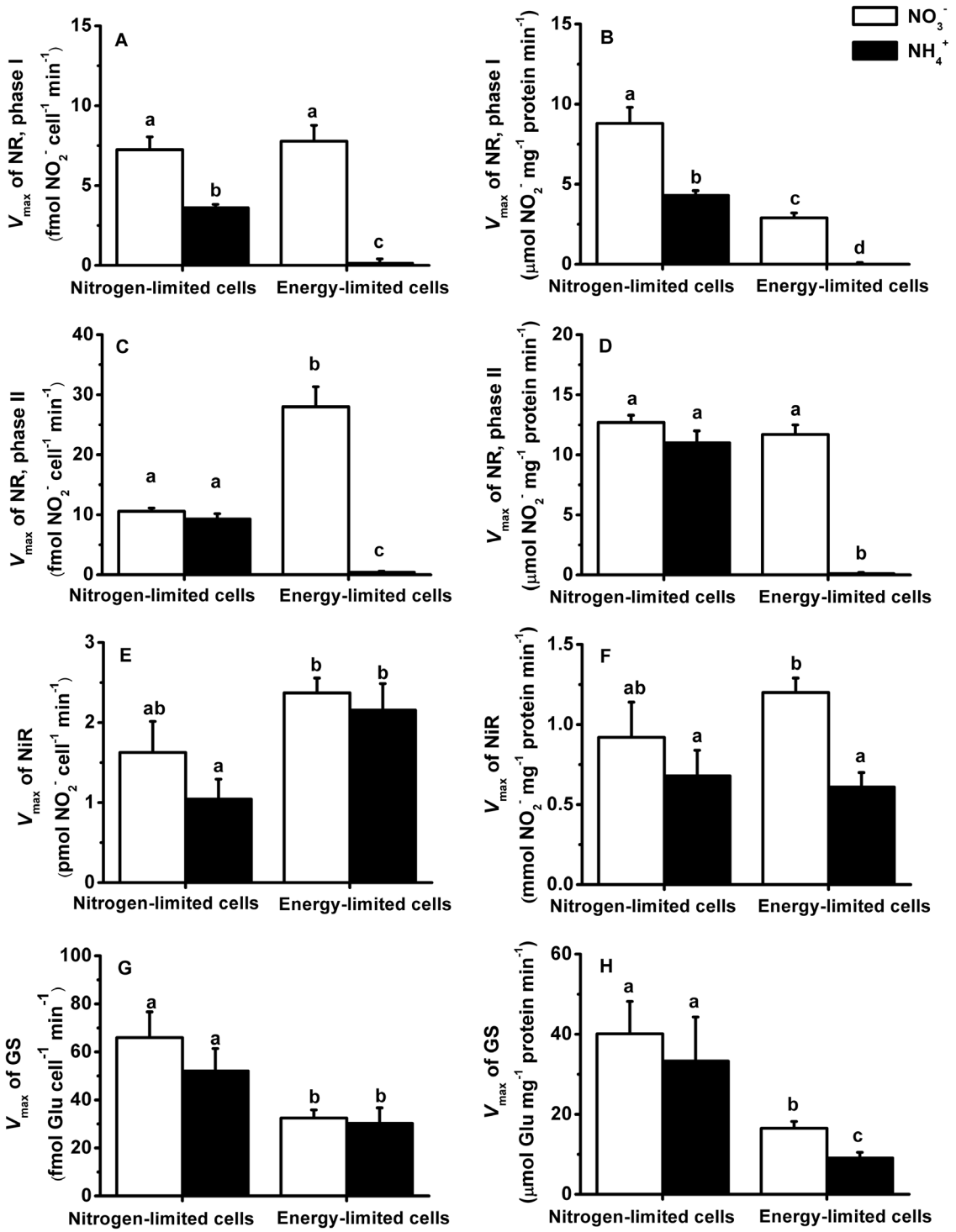


**Fig. 4.** $V_{max}$ of *Synechococcus* sp. UTEX 2380 nitrate reductase (NR; A, B for kinetic phase I; C, D for kinetic phase II), nitrite reductase (NiR; E, F), and glutamine synthetase (GS; G, H). The data are expressed on a per cell basis in (A, C, E, G) and on a protein basis in (B, D, F, H). Different letters indicate significantly different means across all treatments (i.e. between different N sources and between N- and energy-limited cells).

on *N. punctiforme* at limiting N (Aichi *et al.*, 2006). In the case of UTEX 2380, NR is expressed and active in the presence of $NH_4^+$ in N-limited cells, but not at high N when cells are energy limited. We currently lack data on *nrtP* expression, and thus cannot verify whether $NH_4^+$ sensitivity of *nrtP* expression is concentration-dependent.

