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ORIGINAL PAPER

Molecular characterization of nitrate uptake and assimilatory pathway in *Arthrospira platensis* reveals nitrate induction and differential regulation

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Abstract The nitrate assimilation pathway and its regulation in the high-protein neutraceutical cyanobacterium, *Arthrospira* (*Spirulina*), were studied. A complete characterization of the genes of the nitrate uptake and assimilatory pathway in *Arthrospira platensis* strain PCC 7345 was done including cloning, sequencing, phylogenetic analysis and expression studies. Genomic localization studies revealed that their clustering is different from the operons known in other cyanobacteria; only *nrtP* and *narB* are organized together, while *nirA*, *glnA* and *gltS* exist in separate genomic locations. The presence of both types of nitrate transporters (*nrtP*/ABC types) in *A. platensis* is rare, as their occurrence is usually specific to marine and freshwater microorganisms, respectively. The positive effect of nitrate on transcript accumulation of *narB*, *nirA* and *nrtP* genes in N-depleted and N-restored cultures confirmed nitrate induction, which is abolished by the addition of ammonium ions into the medium. Gene expression studies in response to nitrate, nitrite, ammonium and glutamine provided the first evidence of differential regulation of multiple genes of nitrate assimilatory pathway in *Arthrospira*.

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Introduction

Arthrospira platensis is a non-nitrogen fixing, nitrate-utilizing filamentous cyanobacterium best known for its high protein content (70 % by dry weight), apart from its neutraceutical, environmental and biotechnological importance (Vonshak 1997). However, the N metabolic pathway and its regulation responsible for its high protein content remain to be well characterized at the gene or protein level. There are a few studies on the induction, activity, stability and thermotolerance of N-assimilatory enzymes in *Arthrospira* (Jha et al. 2007; Ali et al. 2008; Lochab et al. 2009). The other non-N-fixing cyanobacteria such as *Synechocystis* sp. PCC 6803 that are better studied in terms of N metabolism and its regulation (Reyes et al. 1997; Herrero et al. 2001) do not have such a high protein content or other neutraceutical and environmental applications. Addressing this deficit is also essential to fully exploit the opportunities opened by the recent advances in the genomic sequencing of *Arthrospira* (Fujisawa et al. 2010; Janssen et al. 2010; Carrieri et al. 2011; Cheevadhanarak et al. 2012). *Arthrospira* could also be a model filamentous non-heterocystous cyanobacterium to study regulation of nitrate assimilation and protein content, an area that is also of great importance in crop plants.

The nitrate assimilatory pathway in cyanobacteria generally consists of clusters of related genes encoding the nitrate transport system (*nrtABCD* or *nrtP*) and nitrate assimilatory enzymes (*narB* for nitrate reductase and *nirA* for nitrite reductase). These are usually co-transcribed as a

nir operon and represented as *nirA-nrtABCD-narB* operon (Omata 1995; Frías et al. 1997), though the operon type of arrangement is not universal in all cyanobacteria. The other genes downstream of nitrate assimilation are glutamine synthetase (*glnA*) and glutamate synthase (*gltS*), which have been characterized in a few cyanobacterial species, but not in *Arthrospira*. The distribution of the genes associated with nitrate assimilation in cyanobacterial genomes, including *A. platensis* NIES-39 has been analyzed bioinformatically (Ohashi et al. 2011), but complete annotation of these genes in *A. platensis* awaits their independent molecular characterization.

The genes of cyanobacterial N-assimilatory pathway are regulated by a nitrogen control mechanism mediated by ntcA, a transcriptional activator belonging to CRP (cAMP receptor protein) family, which binds to a palindromic $GTAN_8TAC$ motif and regulates the genes of multiple metabolic and developmental pathways (Herrero et al. 2001). The role of ammonium as an inhibitor of ntcA in unicellular, non-N_2-fixing cyanobacteria has been studied in great detail (Muro-pastor et al. 2001) as compared to filamentous non-N_2-fixing cyanobacteria. At high concentrations, it inhibits uptake and assimilation of nitrate/nitrite (Ohmori et al. 1977; Kobayashi et al. 1997), as well as transcription of N-assimilatory genes through repression of ntcA (Frias et al. 1994; Lee et al. 1999; Lindell and Post 2001; Lindell et al. 2005). However, in environments where nitrate is a predominant N source, it is not clear whether upregulation of N-assimilatory genes can happen independent of ammonium derepression. Nitrate/nitrite is known to be a requirement for expression of N-assimilatory genes in N_2-fixing cyanobacteria, but its role is not so well understood in the case of non-N_2-fixing cyanobacteria, as the regulatory role is mainly attributed to ammonium repression of ntcA (Flores and Herrero 2005). The only nitrite regulation known so far operates through ntcB, a lysR-type transcriptional activator that upregulates the *nirA* operon in *S. elongatus* strain PCC 7942 (Aichi and Omata 1997; Aichi et al. 2004). Further, the role of P11 and pipX regulators in enhancing the 2-OG-dependent ntcA-mediated upregulation of *nirA* operon has been shown in unicellular non-N_2-fixing cyanobacterium *S. elongatus* PCC 7942 (Espinosa et al. 2006; Forchhammer 2004). However, none of these regulators have been studied at any level in *Arthrospira*, and therefore, their role in regulating N metabolism remains to be understood. The difficulty of generating mutants by traditional route in this filamentous multicellular organism necessitates the development of gene tools to study the regulation of N-assimilatory pathway in this organism. The current study is an attempt in this direction.

