

DevT (Alr4674), resembling a Ser/Thr protein phosphatase, is essential for heterocyst function in the cyanobacterium *Anabaena* sp. PCC 7120

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Heterocyst-forming cyanobacteria are able to perform oxygenic photosynthesis and nitrogen fixation simultaneously in the same filament, by restricting the highly O₂-sensitive nitrogenase to specialized cells, the heterocysts. A remarkable change in morphology and metabolism accompanies the differentiation of heterocysts, which only occurs when no source of combined nitrogen is available. In this study, we characterized DevT (Alr4674), a putative protein phosphatase from *Anabaena* PCC 7120. Mutants defective in *devT* are able to form morphologically mature heterocysts, which however cannot fix N₂, and the mutant cannot survive without a source of combined nitrogen. DevT shows homology to phosphatases of the PPP family and displays a Mn²⁺-dependent phosphatase activity that can be inhibited by phosphatase inhibitors and oxidizing conditions. DevT is constitutively expressed in both vegetative cells and heterocysts, and is not regulated by NtcA. The heterocyst regulator HetR may exert a certain inhibition on the expression of *devT*. Under diazotrophic growth conditions, DevT protein accumulates specifically in mature heterocysts. Therefore DevT plays a still unknown role in a late essential step of heterocyst differentiation.

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INTRODUCTION

Protein phosphorylation plays an important role in countless cellular processes, including regulation of enzyme activity, assembly of macromolecules and proteolysis. Most abundant in bacteria is the phosphorylation of proteins at specific His and Asp residues involved in so-called two-component systems. Those systems are based on phospho-relay signal-transduction pathways that allow the cells to respond to environmental changes. In the last two decades

other types of phosphorylation that were thought to be restricted to eukaryotes, the phosphorylation of Ser/Thr and Tyr residues, have been recognized in bacteria (Shi *et al.*, 1998). Many examples of these eukaryotic systems have been found in the increasing number of available bacterial genome sequences. In particular, many protein sequences homologous to Ser/Thr and Tyr kinases and phosphatases have been identified in the diverse group of cyanobacteria (Zhang *et al.*, 1998b, 2005). However, the function of most of these enzymes is still largely unknown. The enormous quantity of two-component systems as well as protein kinases and phosphatases in strains able to undergo cellular differentiation in adaptation to environmental stress has led to the suggestion that protein phosphorylation is related to the cellular complexity of these cyanobacteria (Kennelly & Potts, 1996; Wang *et al.*, 2002). Interestingly, the Ser/Thr kinases from the heterocyst-forming cyanobacterium *Anabaena* sp. PCC 7120 (hereafter referred to as *Anabaena*) contain very complex domain structures. In several cases regulatory domains like the GAF domain or His kinase domains are fused to the Ser/Thr kinase part of these enzymes. In addition, several genes

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Abbreviations: DAB, diaminobenzidine; pNPP, *p*-nitrophenyl phosphate; TTC, 2,3,5-triphenyltetrazolium chloride.

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Two supplementary figures are available with the online version of this paper.

encoding Ser/Thr kinases or phosphatases are clustered with genes for proteins involved in two-component systems, and these protein-modification systems may be part of the same signalling pathway (Wang *et al.*, 2002; Zhang, 1996; Zhang *et al.*, 1998b).

Ser/Thr phosphatases are the counterparts of Ser/Thr kinases and can be divided into two main families, the PPP and the PPM family. Phosphatases of the PPP family are the most frequent eukaryotic Ser/Thr protein phosphatases. Examples are protein phosphatases PP1, PP-2A and PP-2B (calcineurin), which exhibit invariant residues involved in catalysis (Barton *et al.*, 1994). They utilize the same binuclear metal-ion assisted catalytic mechanism, but have strikingly different structures (Barford, 1996). Crystal structures have been determined for PP-1 and PP-2B, showing the conserved metal-binding sites in the catalytic centre: Mn^{2+} and Fe^{2+} in PP-1, Zn^{2+} and Fe^{2+} in PP-2B (reviewed by Cohen, 1989; Shi, 2004). Less-conserved regulatory subunits determine activity, substrate specificity and function (Cohen, 1989). It is not known whether prokaryotic phosphatases of the PPP family show similar distribution into non-catalytic and catalytic domains. The second family of Ser/Thr protein phosphatases is the PPM family with the representative phosphatase PP2C, which differs clearly from the PPP-type in primary sequence, structure and catalytic mechanism (Mumby & Walter, 1993; Virshup & Shenolikar, 2009).

Several filamentous cyanobacteria, such as members of the genera *Nostoc* and *Anabaena*, are able to simultaneously assimilate CO_2 and N_2 and to perform oxygenic photosynthesis in one multicellular filament, despite the fact that the nitrogenase, the enzymic complex responsible for nitrogen fixation, is very sensitive to oxygen. A sophisticated solution to this problem is realized by some filamentous diazotrophic cyanobacteria: they restrict the oxygen-sensitive nitrogen-fixation process to specialized cells termed heterocysts, which differentiate from vegetative cells at semi-regular intervals along the filaments. Heterocysts provide a micro-oxic environment for nitrogenase, while the oxygen-evolving photosystem II is restricted to the vegetative cells (for reviews see Adams & Carr, 1981; Adams, 2000; Aldea *et al.*, 2008; Kumar *et al.*, 2010; Wolk *et al.*, 1994).

During heterocyst differentiation, induced by nitrogen starvation, a thick envelope consisting of an inner laminated and an outer homogeneous layer is deposited outside the Gram-negative cell wall. The laminated layer comprises heterocyst-specific glycolipids and is the main barrier blocking penetration of oxygen from the aerobic environment (Lambein *et al.*, 1973). It is encased by a homogeneous polysaccharide layer, which is formed first (Cardemil & Wolk, 1981). The intracellular organization of thylakoid membranes changes and the honeycomb membrane, hosting the respiratory electron-transport chain, is formed (Wolk *et al.*, 1994).

As shown in many cases, the synthesis of proteins involved in heterocyst differentiation is regulated at the transcriptional level (Golden & Yoon, 2003; Wolk *et al.*, 1994; Xu

et al., 2008). Transcription of genes encoding proteins involved in CO_2 fixation, such as *rbcL* and *rbcS* encoding ribulose biphosphate carboxylase/oxygenase, is repressed in the differentiating cells, whereas expression of genes required for morphological changes or nitrogen fixation is induced (Wolk *et al.*, 1994). The global nitrogen regulator NtcA plays a key role in these regulatory processes as either activator or repressor of several genes (Herrero *et al.*, 2001, 2004; Zhang *et al.*, 2006). The second key regulator is HetR, a protein exhibiting protease and DNA-binding activities *in vitro*, which regulates expression of genes required for heterocyst differentiation, in some cases indirectly via activation of NtcA expression (Black *et al.*, 1993; Buikema & Haselkorn, 1991; Flores & Herrero, 2010; Huang *et al.*, 2004; Khudyakov & Golden, 2004; Muro-Pastor *et al.*, 2002; Zhou *et al.*, 1998).