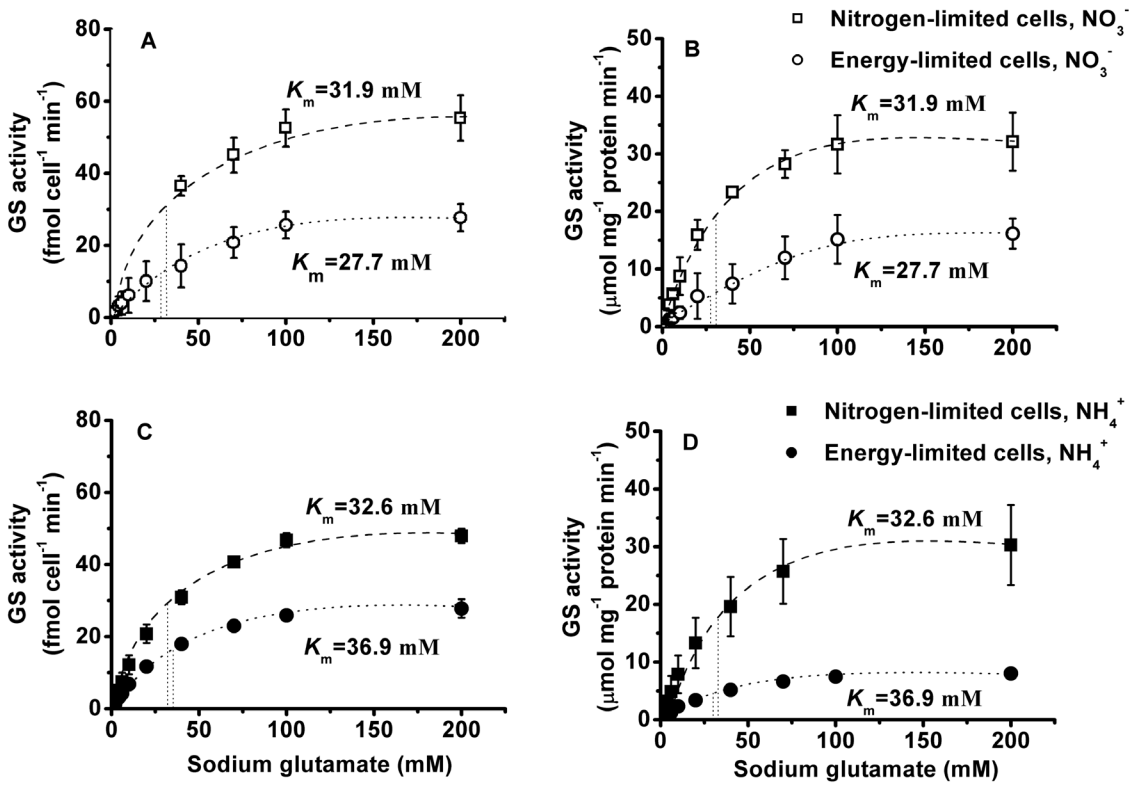


**Fig. 5.** Glutamine synthetase (GS) activity of $NO_3^-$- (A, C) and $NH_4^+$-grown (B, D) *Synechococcus* sp. UTEX 2380 cells as a function of sodium glutamate concentration. The activities were normalized by the number of cells (A, C) or by the amount of protein (B, D). The error bars indicate the standard deviations ($n$=3).