Materials and methods

Strain and growth conditions

Axenic *A. platensis* strain PCC 7345 obtained from the Pasteur Culture Collection (PCC) of the Institüt Pasteur, Paris, was grown photoautotrophically at 27 ± 1 °C in BG-11 and ASN-III medium (1:1, v/v) on an orbital incubator shaker (125 rpm) continuously illuminated by fluorescent tubes (15 μmol m^{-2} s^{-1}) as reported in detail previously (Lochab et al. 2010).

Isolation and cloning of genes

DNA was isolated from 8- to 9-day old culture as described in Lochab et al. (2010). Due to the non-availability of the genomic sequence of *A. platensis* at that time, degenerate primers were designed in-house for *nrtP*, *narB*, *nirA*, *glnA*

Table 1 List of primers used for PCR cloning

Gene	Primer	Primer sequence (5′–3′)	Tm	Amplicon size (kb)
narB	Forward	CTGCTGGGAAGCGAATAAAG	54	2.1
	Reverse	TGGGAAACGTCTCAACATCA		
nrtP	Forward	TGCTAACCGAATTATGGTCTTT	58	1.5
	Reverse	ATCCTACTTGATGATGGTCAGC		
nirA	Forward	AACCGTTATCAGCCACTTGC	55	1.4
	Reverse	GGGAAGCCATGGATAAAACC		
glnA	Forward	GGAGCACGAAARCGCATGG	56	1.5
	Reverse	TTAACGACATGCGAGGAACC		
gltS	Forward	GTCCGAACCCAAAACTCAGA	54	2.3
	Reverse	AGTGCGATCGCTATGGTCTT		
ntcA	Forward	TCCCATGGTCAGGTAAATTGTTGGCTCA	61	0.64
	Reverse	TCCTCGAGGTGTTCCGTCAAATTGGTGG		

and *gltS* from the conserved domains of multiple-aligned (ClustalW) gene sequences from other cyanobacteria, but the primers for full-length cloning of nrtABCD and NtcA were designed by taking sequences from *A. platensis* NIES-39 (Table 1). PCR was performed in a final volume of 25 μl containing: 2.5 μl 10× reaction buffer for Taq DNA polymerase, 0.5 μl dNTP (10 mM of each), 0.6 μl of each primer (10 μM), 1 unit of Taq DNA polymerase (MBI Fermentas). The amplification conditions were varied appropriately, with the following standard conditions: initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 1 min; annealing at 55 °C for 1 min; extension at 72 °C for 1 min kb^{-1} and a final extension at 72 °C for 10 min. The PCR products were purified using a Qiagen Gel Extraction Kit and cloned in pGEM®-T easy cloning vector (Promega, USA). Wherever required, the gel-purified inserts from these PCR clones were used as probes for screening the *Arthrospira* genomic library. Radiolabelled (^{32}P) probes were generated for this purpose by random primer radiolabeling using Mega Prime DNA Labeling System (Amersham Biosciences, USA). A genomic library of the *A. platensis* was prepared using ZAP Express Predigested Vector Kit and ZAP Express Predigested Gigapack Cloning Kits (*Eco*R1/*Xho*1/CIAP-treated), from Stratagene (USA) as per the manufacturer's instructions.

Characterization and sequencing of clones

The clones obtained by PCR cloning and/or by genomic library screening were confirmed by (1) colony PCR, (2) restriction digestion and/or (3) nested PCR as appropriate. The confirmed clones were sequenced on T7 and SP6 sequencing primers and by primer walking from both the ends. Sequences were confirmed by BLAST search, and ORFs were determined and conceptually translated using the tool—TRANSLATE (http://www.expasy.ch/tools/#translate), and the resulting amino acid sequence was used as a query for identification of conserved domains using CD-Search tool at NCBI. The amino acid sequences were included in the entries submitted to GenBank and used for phylogenetic analysis and uploaded to GenBank. The accession numbers of genes obtained by PCR cloning are FJ763143 for *nirB*, FJ763145 for *glnA*, FJ664548 for *gltS*, FJ158615 *nrtP*, FJ158616 for *narB*, HQ450639 for *nrtA*, HQ450640 for *nrtB*, FJ763144 for *nrtC*, HQ450641 for n*rtD*, JX489171 for *ntcA*. The GenBank accession numbers for the full nucleotide sequences of these genomic clones are HM130915 for *nrtP* and *narB*, HM130916 for *nirA*, HM130917 for *glnA* and HM130918 for *gltS* genomic clones, respectively.