In addition to these key players, several protein kinases and phosphatases required for growth on N_2 as nitrogen source have been identified (Kumar *et al.*, 2010; Ohmori *et al.*, 2001; Zhang *et al.*, 2005). The *pknE* gene of *Anabaena* [1] encoding a Ser/Thr kinase clusters with the *prpA* gene encoding a protein phosphatase, and both are required for diazotrophic growth (Zhang *et al.*, 1998a). The genes are upregulated after 8 h of nitrogen depletion (Ehira & Ohmori, 2006). Mutants in these genes show aberrant heterocyst envelope structures and are not able to restore nitrogenase activity after incubation under micro-oxic conditions (Zhang *et al.*, 1998a). A mutant in the *pknD*, gene encoding another Ser/Thr protein kinase, differentiates normal heterocysts but shows reduced growth on N_2 , even though the rate of nitrogen fixation is similar to that of the wild-type *Anabaena* (Zhang & Libs, 1998). This phenotype led to the suggestion that PknD might be involved in the trafficking of the fixed nitrogen from the heterocyst to the neighbouring vegetative cells. The *prpJ1* and *prpJ2* genes, encoding PP2C-type protein phosphatases, were identified as new regulators involved in the initiation of heterocyst differentiation. The activity of PrpJ1 is essential during heterocyst maturation, regulating the synthesis of one of the two heterocyst glycolipid layers. The closely related phosphatase PrpJ2 was proposed to act together with PrpJ1 in upregulating *hetR* (Jang *et al.*, 2007, 2009). In the present work, we describe a novel potential Ser/Thr protein phosphatase, encoded by the *devT* gene (*alr4674*), which is essential for growth on N_2 in the cyanobacterium *Anabaena*.

METHODS

Bacterial strains and growth conditions. *Anabaena* sp. strain PCC 7120 (*Anabaena*) and derived mutant strains were grown in the medium of according to Allen & Arnon (1955) under photoautotrophic conditions as described previously (Fiedler *et al.*, 1998b). Routinely, *Anabaena* strains were grown in Erlenmeyer flasks with constant shaking. Experimental liquid cultures were grown in 750 ml bottles, bubbled with 2% CO_2 -enriched air. During nitrogen step-down experiments, liquid cultures were washed three times in medium without combined nitrogen and resuspended in the same

medium to induce heterocyst formation. Mutant $\alpha 2$ (Ernst *et al.*, 1992), which is a transposon (Tn5-1065)-derived mutant in the *devT* gene (*alr4674*) of *Anabaena*, strain DRpMA54 (a site-directed mutant of *devT*) and *hetR* mutant DR884a (Black *et al.*, 1993) were grown in the presence of 5 mM NO_3^- and 50 μg neomycin ml^{-1} . Mutant $\alpha 2$ (pMA40) was grown in the presence of 50 μg neomycin and 5 μg spectinomycin ml^{-1} . *ntcA* mutant CSE2 (Frias *et al.*, 1994) was grown in the presence of 5 mM NH_4Cl supplemented with streptomycin and spectinomycin, 2.5 μg ml^{-1} each.

Strains of *Escherichia coli* were grown in LB medium containing appropriate antibiotics: kanamycin, ampicillin, spectinomycin (50 μg ml^{-1}), streptomycin or chloramphenicol (25 μg ml^{-1}) (Sambrook *et al.*, 1989). Conjugation between *E. coli* and *Anabaena* was performed by triparental mating using RP-4 as conjugative plasmid and pRL528 as helper plasmid (Elhai & Wolk, 1988b; Wolk *et al.*, 1984).

DNA isolation and manipulation. To isolate plasmid DNA from *E. coli*, the Qiagen plasmid kits were used. DNA from *Anabaena* strains was isolated as described by Cai & Wolk (1990). Restriction digestions and ligations were carried out with enzymes and protocols from New England Biolabs, Fermentas, Eppendorf or Roche. Sequencing was performed with a T7 sequencing kit from Pharmacia using primers complementary to known sequences. To isolate the wild-type *devT* gene, a λ -EMBL3 library of DNA from *Anabaena* (Black & Wolk, 1994) was screened with pRL1630 as a probe as described by Maldener *et al.* (1994). DNA from plaques hybridizing with the probe was digested with *Clal* and cloned into the *Clal* site of plasmid pBlueScript SK+, resulting in plasmid pIM71.

Inactivation of the *devT* gene was done by cloning the C.K3 cassette, conferring neomycin/kanamycin resistance, flanked by *SmaI* sites from the MCS of pRL448 (Elhai & Wolk, 1988a) into the unique *NdeI* site of pIM71, which had been blunted by Klenow treatment, resulting in plasmid pMA53. A 4.1 kb insert was cloned from that plasmid as a *HincII* fragment into *NruI*-digested mobilizable suicide vector pRL277 (Black *et al.*, 1993), resulting in plasmid pMA54. Transfer of pMA54 to wild-type cells of *Anabaena* by triparental mating, following selection on neomycin plates and sucrose, resulted in clones in which the *devT* allele had been replaced by *devT::C.K3* as verified by PCR (data not shown). One chosen clone was the double recombinant DRpMA54.

To carry out complementation of mutants $\alpha 2$ and DRpMA54, first the *devT* gene and its flanking regions were isolated as an 8 kb *Clal* fragment of plasmid pIM71 and cloned into the *Clal* site of shuttle vector pRL1049 (Black & Wolk, 1994), resulting in plasmid pMA40. After transferring this plasmid to mutants $\alpha 2$ and DRpMA54 by triparental mating, exconjugants were selected on spectinomycin and tested for their ability to grow on N_2 as nitrogen source.

To generate a DevT-GFP reporter protein, a DNA fragment consisting of 400 bp of the upstream region of *devT* plus its entire coding region was amplified by PCR using genomic DNA as template with primers DevTGFP1F (5'-AGTACGAATTCTTCGGGATTAAG-AATCTTGGC-3') and DevTGFP1R (5'-CCTATCCCGGGCACAG-ATGGTACTACTG-3'). The PCR product was cloned into the unique *EcoRV* site of plasmid pCSEL19 (Olmedo-Verd *et al.*, 2006) in-frame with the ORF of *gfp*. The resulting plasmid, named pIM331, was used as a template for a second PCR with primers DevTGFP2F (5'-CGGGAGAATTCTTCGGGATTAAGAATC-3') and DevTGFP2R (5'-TCGACCTGCAGGTCTGGACATTTATTG-3'). The PCR product obtained was restricted with *EcoRI/PstI* and cloned into *EcoRI/PstI*-digested pCSEL24, designed for integration into the *nucA* region in the α -megaplasmid of *Anabaena* (Olmedo-Verd *et al.*, 2006), rendering plasmid pIM333. Conjugation to wild-type *Anabaena* resulted in single recombinants, whose genomic structures were confirmed by PCR (data not shown).

RNA isolation and *devT* expression analysis. The protocol for RNA isolation for Northern blot analysis was described by Muro-Pastor *et al.* (1999). In Northern blots, 30 μg RNA per lane was used. The *devT* probe was a 371 bp PCR fragment generated with primers 230 (5'-GTGGGGGATTTTGGTAATGAGTCG-3') and 231 (5'-GAT-AATTGTCTCACAGGCTGCAC-3') and labelled using a ReadyToGo DNA Labelling kit (Amersham) and [α - ^{32}P]dCTP. The *rnpB* probe was used as a control for RNA loading, and was labelled as described by Muro-Pastor *et al.* (1999). After 2 h of prehybridization, the probe was hybridized overnight at 65 °C and the results were visualized with a Cyclone Storage Phosphor System and OptiQuant image analysis software (Packard).

To isolate RNA for RT-PCR analysis, 50 ml cells were harvested by centrifugation and RNA was isolated by hot-phenol pre-extraction and cell lysis combined with the High Pure RNA isolation kit (Roche) following the manufacturer's recommendations.

The RT-PCR analysis of *devT* mRNA abundance under different growth conditions was performed by using the Qiagen OneStep RT-PCR kit according to the manufacturer's description, with 20 ng total RNA per reaction as template. Primers used were oligo230 (5'-GTG-GGGGATTTTGGTAATGAGTCG-3') and oligo217 (5'-GTCCCC-ATTCTGTGGCAG-3') for *devT* amplification, and oligo362 (5'-GCGTTGGCGGTTGCAGAC-3') and oligo130 (5'-TAAGCCGGG-TTCTGTCTCTG-3') for *rnpB*. The mRNA level of the constitutively expressed *rnpB* gene was used as a loading control.