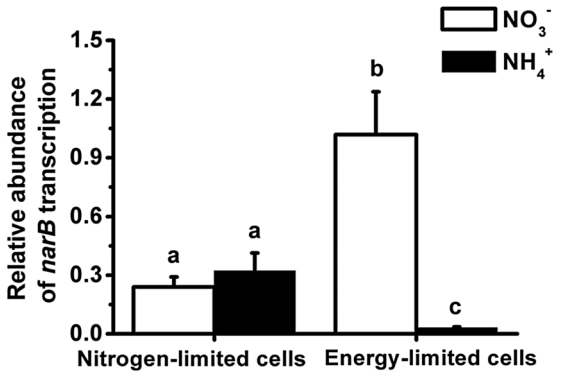


**Fig. 6.** Expression of the nitrate reductase gene (*narB*) of *Synechococcus* sp. UTEX 2380 cultured under either N or energy limitation. Values are reported relative to the *narB* expression in energy-limited cells grown in $NO_3^-$ at 550 μM. The error bars show the standard deviation ($n$=3). Different letters indicate significantly different means across all treatments (i.e. between different N sources and between N- and energy-limited cells) ($P$<0.05).

### *Does the nitrate reductase of* Synechococcus *UTEX 2380 have structural or regulatory anomalies?*

The insensitivity of NR to $NH_4^+$ in cyanobacteria has only been reported in rare cases, such as those of the freshwater β-cyanobacteria *Synechocystis* PCC 6803 (Kobayashi *et al.*, 2005) and *Cyanothece* PCC8801 (previously classified as *Synechococcus* sp. RF-1 in Wang *et al.*, 2003). It was proposed that these NRs are structurally different from the $NH_4^+$ sensitive NRs (Kobayashi *et al.*, 2005; Ohashi *et al.*, 2011). In our strain, only one *narB* gene was found. Its derived NR protein sequence had only 63% similarity with the NR of *Synechocystis* sp. PCC 6803 (although it shares with it biphasic kinetics; Martin-Nieto *et al.*, 1992), whereas its protein sequence was 99% identical to that of the NR of *Synechococcus* sp. PCC 7002 (Supplementary Table S8), which is sensitive to $NH_4^+$ (Sakamoto and Bryant, 1999). It is therefore unlikely that the $NH_4^+$ insensitivity of *Synechococcus* UTEX 2380 NR is associated with structural differences of the enzymes.

As we mentioned earlier, the *nrtP* promoter region, upstream of *narB*, contains typical NtcA and NtcB binding motifs. This is not *per se* sufficient proof of $NH_4^+$ sensitivity, since the binding of NtcA to regulatory sites is mediated by 2-oxoglutarate through PII and PipX proteins (Fig. 7; Llácer *et al.*, 2010; Herrero and Flores, 2018). Abundant 2-oxoglutarate interferes with the interaction of PipX with PII and favors the binding of PipX to NtcA protein, increasing NtcA activity as a gene inducer (Espinosa *et al.*, 2006, 2007, 2014; Llácer *et al.*, 2010; Zhao *et al.*, 2010). It has been reported that in the freshwater *Synechococcus elongatus* PCC7942, when the *glnB* gene, which produces the PII protein, is inactivated,