RNA isolation and RT-PCR

Exponentially growing (8–9 days old) cultures of *A. platensis* were harvested and used directly, or washed twice with N-free medium and resuspended in N-free medium for at least 2 h. Aliquots of the culture were recovered for RNA isolation before reintroducing a source of N, either as nitrate (KNO_3, 20 mM) or nitrite (KNO_2) or ammonium (NH_4Cl) or glutamine, at a final concentration of 4 mM each, either individually or in suitable combinations. The cultures were harvested 2 h after N treatment for RNA

Table 2 Gene-specific primers used for transcript studies by RT-PCR and qRTPCR

Gene	Primer	Primer sequence (5′–3′)	Amplicon size (bp)	Tm
narB	Forward	GTCCTTACTGCGGTGTTGGT	295	56
	Reverse	ATCAGCGCCCATAGTATTGC		
nrtP	Forward	ATTGYTGCCCTCACAGTTCC	218	56
	Reverse	AAATGCTGCACCCAGTATGC		
nirA	Forward	TCATGACCTACTTTTCCGCC	280	56
	Reverse	CCTTTGACTCGATCGCTTGT		
16S *rRNA*	Forward	AAGTCATCATGCCCCTTACG	158	60
	Reverse	AGCGATTCCTCCTTCATGC		
narBq	Forward	ATACTATGGGCGCTGATGCC	159	59
	Reverse	TGATATAACCGGACACGGCG		
nrtPq	Forward	TACGGCCCCAGAATCACCTA	159	60
	Reverse	TTCGGCTACCATGCGGATAC		
nirAq	Forward	TCGCCTCTGAACCCAAAGTC	157	60
	Reverse	ACCCCCAGCCATTTAAGACG		
16S *rRNAq*	Forward	GTGTAGCCTGTATCGACCCG	126	60
	Reverse	CCACATACTCCACCGCTTGT		
ntcAq	Forward	GCGGGGTCTGTCGTCTCGC	131	60
	Reverse	CCATCTCGACCGGGAACCCCA		

isolation or after 8 h for enzyme activity assays. Total RNA was isolated by hot phenol and LiCl method (Pathak and Lochab 2010). 16S rRNA was used as an internal house keeping control for all the conditions, and gene-specific primers for *narB*, *nirA* and *nrtP* were used (Table 2).

Enzyme Assays

The activities of nitrate reductase (NR) and nitrite reductase (NiR) enzymes were assayed in crude extracts as described earlier (Lochab et al. 2009). The specific activity is defined as micromoles of nitrite produced (NR) or consumed (NiR) per minute per milligram of protein.

qRT-PCR analysis

Gene-specific primers for qRT-PCR were designed in-house using primer 3 software (Table 2), based on the gene sequences obtained in this study for *nrtP*, *narB*, *nirA*, *ntcA* and 16S rRNA (housekeeping control). Total RNAs extracted as described above were treated with DNase (Qiagen, Germany) and used as templates for cDNA synthesis with AccuScript High Fidelity 1st Strand cDNA Synthesis Kit (Agilent, USA) according to the manufacturer's instructions. A control without RT was maintained to confirm the absence of DNA contamination in the total RNA. The cDNAs thus generated were used to set up the qRT-PCR using Brilliant III SYBR Green Master Mix (Agilent, USA) as follows: an initial denaturation time of 10 min at 95 °C, 40 cycles of amplification comprising of a denaturation step for 30 s at 95 °C, annealing step for 30 s at 59 °C and the extension step for 30 s at 72 °C. All the primers have been checked for amplification efficiencies before being used for real-time PCR. The melting curves for all the reactions showed single peaks, confirming primer specificity. The non-detectable fluorescence signal in the negative control (without cDNA) confirmed the lack of contamination in any of the reactions. Relative quantification of the targets in each sample was carried out using the signal of 16S rRNA amplicon as a control, and the expression levels of target genes were calculated using the $2^{-\Delta\Delta}C_T$ method (Livak and Schmittgen 2001).

Results

Gene isolation and characterization

Despite the poor nucleotide sequence similarities within cyanobacteria and the constraints imposed by the degenerate primers for the genes of interest, successive rounds of PCR cloning yielded partial gene fragments, but not full-length genes (Fig. S1). Nevertheless, they proved to be very useful as homologous probes to obtain full-length genomic clones from a phage library (Fig. 1a–c). After two rounds of screening of the phage genomic library, 109 clones corresponding to NR, NiR, NrtP, GS and GOGAT were picked, characterized and sequenced as described in "Materials and Methods."

The genes *narB* and *nrtP* were obtained in a single genomic clone of 5.2 kb with separate reading frames of 2,211 and 1,513 nucleotides, respectively (Fig. 1a), coding for proteins of 737 and 504 amino acids. The deduced amino acid sequence of NrtP protein showed high sequence identity with that of *Trichodesmium erythraeum* IMS101 (84 %) and *Lyngbya* sp. PCC 8106 (79 %). Similarly, NarB protein showed >67 % identity with that of most other cyanobacterial species. The highest sequence identities were found with *Lyngbya* sp. PCC 8106 (79) and *Trichodesmium erythraeum* IMS101 (77 %).