Microscopy. Electron microscopy was done as described by Fiedler *et al.* (1998a). In brief, fixation and post-fixation was done with glutaraldehyde and potassium permanganate; ultrathin sections were stained with uranyl acetate and lead citrate. The samples were examined with a Zeiss EM109 electron microscope at 80 kV.

Bright-field micrographs were taken with a Leica microscope equipped with a DFC420 C camera. DAB (diaminobenzidine) staining was performed according to Ernst *et al.* (1992). To observe TTC (2,3,5-triphenyltetrazolium chloride) reduction, cells were supplied with TTC at final concentration of 0.01 % (w/v) for 30 min and subsequently fixed with 10 % glutaraldehyde and stored on ice in the dark until examined (Pay & Kulasoorya, 1972).

Accumulation of DevT-GFP fusion protein was analysed by fluorescence microscopy. Samples were observed using a Leica HCX PL APO 63 \times 1.3 GLYC objective attached to a Leica DM5500B microscope connected to an external light source for fluorescence excitation Leica EL6000 (Leica, Wetzlar, Germany). The wavelength of excitation was 488 nm. Fluorescent emission was monitored by collection across windows of 500–570 nm (GFP imaging) and 630–700 nm (cyanobacterial autofluorescence).

O₂ respiration measurements. To determine O₂ consumption, *Anabaena* cultures were grown to mid-exponential phase. The amount of cells equivalent to 20 μg chlorophyll *a* (measured in methanolic extracts as described by Mackinney, 1941) were collected and concentrated to 2 ml. Subsequently, cells were incubated in constant light (100 μmol photons m^{-2} s^{-1}), and O₂ production was monitored using a Clark-type oxygen electrode (Hansatech Instruments), until it reached a plateau. The cultures were then immediately supplied with 40 μM DCMU, which inhibited O₂ production, and the O₂ consumption ratio was determined.

Expression and purification of His-tagged DevT. A construct of *devT* fused at its 3' end to six codons encoding histidine was prepared as follows. PCR was performed with primers 38 (5'-TTCGCATGCT-GGAAATATACGGCA-3') and 39 (5'-TATGGATCCACAGATG-GTACTAC-3') and plasmid pIM71 as template. The resulting fragment contained the entire ORF of *devT* flanked by *SphI* and

*Bam*HI sites. After digestion with both restriction enzymes, the *devT* fragment was ligated into the *Sph*I and *Bam*HI sites of cloning vector pQE70 (Qiagen), resulting in plasmid pIM88. For overproduction of His₆-tagged DevT (in the following termed DevT-H₆), an overnight culture of *E. coli* M15 containing plasmids pREP4 (providing additional copies of the LacI repressor) and pIM88 was reinoculated 1:60 in 500 ml LB medium (supplemented with 100 µl ampicillin ml⁻¹ and 25 µl kanamycin ml⁻¹) and grown at 37 °C. After the culture had reached an OD₆₀₀ of 0.6, IPTG was added to a final concentration of 1 mM and incubation was continued for 4 h at 37 °C. After harvesting the cells by centrifugation, the cell pellet was resuspended in 1 ml per g cell pellet of lysis buffer (50 mM potassium phosphate buffer pH 7.8, 300 mM NaCl, 10 mM imidazole, 1 mg lysozyme ml⁻¹) and incubated on ice for 30 min. Cells were disrupted by sonication with a Sonifier B-12 from Branson Sonic Power Company. The soluble proteins of the crude extract were applied to a Ni²⁺-NTA column (Qiagen), equilibrated with 50 mM phosphate buffer pH 7.8, 300 mM NaCl, 20 mM imidazole. The same buffer was used for washing. Elution was done with 50 mM phosphate buffer pH 7.8, 300 mM NaCl and 250 mM imidazole. After analysis by SDS-PAGE, the fractions containing pure DevT-H₆ were pooled and dialysed overnight against 10 mM potassium phosphate buffer pH 7.8, 1 mM EDTA, and stored at -20 °C until further use. All steps after crude extract preparation were performed at room temperature to prevent precipitation of DevT-H₆. Most of the expressed protein was found in inclusion bodies, although for purification only the soluble fraction was used. N-terminal sequencing of the purified protein was done by Rainer Deutzmann, Biochemie I, Universität Regensburg, Germany.

Biochemical methods. The amount of protein was determined with the BCA kit from Pierce. SDS-PAGE was performed with the Laemmli buffer system using a Bio-Rad Minicell electrophoresis chamber. Ten micrograms of protein, denatured in SDS loading buffer for 5 min at 95 °C, was applied per lane. Broad-range SDS-PAGE standards from Bio-Rad served as size markers. Coomassie blue was used to stain the proteins.

Size-exclusion chromatography was performed on a Superdex 200 10/300 GL column, equilibrated with gel filtration buffer (10 mM sodium phosphate pH 7.0, 5 mM MgCl₂, 0.5 mM EDTA, 1 mM DTT and 400 mM NaCl). The column was loaded with 500 µg DevT-H₆ in 500 µl gel-filtration buffer and developed on a ÄKTA chromatography system (GE Healthcare) at a flow rate of 0.5 ml min⁻¹, with 0.5 ml fractions collected. Aliquots (20 µl) from each fraction were analysed by SDS-PAGE and Coomassie blue staining to show the presence of DevT-H₆. The Superdex column was calibrated by two independent 500 µl injections corresponding to a total of five proteins: cytochrome *c* (12.4 kDa), carbonic anhydrase (29 kDa), BSA (66 kDa), alcohol dehydrogenase (150 kDa) and β-amylase (200 kDa).

Standard phosphatase activity of DevT-H₆ was assayed in 1 ml volumes in a buffer containing 10 mM Tris/HCl pH 8.2, 2 mM MnCl₂, 1 mM DTT, 50 mM NaCl and 5 mM *p*-nitrophenyl phosphate (pNPP) as substrate. The reaction was started by adding 1–10 µg purified DevT-H₆ and the increase in A₄₀₀ was measured for 15 min in a Kontron Uvicon spectrophotometer. The same reaction omitting DevT-H₆ was prepared as a blank. To determine the optimum temperature for the DevT activity, the reaction was performed in test tubes in heating blocks, stopped after 15 min by chilling on ice and the A₄₀₀ was measured against the blank, incubated under the same conditions. To measure the pH dependence of DevT activity, the pH value was adjusted in the range from 7.4 to 8.6 with Tris/HCl present in the standard buffer. When used, phosphatase inhibitors were present at a final concentration of 10 mM. To measure the effect of H₂O₂ on DevT activity, DevT-H₆ was incubated

in the presence of 0.3, 0.5, 1, 1.5 and 2 mM H₂O₂ in 10 mM HEPES, 1 mM EDTA for 30 min at 30 °C. The reaction was stopped by adding 100 units catalase and the specific activity determined after 10 min at 37 °C.

To determine the time-course of inhibition of DevT by H₂O₂, DevT-H₆ at a concentration of 1 µg µl⁻¹ was incubated with 1 mM H₂O₂ in 10 mM HEPES, 1 mM EDTA pH 8.0 at 30 °C. Every 5 min, 10 µl aliquots were removed and assayed by the pNPP standard test to measure the specific activity of DevT.

To test reactivation of DevT by DTT, 10 µg DevT-H₆ was first inactivated by 1 mM H₂O₂, in 10 mM HEPES, 1 mM EDTA pH 8.0 for 30 min at 30 °C. After removing H₂O₂ by addition of 100 units catalase, DevT activity was recovered by incubation with 10 mM DTT for 15 min at 37 °C. The activity was measured in the pNPP standard test against the control sample (DevT-H₆) treated in the same way.