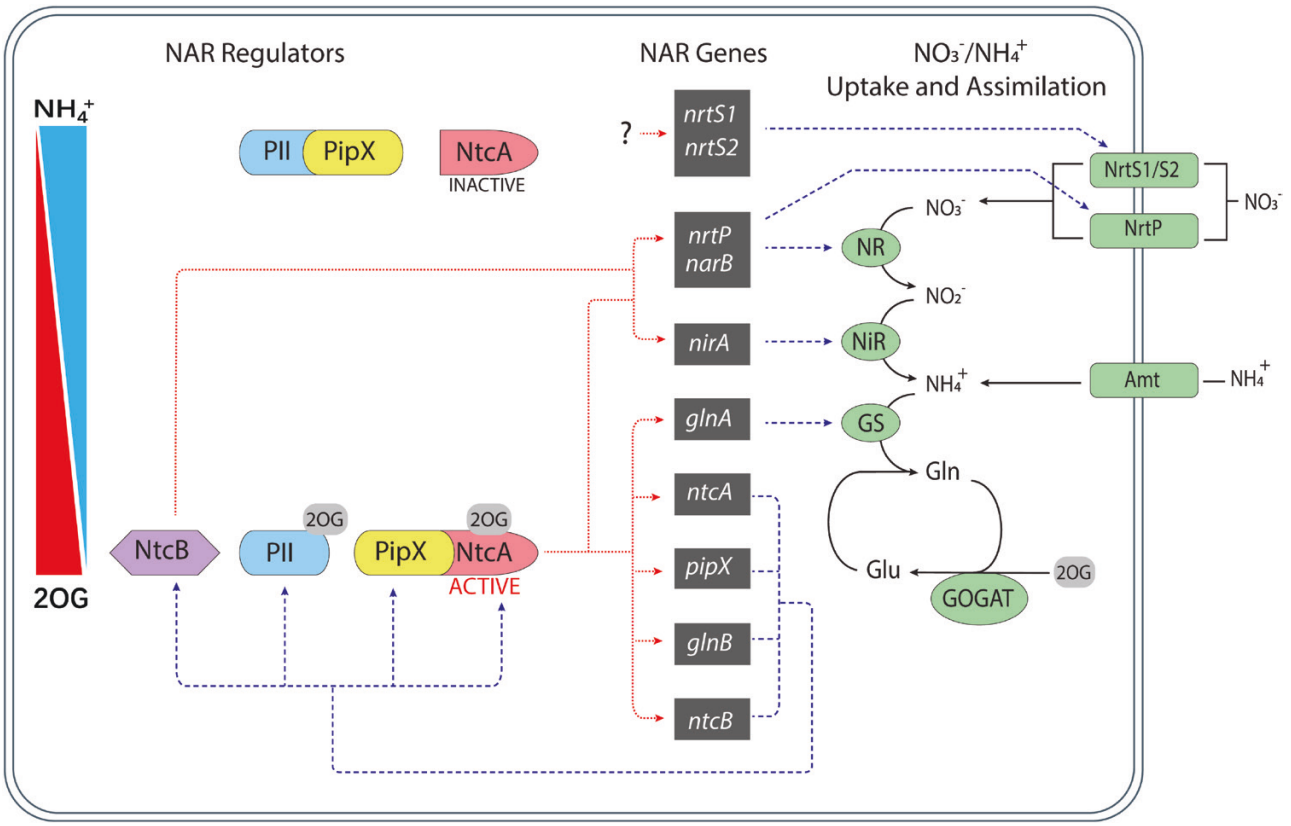


**Fig. 7.** Nitrate assimilation in *Synechococcus* UTEX 2830 is regulated indirectly via 2-oxoglutarate (2OG), the carbon skeleton for glutamate synthesis, depending on intracellular $NH_4^+$. In the presence of high concentration of $NH_4^+$ (cell growth is limited by energy), 2OG is consumed, leading to the segregation of PipX–NtcA–2OG complex and the consequent inactivation of NtcA. This inactivation down-regulates or even completely blocks nitrate assimilation related (NAR) gene expression and nitrate utilization. On the contrary, when the growth is limited by N (irrespective of N source, energy is sufficient), intracellular 2OG accumulates and tends to bind to PII, inducing the release of PipX from the PII–PipX complex. PipX together with 2OG forms the PipX–NtcA–2OG complex, activating NtcA, and the active NtcA promotes NAR gene expression (*nrtP*, *narB*, and *nirA* also require NtcB) and nitrate assimilation. The internal $NH_4^+$ is between these two extremes (high $NH_4^+$ and N limitation) with sufficient $NO_3^-$ present in the culture medium. 2OG, 2-oxoglutarate; Gln, glutamine; *glnA*, GSI gene; *glnB*, PII gene; Glu, glutamate; GSI, type I glutamine synthetase; GOGAT, glutamate synthase; *narB*, NR gene; NiR, nitrite reductase; *nirA*, NiR gene; NR, nitrate reductase; NrtP, nitrate/nitrite permease; *nrtP*, NrtP gene; NrtS1 and NrtS2 (NrtS1/S2), nitrate/nitrite bispecific transporter; *nrtS1* and *nrtS2*, NrtS1 and *nrt*S2 gene; NtcA; global nitrogen regulator; *ntcA*, NtcA gene; NtcB, activator of NAR genes; *ntcB*, NtcB gene; PII, nitrogen regulation protein; PipX, interacting protein; *pipX*, PipX gene.