Analysis of up to 1 kb sequences flanking these ORFs did not reveal any more genes of the N-assimilatory pathway, indicating that they are not arranged in the form of *nir* operon found in other cyanobacteria. Characterization of the genomic clones of other nitrate assimilatory genes of *A. platensis* revealed separate ORFs for *nirA* (1,686 bp), *glnA* (1,422 bp) and *gltS* (4,607 bp), indicating the absence

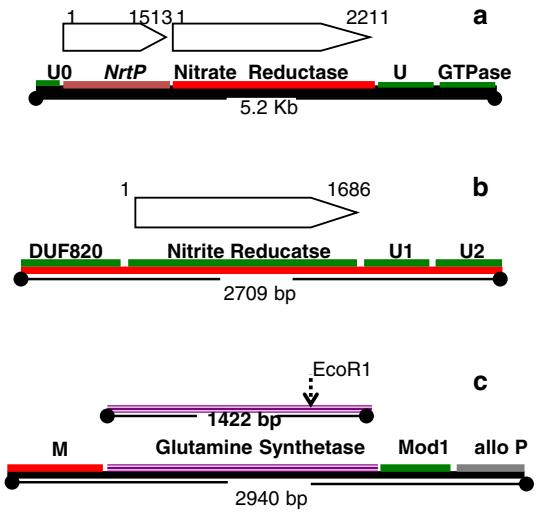

Fig. 1 Schematic maps of the genomic clones of N-assimilatory genes from *A. platensis* PCC 7345. *Numbers* on the *top* of each map denote the size of the gene/ORF and those below denote the size of the genomic clone. The position of the first and last base of the coding region is also denoted and *block arrows* denote the ORFs with their direction of transcription. **a** Map of the 5.2-kb genomic clone encoding hypothetical protein (UO), nitrate/nitrite transporter (*nrtP*), nitrate reductase (*narB*), an unknown protein (U), and a ribosome-associated GTPase. **b** Map of a 2.7 kb genomic clone encoding nitrite reductase (*nirA*), DUF 820 (domain of unknown function protein), U1 & U2 (unknown hypothetical proteins). **c** Map of a 3 kb genomic clone encoding glutamine synthetase (*glnA*), Mod 1 (mod4 gene), allo P (allophycocyanin B-18 subunit gene) and M (similar to methyltransferase family gene)

gtagcactcaa**tac**.....97n.....**atg -** *glnA*
gtgacgcaccc**tac**.....90n.....**atg –** *gltS*
gtatcttcaac**tac**.....63n.....**atg -** *nirA*
gtatcttcaac**tac**.....290n.....**atg -** *nrtP*

Fig. 2 Representation of *ntcA* binding site in the upstream regions of *nrtP*, *nirA*, *glnA* and *gltS* gene in *A. platensis* PCC 7345. The *ntcA* binding sequence ($GTAN_8TAC$) is shown in *bold*. The distance from the ATG site is indicated as number of nucleotides. *glnA* glutamine synthetase, *gltS* glutamate synthase, *nrtP* nitrate/nitrite transporter, *nirA* nitrite reductase

of operon architecture (Fig. 1b, c). The *ntcA* gene from *A. platensis* PCC 7345 showed a high degree of similarity with previously reported *ntcA* gene sequences from other cyanobacteria, such as *A. platensis* NIES-39 (99 %), *Lyngbya* sp. PCC 8106 (96 %), *Leptolyngbya* sp. PCC 7376 (90 %) and *Trichodesmium erythraeum* IMS101 (89 %).

The phylogenetic analyses were done using deduced protein sequences of all the five N-assimilatory genes as well as for the regulator *ntcA*. Phylogenetic trees were constructed using all the 39 species of cyanobacteria whose genomes have been sequenced (http://genome.kazusa.or.jp/cyanobase). Figure (Fig. S2) shows the neighbor-joining phylogenetic tree obtained using Clustal X multiple sequence alignment. It revealed that sequences from all the *Arthrospira* species, including the *A. platensis* strain PCC 7345 used in this study are grouped together. They were also found to be grouped closely with other members of the oscillatoriales like *Trichodesmium erythraeum* IMS101, *Lyngbya* sp. PCC 8106 and *Oscillatoria* sp. PCC 6506 (Fig. 2a–f), which is in good agreement with other studies based on 16srRNA sequences. Moreover, the sequences from freshwater and marine organisms formed different clusters. All these findings remained valid irrespective of the parameters used for construction of the tree. The protein sequences of GS and GOGAT were the most conserved among all the cyanobacteria species, with BLASTp revealing more than 90 % query coverage at an E value cutoff of 0.0. As expected, *Arthrospira* GOGAT also showed more than 57 % identity to plastidic Fd-GOGAT of vascular plants at the amino acid level (data not shown). Overall, our phylogenetic analysis shows very high degree of amino acid sequence similarity of the various genes of N uptake and assimilation among different cyanobacteria adapted to different habitats.

Identification of ntcA binding site

We found *ntcA* binding site sequence $GTAN_8TAC$ in the promoter regions of *nrtP*, *nirA*, *glnA*, *gltS* but not in *narB* (Fig. 2). Of these, *nrtP* and *narB* are clustered together and share a common *ntcA* binding site, suggesting co-transcription/co-regulation as in the case of an operon. However, RT-PCR studies using primers spanning *nrtP* and *narB* failed to produce a single amplicon (data not shown), indicating that they are separately transcribed.

Effect of N metabolites on the transcript levels of NRT, NR and NiR

The effects of different N-sources on the transcript levels of *narB*, *nirA* and *nrtP* were studied by RT-PCR as described in "Materials and Methods." The transcript level of *nrtP, narB and nirA* decreased drastically within 2 h of withdrawing N from the medium (Fig. 3a). They remained low up to 24 h (data not shown), but 8 h was the standardized time point used for measuring the impact of reintroduction of N metabolites on gene expression.

Effect of nitrate

The transcript levels of *narB*, *nirA* and *nrtP* were restored to the control level within 2 h of reintroduction of nitrate (20 mM), clearly indicating nitrate induction of their gene expression (Fig. 3a). Similar induction was also found at the enzyme activity level for NR and NiR (Fig. 4).