Immunodetection of DevT protein. Antiserum against purified DevT-H₆ was produced in rabbits (Biogenes, Berlin). After 90 days of immunization, IgGs from the obtained antiserum were further purified by incubation with purified DevT-H₆ immobilized on a PVDF membrane.

To prepare soluble proteins from *Anabaena*, cells were resuspended in lysis buffer (20 mM HEPES pH 7.5, 1 mM PMSF, 1 mM DTT and 0.5 mM EDTA) and subjected to five 30 s rounds of sonication (45 % duty cycle, 50 % power) in a Branson sonifier 250. Samples were briefly centrifuged at 4 °C, 2 min, 1000 g and the supernatant was taken and used in Western blots. Isolation of heterocyst-specific proteins was carried out essentially as described by Moslavac *et al.* (2007). Isolated heterocysts were resuspended in lysis buffer and samples were passed through a French press (10 cycles at 1300 p.s.i., ~9 MPa). Samples were centrifuged at 4 °C for 5 min at 15 000 g and the supernatant was used in Western blot analysis.

For Western blots, proteins were resolved by SDS-PAGE and transferred to PVDF membranes using a semidry transfer system (Pierce Biotechnologies). Membranes were blocked in TBS (20 mM Tris pH 7.5, 150 mM NaCl) containing 5 % (w/v) BSA. Filters were then incubated with purified anti-DevT antiserum in TBS + 2 % BSA and subsequently treated with an anti-rabbit horseradish peroxidase conjugate (1:15 000) (Amersham Biosciences). Immunoreactive bands were detected using the Lumi-Light Western blotting Substrate (Roche) and recorded with the Gel Logic 1500 imaging system (Kodak).

RESULTS

Phenotypic characteristics of mutant α2

After random mutagenesis of *Anabaena* by the Tn5-derived transposon Tn5-1065, several transposon mutants were identified that were unable to grow on N₂ as sole nitrogen source (Ernst *et al.*, 1992). On the basis of its phenotype, mutant α2 was grouped together with mutants α21 and M7. All three mutants were described as Fox⁻, unable to grow without combined nitrogen, Het⁺, able to form heterocysts, Dab⁻, showing no respiratory activity in heterocysts, Hen⁻, missing layers of the envelope, and Fix⁺, able to fix N₂ under anaerobic conditions (Ernst *et al.*, 1992). In our hands, the filaments of mutant α2 showed a spaced pattern of heterocysts similar to that found in the wild-type as observed by light microscopy and by loss of fluorescence after 24 h of nitrogen starvation

(data not shown). The ultrastructure of filaments from the mutant strain deprived of combined nitrogen for 48 h revealed morphologically mature heterocysts with no conspicuous differences from the wild-type heterocysts (Fig. 1a, b). Both layers of the envelope (glycolipid and polysaccharide layers) were present and the inner membranes were rearranged to form the honeycomb membrane at the poles adjacent to the neighbouring vegetative cells. Both the typical narrow septum between the two cell types and the so-called polar channels are present in the mutant. Therefore, mutant $\alpha 2$ is not impaired in morphological maturation of the heterocysts.

Heterocyst-specific oxidation of DAB, indicating the maturation of the heterocyst protoplast, and reduction of TTC, a sign of heterocyst micro-oxic conditions, were analysed (Fig. 1c). The wild-type strain showed a clear polar DAB staining, indicative of high concentrations of respiratory enzymes located at the honeycomb membranes. By contrast, mutant M7 (a transposon mutant defective in *devA*: Maldener *et al.*, 1994), used here as control strain,

and mutant $\alpha 2$ lacked the polar staining but showed a background of homogeneously precipitated DAB, and were thus considered as DAB⁻, which agrees with previous observations (Ernst *et al.*, 1992). Under our TTC staining conditions, the indicator was reduced rapidly in the heterocysts of the wild-type, resulting in clear formazan crystals, while none were formed in the vegetative cells. The rapid TTC reduction in heterocysts is due to the absence of O₂-evolving photosystem II, the presence of the envelope glycolipid-layer that act as an O₂ barrier and the increased respiration, leading to a reduced amount of O₂ that otherwise may compete with TTC as an electron acceptor. TTC reduction was not observed in mutant M7, probably due to the absence of the laminated layer (Fiedler *et al.*, 1998a). Formazan crystals were hardly detected in heterocysts of mutant $\alpha 2$ and when they appeared, they were significantly smaller than those present in the wild-type heterocysts (Fig. 1c). To investigate whether respiratory activity is high in the mutants following heterocyst differentiation, oxygen consumption of whole filaments was measured after induction of heterocyst differentiation (Table 1). While the wild-type showed a clear increase in respiration during diazotrophic growth, mutants $\alpha 2$ and M7 had comparable low activity after being incubated for 48 h without a source of combined nitrogen. This is in line with the results of DAB and TTC staining, which show, respectively, that the mutants are impaired in respiration and creation of a micro-oxic environment. Since the envelope seems to be fully developed, the inability to provide a micro-oxic environment may be due to insufficient reduction of oxygen by respiration.

Cells of mutant $\alpha 2$ were assayed for their ability to develop nitrogenase activity after nitrogen step-down. Under aerobic conditions, filaments of mutant $\alpha 2$ showed no

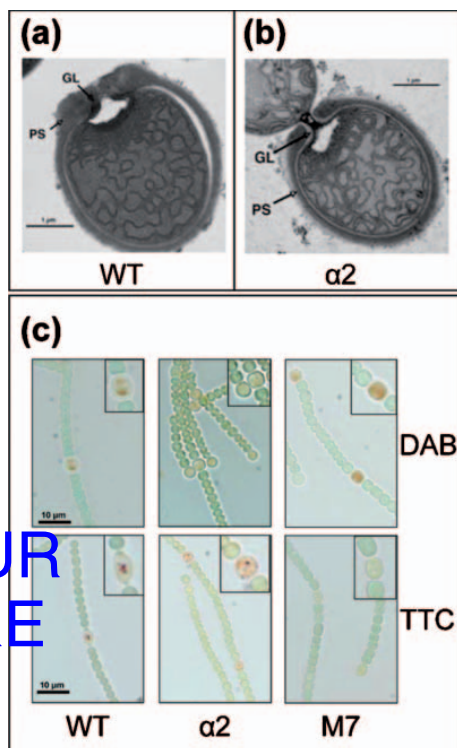


Fig. 1. Analysis of heterocysts by transmission electron microscopy (TEM), DAB staining and TTC reduction. (a, b) Ultrastructure of terminal heterocysts of the wild-type (a) and the $\alpha 2$ mutant (b) as visualized by TEM. PS and GL are polysaccharide and glycolipid layers, respectively. (c) Filaments from wild-type (WT), *devT* ($\alpha 2$) and *devA* (M7) mutant strains, incubated for 48 h under diazotrophic conditions showing DAB staining (DAB) and formazan crystals in heterocysts (TTC). Each picture contains a magnified view of a selected heterocyst as an inset.

Table 1. O₂ respiration in *Anabaena* wild-type, $\alpha 2$ and M7 mutants

The wild-type strain was grown in the presence of nitrate until the culture reached an OD₆₀₀ of 0.5–0.6; then cells were collected and assayed as described in Methods. In addition, cultures of wild-type and mutant strains were grown with nitrate to similar optical densities (~0.5), then harvested, washed with medium lacking any source of combined nitrogen and further incubated in medium lacking any source of combined nitrogen for 48 h prior carrying out the measurements.