$NH_4^+$ sensitivity disappears (Lee *et al.*, 1998). We may thus expect that a non-functional *glnB* gene may contribute to generating the phenotype we observed. However, no obvious defect was observed in the *glnB* gene of *Synechococcus* sp. UTEX 2380.

### *Proposed mechanism for metabolic regulation of N assimilation*

Although no obvious peculiarities could be identified in the NAR genes of *Synechococcus* UTEX 2380, $NH_4^+$ sensitivity of NR in this cyanobacterium is suppressed under N limitation (Figs 4, 6). The high 2-oxoglutarate concentration attained under N limitation (Jiang *et al.*, 2000; Ludwig and Bryant, 2012) appears to favor *narB* gene induction, even in the presence of $NH_4^+$. The fact that a NtcA binding site is present upstream of the *ntcA* gene itself is suggestive of an autocatalytic effect of NtcA on its own expression (Jiang *et al.*, 2000), which may amplify the induction of this gene under high 2-oxoglutarate. It is noteworthy, however, that NtcA binding to its target motif is under the control of redox status, and only when the thiol groups of NtcA are in the reduced state (e.g. cells in N limitation) may this autocatalysis of NtcA function (Alfonso *et al.*, 2001). Besides, we found that PipX, a coactivator of *ntcA*, is also promoted by NtcA. Consequently, these would enhance *narB* (and possibly other NAR genes) expression (Luque *et al.*, 1994, 2004). It is only when the reduced N in the cell is high (see Giordano and Raven, 2014 for a detailed discussion on N algal metabolism) and the level of 2-oxoglutarate falls that the interference of 2-oxoglutarate with the interaction of PipX with NtcA would down-regulate, and possibly block, the expression of *narB* (Espinosa *et al.*, 2014). High $NO_3^-$ availability would not have the same effect as high $NH_4^+$ concentration due to the presence, in addition to the NtcA regulatory system,

of a $NO_2^-$-dependent induction system, based on the NtcB protein (Aichi and Omata, 1997; Aichi *et al.*, 2001), which would maintain the expression of *narB* when $NO_2^-$ is produced from $NO_3^-$ reduction. NtcB binding motifs are present in the promoter region of both the *nrtP–narB* operon and the *nirA* operon, in *Synechococcus* UTEX 2380. In the case of the *nirA* operon of *Synechococcus elongatus* PCC 7942, it was shown that it ceases to respond to $NO_2^-$ if the NtcB system is disrupted (Aichi and Omata, 1997); however, a complete functional NtcA system is required for NtcB function (Maeda *et al.*, 1998; Ohashi *et al.*, 2011 and references therein).

The regulatory mechanism proposed above (Fig. 7) makes ecological sense, because it would allow cells not to discriminate in favor of N as $NO_3^-$ in the presence of $NH_4^+$ under severe N limitation. N limitation is frequent in the open ocean, where $NH_4^+$ must be present at appreciable concentrations (although low), demonstrated by the fact that many oceanic *Prochlorococcus* strains are unable to use $NO_3^-$ and are only able to use $NH_4^+$ (Moore *et al.*, 2002). In such an environment, an $NH_4^+$-triggered impediment to $NO_3^-$ assimilation would most likely lead to N starvation. Furthermore, under these conditions, the use of $NH_4^+$ rather than $NO_3^-$ would bring no advantage, since energy would not be controlling growth (Ruan and Giordano, 2017; Ruan *et al.*, 2017; Ruan *et al.*, 2018). An $NH_4^+$-mediated control only becomes relevant in N-rich and energy-limiting environments (Ruan and Giordano, 2017; Ruan *et al.*, 2017).