Effect of nitrite, ammonium and glutamine

Similar experiments were conducted on the effects of downstream N metabolites, nitrite, ammonium and glutamine using N-depleted cultures, in the presence and absence of nitrate. Two patterns of response to nitrite emerged, depending on the genes involved (Fig. 3b). In the case of *nrtP* and *nirA*, nitrite enhanced their gene expression to levels comparable to that of nitrate alone, or a combination of nitrite and nitrate. In the case of *narB*, nitrite alone had no inductive effect and instead inhibited nitrate induction of gene expression when provided in combination. Glutamine had no effect at the RNA level (Fig. 3c), while ammonium had significant inhibitory effect on the expression of these genes, either on its own or on nitrate response (Fig. 3d). However, at the level of enzyme activity, all three downstream N metabolites had inhibitory effects on NR and NiR activities (Fig. 5).

Ammonium inhibition of N-assimilatory genes

A downstream metabolite may have an independent effect of its own, on the expression of a N-assimilatory gene, regardless of nitrate induction. In order to demonstrate this, the independent effect of ammonium was studied without N-depletion and nitrate induction. For this purpose, cultures growing in nitrate-containing media were harvested and resuspended in fresh medium containing different concentrations of ammonium (0.04–4 mM NH_4Cl), with or without

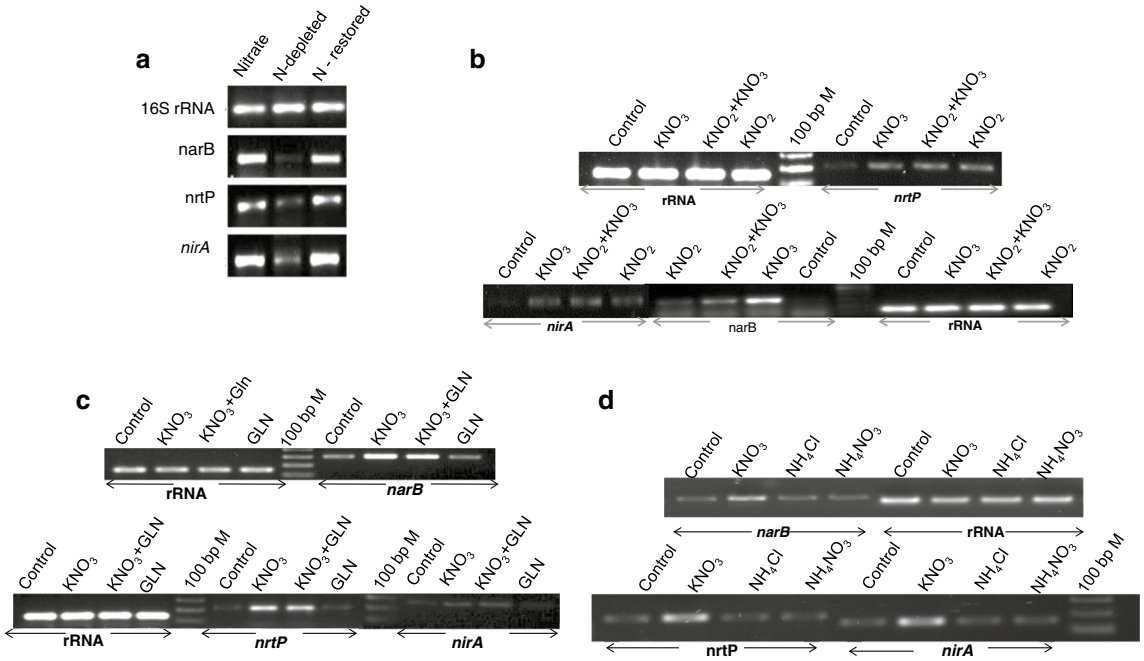

Fig. 3 Effect of N metabolites on the transcript levels of *narB*, *nrtP* and *nirA* from *A. platensis* PCC 7345. Mid-log phase cultures growing in medium containing 20 mM nitrate were harvested, washed and resuspended in nitrate-free medium. After incubation of these N-depleted cultures for 8 h, N was reintroduced, either as 20 mM nitrate (KNO_3) or 4 mM each of nitrite (KNO_2) or ammonium (NH_4Cl) or glutamine (GLN), either individually or in combination with nitrate (N-restored). RNA was isolated from each of these cultures 2 h after N restoration and subjected to RT-PCR analysis using 16S rRNA as a housekeeping control. *narB* nitrate reductase, *nrtP* nitrate/nitrite transporter, *nirA* nitrite reductase. **a** Effect of nitrate; effect of nitrite (**b**), or ammonium (**c**) or glutamine (**d**) alone or in combination with nitrate, for which the N-depleted condition described above is denoted as "control"

nitrate (20 mM KNO_3) for 2 h. RNA isolation and RT-PCR analysis for *nrtP*, *nirA* and *narB* gene expression revealed their clear inhibition by ammonium (alone or with nitrate) (Fig. 6). This shows the independent effect of ammonium on the expression of upstream N-assimilatory genes, which is known in other organisms (Herrero et al. 2001).