<i>Anabaena</i> strain	O ₂ consumption [nmol O ₂ . (mg Chla h ⁻¹) ⁻¹]*
WT (NO ₃)	12.15 ± 1.05
WT (N ₂)	50.94 ± 4.74
$\alpha 2$ (N ₂)	19.54 ± 3.48
M7 (N ₂)	19.97 ± 0.97

*Values are means ± SD of two independent experiments. Chla, chlorophyll *a*.

reduction of acetylene. However, after incubation under anaerobic conditions (argon, DCMU treatment) they developed about 45% of the activity of the wild-type under the same conditions. These data were similar to those reported by Ernst *et al.* (1992).

Recovery and characterization of the mutated genomic region of mutant $\alpha 2$

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Plasmid pRL1630 is the circularized *Clal* fragment recovered from mutant $\alpha 2$, bearing the transposon Tn5-1065 flanked by *Anabaena* DNA; it was obtained from C. Peter Wolk, Michigan State University, East Lansing, USA. The gene where the transposon had inserted was identified by using primers complementary to the ends of the transposon for sequencing. A 9 bp direct repeat (5'-CCGCCAGTG-3') was created upon transposition into the gene. After screening of a λ library of *Anabaena* chromosomal DNA (Black & Wolk, 1994) with pRL1630 as a probe, the wild-type copy of the gene was cloned on a 7753 bp *Clal* fragment into vector pBlueScript SK+, resulting in plasmid pIM71. Further subcloning and sequencing revealed an ORF of 957 bp that we named *devT* (GenBank accession no. CAB57788). *devT* would encode a protein with molecular mass of 35.33 kDa (319 residues, isoelectric point 5.48) assuming the first ATG to be the start codon. Thirty base pairs downstream from that codon, a second putative start codon is present. Putative ribosome-binding sites are located upstream from each of those sites. A sequence similar to *serW* from *E. coli* encoding tRNA^{Ser} is found 80 bp downstream of *devT*. In the annotated genome of *Anabaena* PCC 7120 (Kaneko *et al.*, 2001), *devT* corresponds to gene *alr4674*. *In silico* analysis of the *devT* chromosomal region does not show additional ORFs that could form a transcription unit with *devT*, except for the tRNA-Ser downstream of *devT* (Fig. 2a).

Reconstruction of the DevT mutant in the wild-type of *Anabaena* was achieved by site-directed mutagenesis of *devT*. The neomycin-resistance cassette C.K3 (Elhai & Wolk, 1988a) was cloned in the opposite orientation with respect to *devT* transcription into the unique *NdeI* site of *devT* (Fig. 2a). After transfer into *Anabaena* cells, double

recombinants were selected by a positive selection method based on the use of the conditionally lethal gene *sacB* (Cai & Wolk, 1990). Disruption of the gene was confirmed by Southern blot analysis and PCR (data not shown). The recombinant strain DRpMA54 showed the same phenotype as the original mutant $\alpha 2$ and was not able to grow on N₂, although heterocysts were formed upon nitrogen starvation. The ultrastructure observed by transmission electron microscopy was similar to that of mutant $\alpha 2$ and the wild-type (data not shown). pDU1-based shuttle vector pMA40, harbouring the entire ORF of *devT* including flanking regions, was transferred into strains DRpMA54 and $\alpha 2$, resulting in complemented strains that had recovered the ability to grow on N₂ as sole nitrogen source (shown for $\alpha 2^C$ in Fig. 2b).

DevT is a Ser/Thr phosphatase conserved in the photosynthetic lineage

The deduced amino acid sequence of DevT shows homology to the family of phospho-Ser/Thr phosphatases, which belongs to the superfamily of phosphoesterases (Koonin, 1994). A region of 216 amino acids (residues 21–237) shows high similarity to the catalytic domain of PPP-type Ser/Thr protein phosphatases and contains the three conserved motifs of PPP-type phosphatases (see Supplementary Fig. S1, available with the online version of this paper) (Barton *et al.*, 1994; Shi *et al.*, 1998; Shi, 2009). DevT contains the conserved residues involved in metal binding (motifs I and II) and the catalytic histidine residue (motif III), respectively (Barford, 1996). While motif I of DevT matches exactly the consensus sequence, motif II is less conserved in DevT. However, it contains the Gly and Asp residues of the consensus sequence GD(LYF)V(DA)RG, proposed by Barton *et al.* (1994). The RG residues in this motif are not as highly conserved as the GD residue pair. Amino acid residues between the three motifs do not show much homology among different phosphoesterases. The previously identified PPP-type phosphatase PrpA from *Anabaena* shows low similarity to DevT besides the conserved residues (Zhang *et al.*,

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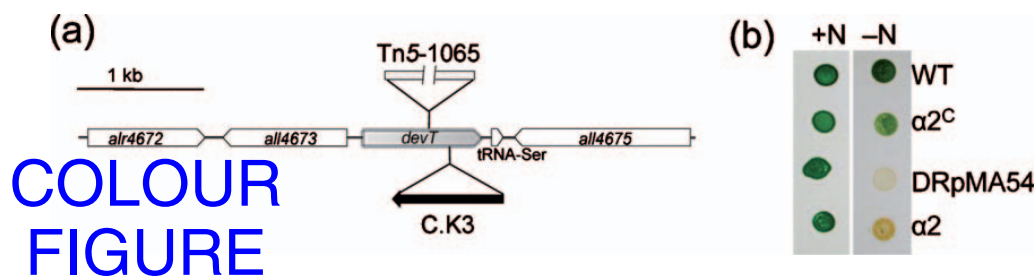


Fig. 2. The *devT* gene cluster and Fox phenotype of *devT* mutant strains. (a) Genomic organization of the chromosomal region containing the *devT* gene (*alr4674*) and the two strategies to inactivate *devT*: via random insertion of transposon Tn5-1065 and via insertion of the C.K3 resistance cassette (mutants $\alpha 2$ and DRpMA54, respectively). (b) Growth on Allen & Arnon agar plates containing 5 mM NaNO₃ (+N) or lacking combined nitrogen (-N) of wild-type (WT), *devT* ($\alpha 2$ and DRpMA54) and *devT*-complemented ($\alpha 2^C$) strains.

[12]

1998a). This is also the case for the PPP-type phosphatase PP1-cyano1 from the cyanobacterium *Microcystis aeruginosa* (Shi & Carmichael, 1997) and for eukaryotic representatives of this protein family (Supplementary Fig. S1).

However, BLAST analysis indicated the presence of very close homologues of DevT present exclusively in 51 cyanobacterial genomes and significant matches on several, but not all, plant genomes, thus suggesting a common ancestor for all these proteins. Cyanobacterial DevT-like sequences were found in filamentous as well as unicellular strains such as *Synechococcus* and *Synechocystis*. Among filamentous strains, matches were found in heterocyst-forming strains as well as in non-differentiating strains such as *Lyngbya* and *Trichodesmium*. Our phylogenetic analysis showed that DevT homologues from the marine picocyanobacteria (including *Prochlorococcus* strains) form a rather distantly related cluster from the rest of sequences, which can be grouped in either plant or cyanobacterial (unicellular and filamentous strains) sequences (see Supplementary Fig. S2). This phylogenetic tree of cyanobacterial DevT homologues resembles recently proposed phylogenetic relationships of cyanobacteria (Gupta & Mathews, 2010).

Biochemical characterization of DevT

The *devT* gene was cloned into the pQE70 vector (yielding plasmid pIM88) to produce a recombinant DevT protein with its C-terminus fused to six histidine residues. After overproduction (see Methods), most of the protein was in the insoluble pellet of the crude cell extracts. Nevertheless, the soluble fraction contained enough DevT protein to be purified on a Ni^{2+} -NTA column (Fig. 3a). To analyse the native molecular mass of DevT- H_6 , size-exclusion chromatography on a Superdex G200 10/300 GL column was performed. The purified fusion protein eluted in one peak corresponding to a molecular mass of about 36 kDa, which would correspond to monomeric DevT protein in solution (Fig. 3b). N-terminal sequencing of the N-terminus of the DevT purified protein gave an amino acid sequence exactly matching that of the predicted protein, if the assumed first start codon (ATG) is used in *E. coli*.