### *Impact of energy limitation on N metabolism*

NR was not expressed at high $NH_4^+$ concentration in *Synechococcus* UTEX 2380. However, under energy limitation NiR and GS were always present and active, though their activities were modulated by growth conditions (Figs 4, 5; Supplementary Table S9). It is known that NiR (especially) and GS activity are often constitutively high to allow the prompt removal from the intracellular environment of the potentially harmful $NO_2^-$ and $NH_4^+$, and are little responsive to the chemical form in which N is available outside the cell (Bird and Wyman, 2003; Wyman and Bird, 2007). The fact that the *nirA* gene is preceded in the genome by NtcA and NtcB binding sites suggests that it occupies a distinct operon from *narB*; this may play a role in the finding that, at high N, NiR does not show $NH_4^+$ sensitivity. In support of this idea (although, admittedly, not conclusively), in organisms in which the genes encoding NR and NiR are in the same operon, such as *Synechococcus elongatus* PCC 7942, both are $NH_4^+$ sensitive (Forchhammer and de Marsac, 1995; Ohashi *et al.*, 2011); on the contrary NR and NiR appear to respond independently to the N-source in *Synechococcus* WH 8103, where the two genes are located in distinct operons (Bird and Wyman, 2003). In the case of GS, the situation is complicated by the presence of two genes, *glnA* and *glnN*. *GlnA* appears to be under the control of the NtcA system, but not under that of the NtcB system, whereas neither *ntcA* nor *ntcB* binding motifs were found upstream of the *glnN* gene (see Fig. 2; Jiang *et al.*, 2000).

### *The nitrate reductase of* Synechococcus *sp. UTEX 2380 shows biphasic kinetics*

At the end of this paper, it seems useful to spend a few words on the biphasic (multimodal *sensu* Nissen and Martín-Nieto, 1998) kinetics of *Synechococcus* sp. UTEX 2380 NR. Biphasic NRs have already been reported for cyanobacteria, but always in freshwater or soil nitrogen fixers (Martin-Nieto *et al.*, 1992; Wang *et al.*, 2003, 2011). Up to this point in time, *Synechococcus* sp. UTEX 2380 is the only non-diazotrophic and marine cyanobacterium with a biphasic NR. The $K_m$ for nitrate in phase I ($K_{mI}(NO_3^-)$=24–31 μM) and phase II ($K_{mII}(NO_3^-)$=2.14–4.44 mM) (Supplementary Table S9) were lower than those reported for other biphasic NRs ($K_{mI}$=50–550 μM; $K_{mII}$=4.2–22 mM) (Martin-Nieto *et al.*, 1992; Nissen and Martín-Nieto, 1998; Wang *et al.*, 2003, 2011), and cells cultured under N limitation had a higher affinity for $NO_3^-$ than cells cultured in N-replete conditions (Supplementary Table S9). *Synechococcus* sp. UTEX 2380 possesses only one gene encoding NR (*narB*). The biphasic kinetics must therefore be due to allosteric regulation. This seems to be the case for all the biphasic cyanobacterial NR studied so far in sufficient detail (Martin-Nieto *et al.*, 1992; Wang *et al.*, 2003, 2011). The biphasic NR of *Cyanothece* sp. PCC8801 (previously recognized as *Synechococcus* sp. RF-1; Wang *et al.*, 2003) and *Synechocystis* PCC 6803 (Kobayashi *et al.*, 2005) are $NH_4^+$-insensitive at high N (4 mM N), unlike the NR of our *Synechococcus* sp. UTEX 2380, which is insensitive only under N limitation. Whether strains with biphasic kinetics have a common phylogeny is unclear. Nissen and Martín-Nieto (1998) pointed out that the strains with biphasic NR they analysed were all heterocystous $N_2$ fixers and phylogenetically related. For each of the Martin-Nieto *et al.* (1992) species with an available Rubisco sequence, our phylogenetic analysis indicates that biphasic species—both filamentous and unicellular—are placed among the β-cyanobacteria, while the single monophasic species was an α-cyanobacterium. *Synechococcus* sp. UTEX2380, identified herein as a β-cyanobacterium, is not a $N_2$ fixer and does not have heterocysts. Therefore, if biphasic NRs follow a common phylogenetic trajectory, *Synechococcus* sp. UTEX 2380 may have lost its *nif* genes.