Effect of N source on ntcA expression

The experiments were performed exactly as described above for ammonium effect. The *ntcA* transcript is expressed at basal levels when grown in nitrate-containing medium and at maximal levels following N-depletion, while it decreases when ammonium is supplied to the N-depleted cultures (Fig. 7). The effects of nitrite and glutamine on *ntc*A expression were more or less similar to that of nitrate described above (Fig. 7).

Discussion

Molecular characterization of nitrate assimilatory pathway remains an under-explored area of research in the non-N-fixing filamentous cyanobacterium *A. platensis*, despite its importance in nutritional and environmental biotechnology. We report here the first comprehensive characterization of the genes of nitrate uptake, assimilation and regulation in *A. platensis*, using the type strain PCC 7345. The full-length genes of *nrtP*, *narB*, *nirA*, *glnA* and *gltS* and the regulator *ntcA* have been cloned, sequenced and their phylogeny, genomic organization and transcriptional regulation by nitrate and its downstream metabolites have been determined.

Cloning and sequence analysis

Full-length genomic clones were obtained by screening a phage genomic library using homologous probes generated through PCR cloning, and the sequences were confirmed by BLAST. Phylogenetic analysis with all these sequences revealed that *Arthrospira* belongs to a distinct evolutionary branch and it clustered with other members of oscillatoriales (Fig. S2).

The sequences of *nrtP*, *nrt A, B, C* and *D*, as well as those of GS (*glnA*) and GOGAT (*gltS*) reported here corroborate the genomic sequence and putative annotation data published earlier (Fujisawa et al. 2010; Janssen et al. 2010; Carrieri et al. 2011; Cheevadhanarak et al. 2012). However, the sequences of NR (*narB*) reported in our study have been vaguely annotated as molybdenum oxidoreductase in the publicly available genome sequences of *A. platensis*

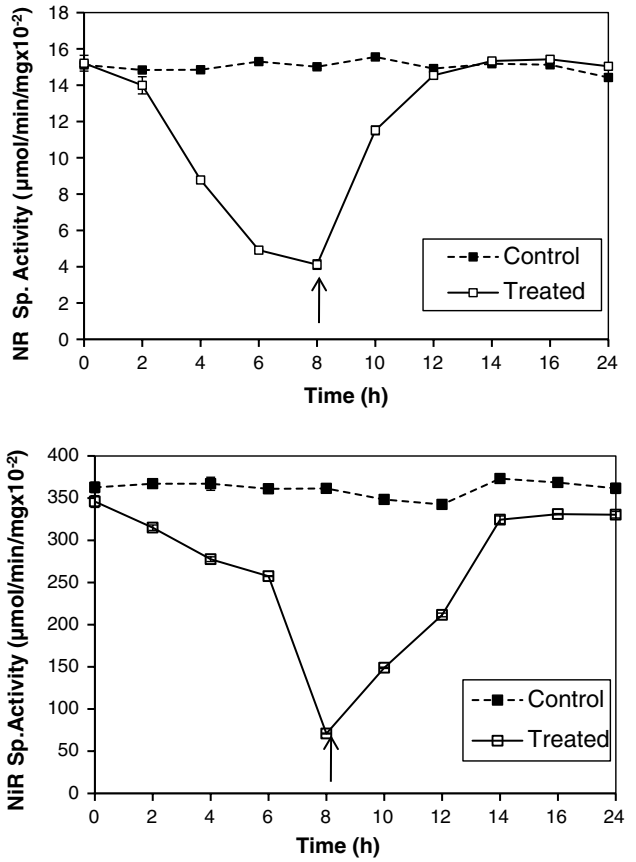

Fig. 4 Effect of nitrate removal and reintroduction on the activities of NR (*upper panel*) and NiR (*lower panel*) of *A. platensis* PCC 7345. Mid-log phase cultures growing in normal medium that contains 20 mM nitrate (Control) were harvested, washed and resuspended in nitrate-free medium for 8 h before reintroducing nitrate (Treated) and incubated further. Crude extracts from aliquots of these cultures were assayed for NR and NiR enzyme activities every 2 h. They were expressed as micromoles of nitrite produced (NR) or consumed (NiR) per minute per milligram of protein. The curves were plotted using mean values of internal triplicates from three different experiments, along with standard *error bars*

