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.

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 phosphorelay 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) exhibit 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
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 microoxic 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 bisphosphate 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 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). These genes are upregulated after 8 h of nitrogen depletion (Ehira et al., 2003). 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 (acr4674), 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 liquid medium 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 s2 (Ernst et al., 1992), which is a transposon (Tn5-1065)-derived mutant in the *devT* gene (alr-6747) of *Anabaena*, strain DRpMA54 (a site-directed mutant of *devT*) and *hetR* mutant DR884a (Black & Wolk, 1993) were grown in the presence of 5 mM NO₃⁻ and 50 μg neomycin ml⁻¹. Mutant s2 (pMA40) was grown in the presence of 50 μg neomycin and 5 μg spectinomycin ml⁻¹. ntcA mutant CSE2 (Frias et al., 1989) was grown in the presence of 5 mM NH₄Cl 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 conjunctive 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 DevTGFP1F (5'-AGTACGAATTCTTCGGGATTAAG-3') and DevTGFP2R (5'-ACTTCTGTGGCAG-3'). The PCR product was cloned into the unique *EcoRI* site of plasmid pIM71, resulting in plasmid pIM71.

Inactivation of the *devT* gene was done by cloning the C.K3 cassette, conferring neomycin/kanamycin resistance, flanked by *Smal* sites from the MCS of pRL448 (Elhai & Wolk, 1988a) into the unique *Ndel* site of pIM71, which had been blunt by Klenow treatment, resulting in plasmid pMA53. A 4.1 kb insert was cloned from that plasmid as a *Clai* fragment into the *Ndel*-digested mobilizable suicide vector pRL277 (Black & Wolk, 1994), resulting in plasmid pMA54. Transfer of pMA54 to wild-type cells of *Anabaena* by triparental mating, followed by 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 s2 and DRpMA54, first the *devT* gene and its flanking regions were isolated as an 8 kb *Clai* fragment of plasmid pIM71 and cloned into the *Clai* site of shuttle vector pRL1049 (Black & Wolk, 1994), resulting in plasmid pMA40. After transferring this plasmid to mutants s2 and DRpMA54 by triparental mating, exconjugants were selected on spectinomycin and tested for their ability to grow on N₂ as nitrogen source.

**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'-GTGGGGGATTGGTTGAAATGCTG-3') and 231 (5'-GATTATGTCATCCAGGCTGCAC-3') and labelled using a ReadyToGo DNA Labelling kit (Amersham) and [α-32P]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'-GGGATGGTTTGTGAAATGCTG-3') and oligo217 (5'-GTCGCC-ATTCTGTGGCAGC-3') for *devT* amplification, and oligo362 (5'-GGCTTTGGCGGTTCGAC3'-3') and oligo130 (5'-TAAACCGGG-TTCGTGGTCTCTG-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. (1998). 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 (Fay & Kulasooryia, 1972).

Accumulation of *DevT–GFP* fusion protein was analysed by fluorescence microscopy. Samples were observed using a Leica HCX PL APO 63×/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 and nitrogenase activity 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. Nitrogenase activity was measured as described by Valladares et al. (2007).

**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 39 (5'-TTCGCAATGCTGGAATATACCGGCA-3') and 39 (5'-TAATGCCAGTGACTTAC-3') and plasmid pIM71 as template. The resulting...
The soluble proteins of the crude extract were applied to a min
independent 500 ml injections corresponding to a total of five
fragments contained the entire ORF of devT flanked by SplI and
BamHI sites. After digestion with both restriction enzymes, the devT
fragment was ligated into the SplI and BamHI sites of cloning vector
pQE70 (Qiagen), resulting in plasmid pLM88. 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 pLM88 was reincubated
1:60 in 500 ml LB medium (supplemented with 100 ml ampicillin
ml⁻¹ and 25 ml 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 sequen-
cing 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 an AKTA chromatog-
raphy 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, 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 15000 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
(Peqlab 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 (1:15000) (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 a2**

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 a2 was grouped together with mutants a21 and
M7. All three mutants were described as Fox¹, unable to
grow without combined nitrogen, Het⁴, able to form
heterocysts, Dab⁸, showing no respiratory activity in
heterocysts, Hen⁹, missing layers of the envelope, and
Fix⁶, able to fix N₂ under anaerobic conditions (Ernst
et al., 1992). In our hands, the filaments of mutant a2
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 a2 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 a2 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 acts 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., 1998). Formazan crystals were hardly detected in heterocysts of mutant a2 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 a2 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 a2 were assayed for their ability to develop nitrogenase activity after nitrogen step-down. Under

![Fig. 1. Analysis of heterocysts by transmission electron microscopy (TEM) (a, b), and by DAB staining and TTC reduction (c). Ultrastructure of terminal heterocysts of the wild-type (a) and the a2 mutant (b) as visualized by TEM. PS and GL are polysaccharide and glycolipid layers, respectively. (c) Filaments from wild-type (WT), devT (a2) 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.](image)

<table>
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<th>Table 1. O2 respiration in Anabaena wild-type, a2 and M7 mutants</th>
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<td>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 to carrying out the measurements.</td>
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<td>Anabaena strain</td>
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<td>WT (NO3−)</td>
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<td>a2 (N₂)</td>
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<td>M7 (N₂)</td>
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*Values are means ± SD of two independent experiments. Chl a, chlorophyll a.
DevT, a phosphatase required for heterocyst function

Recovery and characterization of the mutated genomic region of mutant α2

Plasmid pRL1630 is the circularized Clal fragment recovered from mutant α2, 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 insertion 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+. 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 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 α2 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 α2 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 α2, resulting in complemented strains that had recovered the ability to grow on N2 as sole nitrogen source (shown for α2C 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 217 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 do not act as highly conserved as the GD residue pair. Amino acid residues between the three motifs do not show much conservation among different phosphoesterases. The previously identified PPP-type phosphatase PrpA from Anabaena shows low similarity to DevT.

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 α2 and DRpMA54, respectively). (b) Growth on Allen & Arnon agar plates containing 5 mM NaN3O3 (+N) or lacking combined nitrogen (−N) of wild-type (WT), devT (α2 and DRpMA54) and devT-complemented (α2C) strains.
Besides the conserved residues (Zhang et al., 1998a). This is also the case for the PPP-type phosphatase PP1-cyanol 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 Ni²⁺-NTA column (Fig. 3a). To analyse the native molecular mass of DevT-H₆, 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 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 phosphatase activity in vitro using the commonly used chromogenic substrate pNPP. The purified DevT-H₆ protein did indeed show phosphatase activity with pNPP as substrate, but only in the presence of Mn²⁺ (Fig. 4a). The estimated *Kₘ* for Mn²⁺ was 23.6 μM (estimation by Hanes–Woolf plot of the direct plot shown in Fig. 4b) and the apparent kinetic constants of the DevT activity towards pNPP corresponded to a *Kₘ* of 2.5 mM and a *Vₘₐₓ* of 0.8 μmol pNPP min⁻¹ mg⁻¹ (Hanes–Woolf 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 able to dephosphorylate phosphorylated PII protein from *Synechococcus elongatus* in vitro nor ³²P-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
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 (Frias 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. 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
described by Olmedo-Verde 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 *Lymnbya* 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...
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 α2 (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 α2 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

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.
constant low level whereas the protein significantly accumulated. Indeed, transcription of the \textit{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 HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. WhetherHetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts. Whether HetR constitutes an additional point of transcriptional regulation of HetR, but – by an unknown mechanism – might be stabilized in mature heterocysts.

Considering the phenotypic features of the \textit{het} \textit{A} 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 \textit{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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**REFERENCES**


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