β-Cyanobacteria include freshwater, coastal, and terrestrial species (e.g. in desert crusts and in lichens), while the α-cyanobacteria include only marine strains of *Synechococcus* and *Prochlorococcus*. This is relevant to our work because α- and β-cyanobacteria also differ for features related to N acquisition and assimilation, although some characteristics (e.g. the type of $NO_3^-$ transporter) appear to be more influenced by ecology than by phylogeny, i.e. marine strains of both α- and β-cyanobacteria tend to have NrtP transporters only, whereas

the freshwater strains are usually equipped with ABC-type transporters, with rare exceptions (*Cyanothece* sp. and *Arthrospira platensis*) (Ohashi *et al.*, 2011 and references therein). This, however, requires further confirmation by increasing the number of species analysed; this work also provides a contribution in this direction. It is noteworthy that different from α-cyanobacteria with NtcA as the sole transcription activator for NAR genes, β-cyanobacteria own a dual activation mechanism involving both NtcA and NtcB. NctA *per se* cannot fully activate NAR genes (Aichi and Omata, 1997; Maeda *et al.*, 1998; Aichi *et al.*, 2001); the coactivator NtcB plays an essential role, and the nitrate assimilation capability of its mutant may decrease by 90%, e.g. in *Synechococcus* sp. PCC7002 (Sakamoto *et al.*, 2008). However, whether and how much this regulator affects $NH_4^+$ sensitivity is not clear. Since NtcB is also subjected to NtcA regulation, we may thus expect that the control of $NH_4^+$ over nitrate acquisition and assimilation in β-cyanobacteria is mainly via NtcA as described above, albeit with a different organization of NAR genes. Besides, there is relatively little related research available on α-cyanobacteria (Bird and Wyman, 2003; Wyman and Bird, 2007), and it is still very hard to tell the difference in $NH_4^+$ sensitivity between α- and β-cyanobacteria.

The ecological relevance of biphasic kinetics is not obvious: millimolar concentrations of $NO_3^-$ are unlikely to occur in the environment, except in rare cases (Bruland *et al.*, 2008). This may become relevant if NR operates in an intracellular environment where $NO_3^-$ is accumulated at high concentration. There is evidence that some cyanobacteria are capable of accumulating nitrate intracellularly at concentrations as high as 0.2–1.5 mM (Lara *et al.*, 1987; Kobayashi *et al.*, 1997), a range compatible with the phase II kinetics of the biphasic NRs. Data are insufficient to say whether organisms with biphasic NR are capable of high $NO_3^-$ accumulation.

## Supplementary data

The following supplementary data are available at *JXB* online.

Fig. S1. GS activity as a function of $NH_2OH$ concentration, on per protein and per cell basis, in *Synechococcus* sp. UTEX 2380.

Table S1. Primers used in this study.

Table S2. PCR protocol for *narB* (nitrate reductase) gene cloning.

Table S3. Parameters of RT-qPCR used for the quantification of *narB* (nitrate reductase) gene transcript abundance, based on the $2^{-\Delta\Delta CT}$ method.

Table S4. NrtS (Syc1385_c) protein sequences for hidden Markov model construction (from Maeda *et al.*, 2019).

Table S5. *pipX* (DUF3539 Superfamily) accession numbers for hidden Markov model construction.

Table S6. Gene accession numbers for Rubisco phylogenetic analysis.

Table S7. Growth rate, protein and N content of N- or energy-limited *Synechococcus* UTEX 2380 cultured in the presence of either $NO_3^-$ or $NH_4^+$.

Table S8. Similarity to the sequences of *Synechococcus* sp. UTEX 2380 of nitrate assimilation enzymes, transporters, and regulators genes of related cyanobacterial strains.

Table S9. $V_{max}$ and $K_m$ of nitrate reductase (NR), nitrite reductase (NiR), glutamine synthetase (GS) in *Synechococcus* sp. UTEX LB 2380, under nitrogen or energy limitation.

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## Author contributions

MG formulated the hypothesis and designed the experiments, and provided funding for the work. ZR carried out the experiments. FH carried out the RT-qPCR measurements. MG, JAR and ZR analysed the results. ZR and CAG analysed and interpreted the genomic data. MG wrote/revised the paper with contributions from ZR, CAG, and JAR.

## Conflict of interest

The authors declare no conflict of interest.

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## Data availability

The data supporting the findings of this study are available from the corresponding author, ZR, upon request.

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