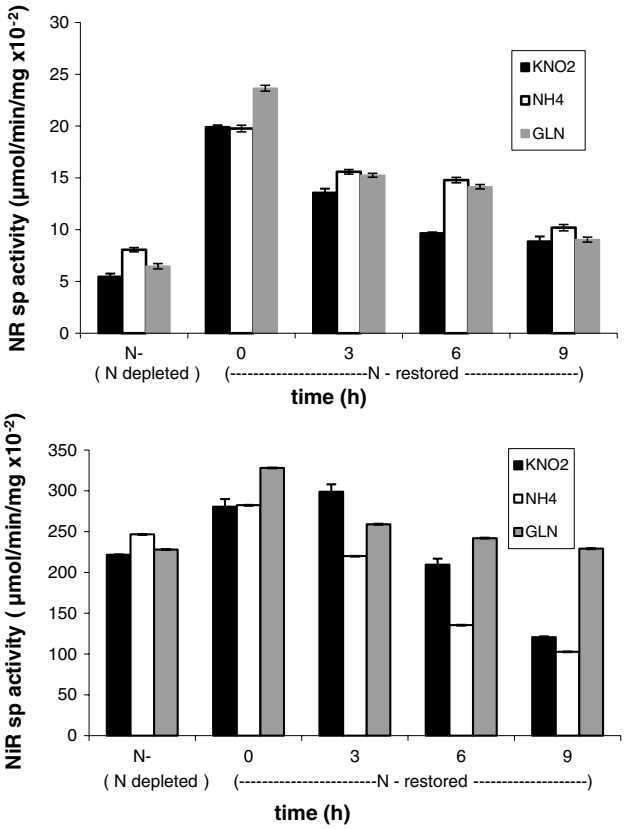

Fig. 5 Effect of nitrite, ammonium and glutamine on NR (*upper panel*)- and NiR (*lower panel*)-specific activities of *A. platensis* PCC 7345. Mid-log phase cultures growing in normal medium that contains 20 mM nitrate were harvested, washed and resuspended in nitrate-free medium for 8 h (N-depleted) before reintroducing nitrate (N-restored) in combination with 4 mM each of nitrite (KNO_2) or ammonium (NH_4Cl) or glutamine (Gln) and incubated for 8 h. Crude extracts of these cultures were assayed for NR and NiR enzyme activities, which were expressed as micromoles of nitrite produced (NR) or consumed (NiR) per minute per milligram of protein. The curves were plotted using mean values of internal triplicates from three different experiments, along with standard *error bars*

strain Paracaca, *Arthrospira* sp. PCC 8005 and *Arthrospira maxima* CS-328, whereas some other gene has been misannotated as NR in the genome sequence of *A. maxima* CS-328. In case of NiR, the sequence reported in our study matches with only one of the two gene sequences annotated as NiR in the *A. platensis* NIES genome (Fujisawa et al. 2010). The other gene may have been misannotated as putative NiR, as there is no sequence similarity with the fd-NiR. The discrepancies in the published annotations are more significant in the case of *ntcA* gene. It is annotated as global nitrogen regulator in *Arthrospira* sp. PCC 8005 and as Crp/FNR family transcriptional regulator in rest of the *Arthrospira* genomes (Janssen et al. 2010). Thus, our independent cloning, sequencing and phylogenetic analysis of all the components of nitrate uptake, assimilation and regulation, not only corroborates some of the published sequences and putative annotations, but also points out misannotations and incomplete annotations and contributes to resolving them.

Genomic localization and clustering analysis

The genes of nitrate uptake and assimilation are often clustered as operons in cyanobacteria. The *nir* operon in freshwater cyanobacteria like *Synechococcus elongatus* strains PCC 6301 and PCC 7942 and *Anabena* sp. strain PCC 7120 is comprised of the genes *nirA-nrtABCD-narB* (Ohashi et al. 2011), whereas marine cyanobacteria like *Trichodesmium* sp. have *nrtP* in place of *nrtABCD* in the operon (Ohashi et al. 2011; Wang et al. 2000).

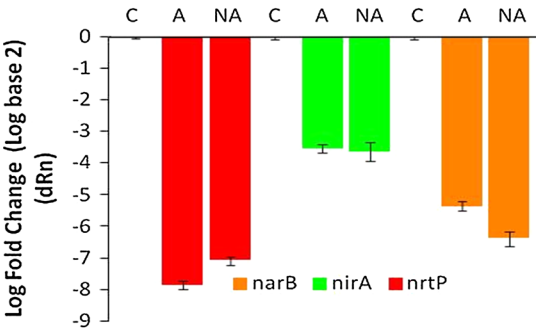

Fig. 6 Effect of ammonium on the transcript levels of *narB*, *nrtP* and *nirA* from *A. platensis* PCC 7345. Mid-log phase cultures growing in 20 mM nitrate (C) were harvested and resuspended in a medium containing either 4 mM ammonium (A) and/or 20 mM nitrate (NA). RNA was isolated after 2 h under each of the above conditions and subjected to qRT-PCR using 16S rRNA as a housekeeping control. *narB* nitrate reductase, *nrtP* nitrate/nitrite transporter, *nirA* nitrite reductase

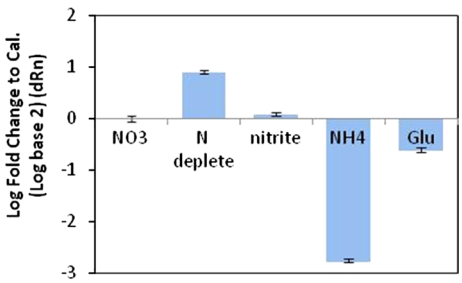

Fig. 7 Effect of N metabolites on the transcript levels of *ntcA* from *A. platensis* PCC 7345. Mid-log phase cultures growing in 20 mM nitrate (C) were washed, resuspended in nitrate-free medium for 8 h and supplied with either 20 mM nitrate, and 4 mM each of nitrite, ammonium and glutamine. RNA was isolated after2 hunder each of the above conditions and subjected to qRT-PCR using 16S rRNA as a housekeeping control