Given that the deduced amino acid sequence contains the metal-binding and active-site residues of PPP-type phosphatases, we analysed whether DevT shows phosphorylase activity *in vitro* using the commonly used chromogenic substrate pNPP. The purified DevT- H_6 protein did indeed show phosphatase activity with pNPP as substrate, but only in the presence of Mn^{2+} (Fig. 4a). The estimated K_m for Mn^{2+} was 23.6 μM (estimation by Hanes–Woelf plot of the direct plot shown in Fig. 4b) and the apparent kinetic constants of the DevT activity towards pNPP corresponded to a K_m of 2.5 mM and a V_{max} of 0.8 $\mu\text{mol pNPP min}^{-1} \text{mg}^{-1}$ (Hanes–Woelf plot, not shown). However, DevT was not able to remove the phosphate from a peptide containing phosphothreonine RRA(pT)VA or phosphoserine RRA(pS)VA (Promega). Furthermore, DevT was not

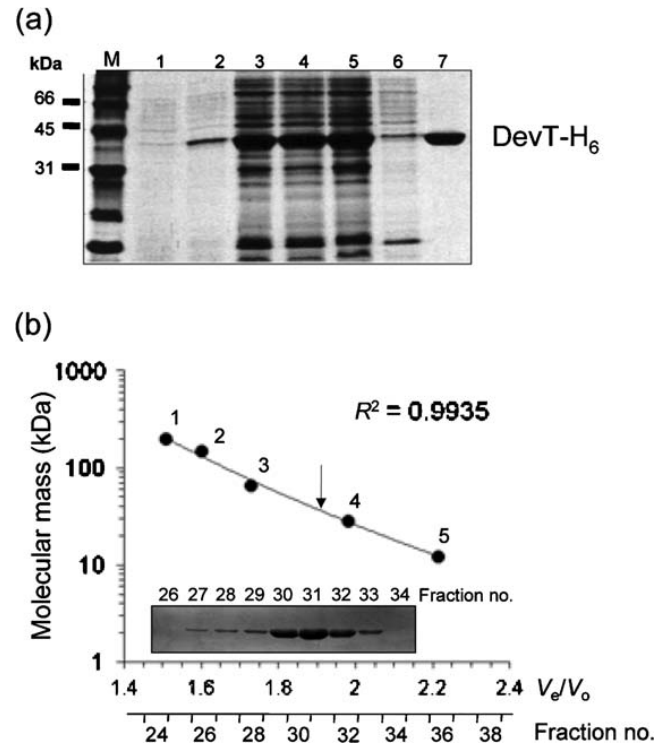


Fig. 3. Purification of recombinant DevT and gel filtration. (a) Expression of DevT- H_6 in *E. coli* cells in either the absence (lane 1) or the presence (lane 2) of IPTG. Crude protein extracts of cells induced by IPTG at 37 °C (lane 3) and 25 °C (lane 4). Proteins present in the pellet (lane 5) and the soluble fraction (lane 6). DevT- H_6 after Ni^{2+} -NTA purification (lane 7). Proteins were separated by SDS-PAGE and stained with Coomassie blue. Lane M, protein size marker. (b) Determination of the apparent molecular mass of DevT by gel-filtration analysis. The gel-filtration column was calibrated with the molecular mass standards from Sigma: 1, β -amylase; 2, alcohol dehydrogenase; 3, BSA; 4, carbonic anhydrase; 5, cytochrome c. The elution peak of DevT- H_6 is represented by an arrow. The elution volume (V_e) is shown as a fraction of the void volume (V_0). The fraction numbers corresponding to fraction numbers shown in the inset are given below the plot. The inset shows a Coomassie-stained SDS-polyacrylamide gel of the indicated fractions collected after gel filtration of DevT- H_6 .

able to dephosphorylate phosphorylated PII protein from *Synechococcus elongatus in vitro* nor ^{32}P -casein (not shown).

DevT activity was maximal at 55 °C and at a pH of 8 (data not shown). The ability of phosphatase inhibitors to influence DevT activity was analysed (Fig. 4c). Addition of the competitive inhibitor inorganic phosphate and the metal chelator EDTA resulted in complete inhibition, but vanadate, NaF and tartrate also decreased DevT activity.

Because it is known that some protein phosphatases are redox regulated (Rusnak & Reiter, 2000), we measured pNPP hydrolysis after incubation of purified DevT protein under different redox conditions. Oxidation of DevT by

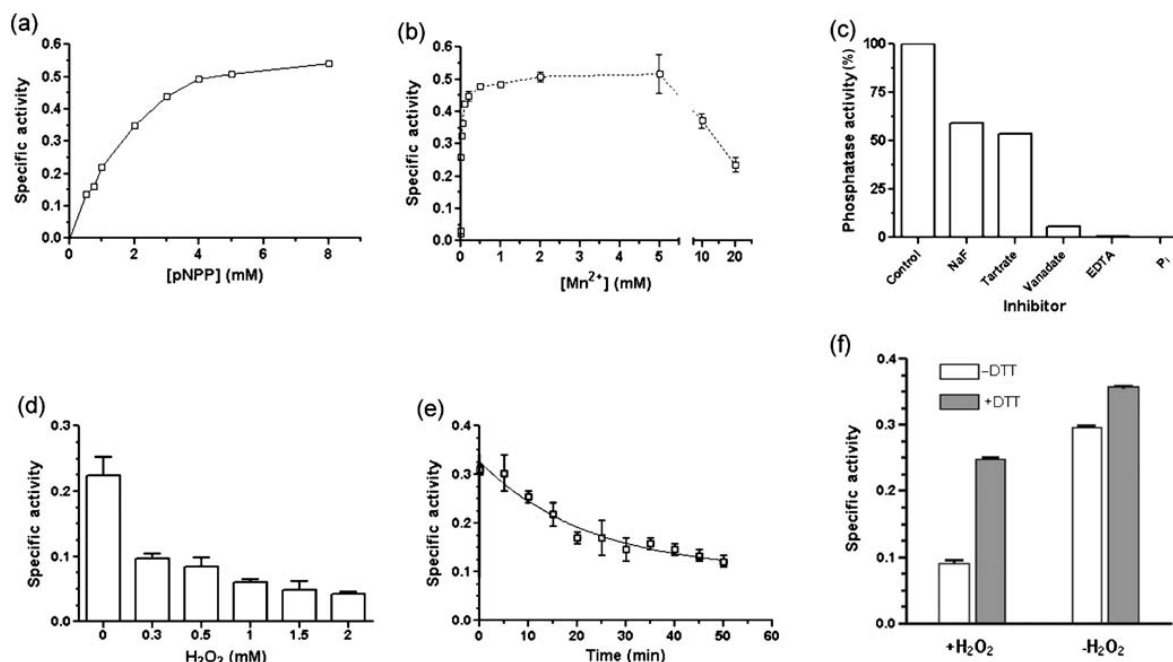


Fig. 4. Enzymic activity of purified recombinant DevT from *Anabaena*. (a) Hydrolysis of pNPP by DevT-H₆. The specific activity (μmol min⁻¹ mg⁻¹) is plotted against different concentrations of pNPP. A representative experiment is shown. (b) Stimulation of reactivity towards pNPP by Mn²⁺. (c) Effect of different phosphatase inhibitors on DevT activity. Activity for each inhibitor is expressed as a percentage of the control reaction with no inhibitor (means of three experiments). (d) Effect of different H₂O₂ concentrations on DevT activity. (e) Time-course of inhibition of DevT activity after incubation with 1 mM H₂O₂. (f) Inhibition of DevT activity by H₂O₂ and subsequent reactivation by DTT (for details see Methods).

either incubation in the presence of increasing concentrations of H₂O₂ or prolonged exposure to H₂O₂ resulted in a significant decrease of activity (Fig. 4d, e), with a half-maximal inhibitory concentration (IC₅₀) of 250 μM H₂O₂. However, subsequent incubation with the reducing agent DTT restored the pNPP dephosphorylation activity (Fig. 4f).