Interestingly, our results reveal that while *nrtP* and *narB* are organized together (Fig. 1), *nrtABCD*, *nirA*, *glnA* and *gltS* exist in separate genomic locations, indicating that *A. platensis* does not follow the operon architecture typical of other cyanobacteria. This is in spite of the fact that it belongs to the same order (Oscillatoriales) as *Trichodesmium*. Such differences in gene clustering and operon architecture are also found in some other cyanobacteria like *Microcystis aeruginosa* NIES-843 (Ohashi et al. 2011) and a marine *Synechococcus elongatus* strain PCC 7002 (Sakamoto et al. 1999). As such examples accumulate, they indicate that the genes of the N-assimilatory pathway neither are clustered in all cyanobacteria nor follow a conserved pattern even when they are clustered. These findings are at variance with the suggestion of a possible selective advantage to clustering of nitrate assimilatory genes in freshwater cyanobacteria (Slot and Hibbett, 2007). It is interesting and significant that *A. platensis* PCC 7345 has both types of transporters, *nrtP* as well as *nrtABCD*, which are otherwise known to be specific to marine and freshwater organisms, respectively, barring a few exceptions (Luque and Forchammer 2008; Ohashi et al. 2011). This may reflect the evolutionary adaptation of *A. platensis* to suit nitrate uptake in both types of environments, considering that there is no such habitat specificity in the other genes *nirA, narB, glnA and gltS* as indicated by our phylogenetic analysis (Fig. S2).

The genes encoding the transporters and enzymes of nitrate assimilation are known to be suppressed by ammonium at the transcriptional level and its withdrawal from the medium is considered sufficient for their expression in non-N_2-fixing cyanobacteria (Herrero et al 2001; Flores and Herrero 2005). But the question whether nitrate/nitrite have any specific role in upregulating their expression is not well understood, perhaps because such experiments were conducted in ammonium-grown cultures and the role of other N metabolites was interpreted in terms of the presence or absence of ammonium. In any case, there is no literature on the expression of any of the genes of nitrate uptake, assimilation and N metabolism in *Arthrospira* species, mainly due to the lack of the gene tools needed for such studies.

In our experiments, nitrate is the only source of N in the media and the roles of other N metabolites were studied in the presence or absence of nitrate. Our transcript studies clearly showed for the first time that nitrate upregulates the gene expression of *narB, nirA* and *nrtP* in *A. platensis*. Their transcript levels dropped drastically when the cultures were harvested and resuspended in N-free media and recovered when nitrate was reintroduced (Fig. 3a). Similarly, nitrate induction was also found at the enzyme activity level for NR and NiR (Fig. 4). We earlier suggested nitrate induction of NR activity based on a different strain of *A. platensis* (Jha et al. 2007). The present study not only confirms it at the transcriptional level in the type strain PCC7345, but also provides the first evidence for nitrate induction of multiple genes of nitrate assimilatory pathway in *Arthrospira* or in any other filamentous, non-N_2-fixing, nitrate-utilizing cyanobacterium.

Our transcript studies also revealed that nitrite induces *nrtP* and *nirA* gene expression in a manner similar to nitrate, but inhibits induction of *narB* by nitrate (Fig. 3b). Upregulation of *nir* operon by nitrite is known in *Synechococcus* sp. strain PCC 7942 and *Plectonema boryanum* (Kikuchi et al. 1996; Aichi et al. 2004). Nitrite inhibition of nitrate induction is not known in non-N_2-fixing filamentous cyanobacteria, because nitrate induction itself was not known earlier. However, this is a well-known phenomenon in higher plants like maize (Raghuram and Sopory 1999) and rice (Ali et al. 2007).

Ammonium showed clear inhibitory effect on the nitrate induction of gene expression of *nrtP*, *narB* and *nirA*

(Figs. 3d, 6), as well as the enzyme activities of NR and NiR (Fig. 5), while glutamine had no effect (Fig. 3c). In N-depleted cultures, the fall in the activities of the above enzymes is further accentuated by the addition of ammonium ions. Therefore, in addition to corroborating the well-known ammonium repression of the above genes/enzymes in other cyanobacteria (Muro-pastor et al. 2001; Flores and Herrero 2005), our results comprehensively document the differential responses to other N metabolites. They may be explained in part by the lack of operon architecture.

Our cloning and characterization of *ntcA* gene encoding the global nitrogen regulatory protein (Fig. S1b) and our finding of its binding elements in the promoter regions of *nrtP, nirA, glnA* and *gltS* of *A. platensis* (Fig. 2) pave the way for a more detailed characterization of the mechanisms of N regulation in this organism. The *ntcA* gene was expressed at maximum levels in N-depleted cultures and it fell to minimum levels within 90 min of addition of ammonium salt to the medium; nitrate addition had a similar, albeit less drastic effect (Fig. 7). This indicates that *ntcA* induction of nitrate uptake and assimilation operates at the highest level during N-depletion and falls to intermediate level with nitrate nutrition and to lowest level with ammonium nutrition. Thus, *ntcA* plays a key role in sensing the nitrogen status of the cell in terms of both the form and amount of N and regulates the genes of N-assimilatory pathway accordingly. This was further verified by using ammonium-grown cultures and by supplementing them with nitrate or nitrite, when an increase in the *ntcA* gene expression was observed. Similar results were reported earlier in *Synechococcus*-WH7803 (Lindell and Post 2001; Lindell et al. 2005).

Further studies are in progress to understand the mechanism of regulation of gene expression by *ntcA* and other regulators, LysR, PII and ntcB, which we have cloned recently (*ntcB*- JX489170, *PII*- JX431864, *lysR*- JX431863) from *A. platensis*.

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