The *devT* gene is constitutively expressed but DevT protein accumulates only during diazotrophic growth

Since mutant α2 was impaired in diazotrophic growth, we investigated whether *devT* expression is differentially regulated upon nitrogen deprivation and whether it is affected by the two master regulators of heterocyst differentiation, NtcA and HetR. Northern blot analysis was performed using total RNA isolated from filaments of the wild-type *Anabaena* strain, *ntcA* mutant CSE2 (Frías *et al.*, 1994) and *hetR* mutant DR884a (Black *et al.*, 1993) at different time intervals after nitrogen step-down. No substantial difference in the abundance of the mRNA specifically hybridizing to the *devT* probe could be observed during nitrogen step-down in wild-type cells (Fig. 5a, WT lanes). Correspondingly, neither upregulation nor downregulation of *devT* was observed by RT-PCR in wild-type cells starved for nitrogen for up to 48 h (Fig. 5b). *ntcA* and *hetR* mutants showed similar and a moderate

increase of *devT* expression, respectively, in comparison with wild-type (Fig. 5a, NtcA⁻ and HetR⁻ lanes).

Immunoblot analyses were carried out to analyse DevT protein levels in wild-type *Anabaena* cells deprived of combined nitrogen. The polyclonal antisera prepared against DevT detected a protein in wild-type extracts that was absent from mutant α2 (data not shown). The estimated mass of this protein, roughly 36 kDa, is in agreement with the value predicted from the deduced amino acid sequence of DevT. Total soluble protein was isolated from filaments of cells shifted from ammonia-supplemented to combined nitrogen-free medium, with samples collected at different time points (Fig. 6a). DevT was detected in extracts from wild-type whole filaments deprived of nitrogen for 48 h, but not at 9, 24 or 34 h. When proteins from isolated heterocysts were separated by SDS-PAGE and blotted, DevT was first noticeable after 24 h and increased strongly during the next 24 h (Fig. 6b).

Heterocyst-specific localization of DevT

Localization of DevT in the diazotrophic filaments of *Anabaena* wild-type strain was examined by using the green fluorescent protein (GFP) as a reporter. The *devT*-*gfp* fusion was inserted into a neutral site (*nucA*-*nuiA* region) located in the α-megaplasmid, essentially as

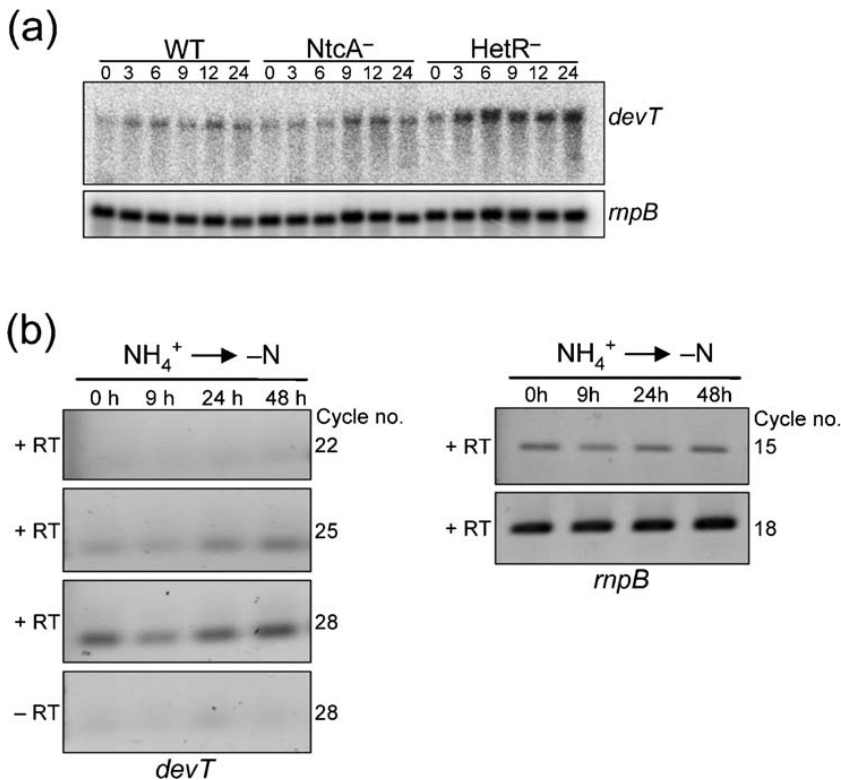


Fig. 5. Expression of the *devT* gene. (a) Expression of *devT* in the wild-type (WT), *ntcA* (*NtcA*⁻) and *hetR* (*HetR*⁻) mutants, analysed by Northern blotting with RNA isolated from whole filaments grown with ammonium and incubated without combined nitrogen for the times indicated above the lanes (in hours). The filter was subsequently hybridized with a probe for the *mpB* gene (see Methods for details). (b) RT-PCR analysis of *devT* transcripts from wild-type *Anabaena* under different growth conditions. The cells from which RNA was prepared were first grown in ammonia-supplemented medium, then incubated with medium lacking combined nitrogen and collected at indicated times. The left panel shows *devT* amplification (+RT) after 22, 25 and 28 cycles. A control where reverse transcriptase was omitted (-RT) is also shown. The right panel shows amplification of the constitutively expressed *mpB* gene after 15 and 18 cycles.

described by Olmedo-Verd *et al.* (2006). The selected clone (strain WT-333) bore a copy of the *devT-gfp* construct, integrated in the α -megaplasmid, and retained an intact copy of the wild-type *devT* region, as verified by PCR with specific oligonucleotides (data not shown). DevT-GFP accumulated in fully developed heterocysts, which showed a decreased autofluorescence of photosynthetic pigments. Faint green fluorescence in heterocysts from the reporter

strain could be observed 28 h after nitrogen step-down (Fig. 7). Subsequently, heterocysts showed a progressive increase of GFP fluorescence, with the highest level in heterocysts of steady-state N₂-fixing cultures (Fig. 7). These results suggest that the accumulation of DevT takes place specifically in mature, presumably older, heterocysts, at least up to a certain heterocyst age.

DISCUSSION

In this study, we characterized *devT* (*alr4674*) from *Anabaena*, which encodes a PPP-family phosphatase involved in heterocyst function. Orthologues of DevT were found in the available genomes of cyanobacteria and certain genomes of plants and the green alga *Chlamydomonas*. Cyanobacterial DevT sequences included heterocyst-forming (as *Anabaena variabilis* ATCC 29413), nitrogen-fixing non-heterocystous (as *Trichodesmium erythraeum*) strains and filamentous non-fixing (as *Lyngbya* sp. PCC 8106) as well as unicellular strains (as *Synechocystis* sp. PCC 6803), which do not fix N₂. The conservation of DevT in strains with different cell structure and metabolism suggests different functions for DevT in these organisms. Since close DevT homologues are only present in organisms that perform oxygenic photosynthesis, its role could also be related to redox control and oxygen stress.

DevT shows a manganese-dependent phosphatase activity, and the effect of various inhibitors of different families of protein phosphatases together with sequence-based

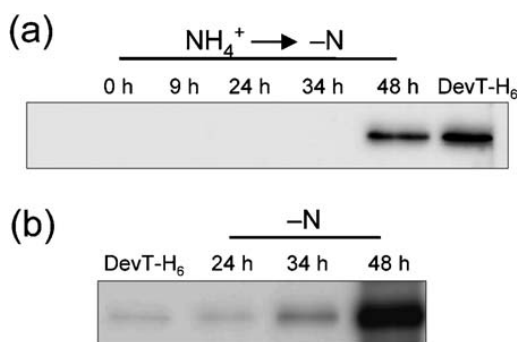


Fig. 6. Accumulation of DevT. Abundance of DevT protein in wild-type *Anabaena* during nitrogen step-down. (a) Ten micrograms of total protein was subjected to SDS-PAGE and the presence of DevT was detected by immunoblotting using antiserum against DevT-H₆. (b) As in (a), but protein was obtained from isolated heterocysts and the exposure time for chemiluminescence was decreased.

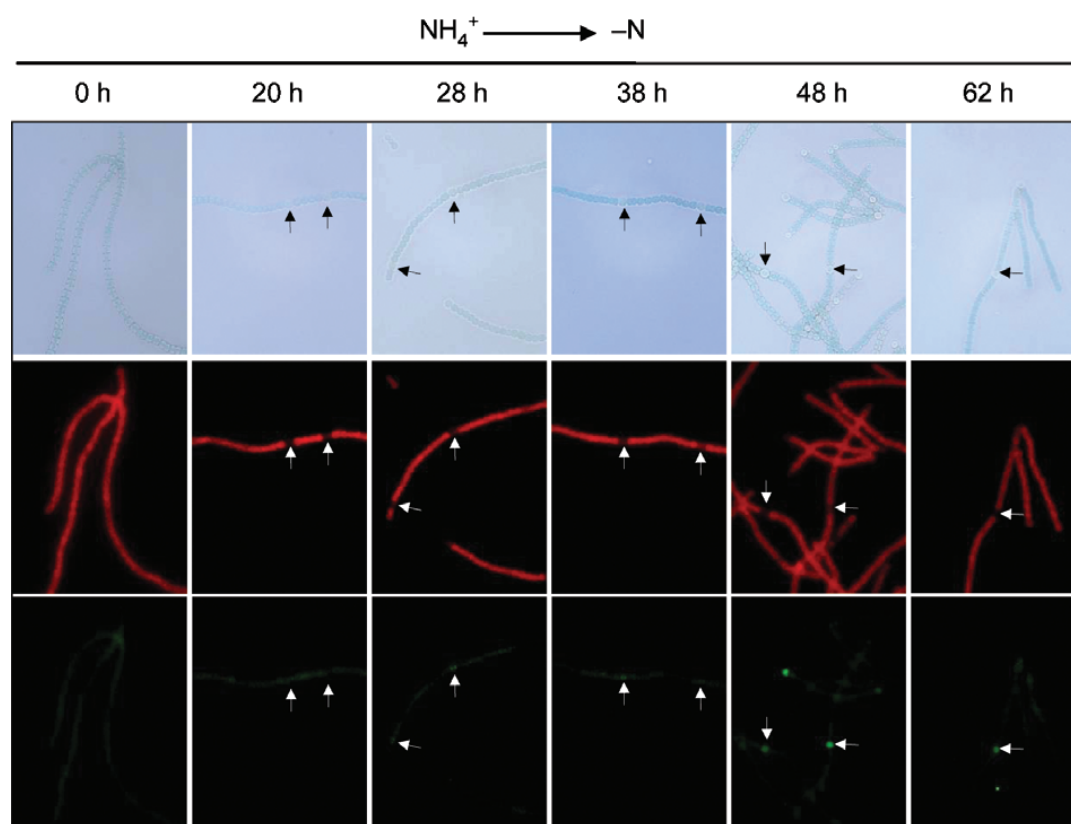
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FIGURE

Fig. 7. Specific accumulation of the DevT-GFP translational fusion in heterocysts. Accumulation of DevT in heterocysts after nitrogen step-down, as reported by the GFP-based fluorescence at indicated time points of *Anabaena* bearing a *devT-gfp* fusion integrated into the α -megaplasmid. Arrows indicate the position of heterocysts. Bright-field (top), red autofluorescence (middle) and GFP fluorescence (bottom) images are shown.

similarities of the catalytic domain are consistent with DevT being a member of the PP1 family. pNPP hydrolysis was observed using recombinant DevT, but the protein was not able to remove the phosphate from peptide substrates nor from casein. Several known serine/threonine phosphatases, such as PP2A, PP2B and PP2C, dephosphorylate those substrates. However, the artificial phospho-peptides used in this study are poor substrates for PP1 phosphatases given their more stringent structural requirements, and this could also be the case for the lack of activity of DevT-H₆. Also, phosphorylated PII protein was not a substrate for DevT-H₆. Further research should clarify whether DevT can act on targets containing phospho-Thr or phospho-Ser. Due to conservation in the primary sequences of phosphoesterases it cannot be excluded that DevT acts on non-proteinaceous substrates, such as cyclic-nucleotide phosphates, polynucleotides, etc. (Koonin, 1994).

In the last two decades of heterocyst research, the approach of reverse genetics by transposon mutagenesis has proved to be a straightforward tool for identifying new genes involved in this prokaryotic differentiation process (e.g. Ernst *et al.*, 1992; Fan *et al.*, 2005). Transposon mutant $\alpha 2$ (a *devT* mutant), identified previously by its inability to

grow on N₂ as sole nitrogen source, has been analysed in depth here. The ultrastructure of the non-functional heterocysts of the mutant showed no differences from that of the wild-type, in contrast to the first description by (Ernst *et al.*, 1992). Hence the *devT* mutant could be altered in regulatory or metabolic processes. In this respect, DAB and TTC staining as well as respiration measurements suggest that the drop in O₂ concentration that takes place during heterocyst formation does not occur efficiently in the *devT* mutant, leading to a defect in nitrogenase activity that results in the inability to grow diazotrophically. It should be noted that mutant $\alpha 2$ showed a severe decrease of nitrogenase protein levels in the presence of air compared to anaerobic conditions, the latter conditions partially restoring nitrogenase activity and levels (Ernst *et al.*, 1992).

DevT protein was shown to be upregulated and accumulated only during diazotrophic growth, specifically in heterocysts. While the mRNA of *devT* stayed at a similar low level during nitrogen step-down, low levels of DevT protein were initially detected at a time when heterocysts became mature and functional, about 24 h after step-down. After 48 h, *devT* mRNA levels remained at a

constant low level whereas the protein significantly accumulated. Indeed, transcription of the *devT* gene was constitutive under tested conditions, independent of NtcA, while HetR exhibited a certain negative effect. Taken together, our data suggest that DevT might be post-transcriptionally regulated, and there might be a mechanism that controls stability of the DevT protein. DevT protein could be easily degraded in vegetative cells and immature heterocysts (proheterocysts), but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of *devT* directly by binding to regulatory DNA elements or indirectly requires further investigation.

Considering the phenotypic features of the $\alpha 2$ mutant and the late accumulation of DevT during heterocyst formation, DevT seems to be a factor involved in late steps of heterocyst maturation (approx. 24 h after initiation of heterocyst formation), presumably transmitting signals, via dephosphorylation, that are essential to achieve functionality in mature heterocysts. Our data clearly indicate that the phenotype of the *devT* mutant is related to oxygen stress. In order to understand the role of DevT, the signals transmitted by DevT as well as its putative target or targets need to be addressed.

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