Anabaena sp. strain PCC 7120 *conR* contains a LytR-CpsA-Psr domain, is developmentally regulated, and is essential for diazotrophic growth and heterocyst morphogenesis

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The *conR* (all0187) gene of the filamentous cyanobacterium *Anabaena* (*Nostoc*) sp. strain PCC 7120 is predicted to be part of a family of proteins that contain the LytR-CpsA-Psr domain associated with septum formation and cell wall maintenance. The *conR* gene was originally misannotated as a transcription regulator. Northern RNA blot analysis showed that *conR* expression was upregulated 8 h after nitrogen step-down. Fluorescence microscopy of a *P_{conR}-gfp* reporter strain revealed increased GFP fluorescence in proheterocysts and heterocysts beginning 9 h after nitrogen step-down. Insertional inactivation of *conR* caused a septum-formation defect of vegetative cells grown in nitrate-containing medium. In nitrate-free medium, mutant filaments formed abnormally long heterocysts and were defective for diazotrophic growth. Septum formation between heterocysts and adjacent vegetative cells was abnormal, often with one or both poles of the heterocysts appearing partially open. In a *conR* mutant, expression of *nifH* was delayed after nitrogen step-down and nitrogenase activity was approximately 70 % of wild-type activity, indicating that heterocysts of the *conR* mutant strain are partially functional. We hypothesize that the diazotrophic growth defect is caused by an inability of the heterocysts to transport fixed nitrogen to the neighbouring vegetative cells.

Received 13 October 2010
Revised 16 November 2010
Accepted 17 November 2010

INTRODUCTION

The filamentous cyanobacterium *Anabaena* (also *Nostoc*) sp. strain PCC 7120 (hereafter *Anabaena* PCC 7120) is capable of fixing carbon dioxide by oxygenic photosynthesis and fixing molecular nitrogen when a combined nitrogen source such as ammonium or nitrate is not available. These two processes are incompatible because nitrogenase is oxygen-sensitive and oxygen is produced as a by-product of photosynthesis. *Anabaena* PCC 7120 spatially separates these processes by undergoing multicellular development in which 5–10 % of vegetative cells differentiate into specialized cells called heterocysts, which switch from photosynthesis to nitrogen fixation and supply the filament with fixed nitrogen as amino acids (Aldea *et al.*, 2008; Flores & Herrero, 2010; Kumar *et al.*, 2010).

Heterocyst differentiation involves morphological and physiological changes that produce the micro-oxic envi-

ronment necessary for nitrogen fixation (Flores & Herrero, 2010; Kumar *et al.*, 2010). This micro-oxic environment is generated by the inactivation of the oxygen-producing photosystem II, an increase in respiration and the deposition of a multilayered cell envelope consisting of an inner layer of glycolipids and an outer layer of polysaccharides that diminishes O₂ entry into the cell. Heterocysts are present in a developmental pattern of single heterocysts separated by 10–15 vegetative cells that is regulated by cell–cell signalling involving the products of the *patS* and *hetN* genes, as well as fixed nitrogen produced by heterocysts (Borthakur *et al.*, 2005; Kumar *et al.*, 2010; Risser & Callahan, 2009; Yoon & Golden, 1998, 2001; Zhang *et al.*, 2006). Heterocysts supply vegetative cells with fixed nitrogen, and vegetative cells supply fixed carbon to the heterocysts (Popa *et al.*, 2007). Transport of metabolites between cells along each filament appears to involve both cytoplasmic connections between adjacent cells and possibly a continuous periplasm (Flores & Herrero, 2010; Mariscal *et al.*, 2007; Mullineaux *et al.*, 2008; Zhang *et al.*, 2008).

The early stages of heterocyst differentiation require increased levels of the regulators NtcA and HetR, which

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Abbreviation: DIC, differential interference contrast [microscopy].

are mutually dependent on each other for upregulation (Muro-Pastor *et al.*, 2002). NtcA is an autoregulatory transcription factor that belongs to the cAMP receptor protein (CRP) family (Herrero *et al.*, 2004). HetR has DNA-binding activity that is inhibited *in vitro* by a synthetic pentapeptide corresponding to the C-terminal region of the PatS product (Huang *et al.*, 2004). As cells undergo heterocyst differentiation they reach a point at which the regulation becomes irreversible. For *Anabaena* PCC 7120, proheterocysts become committed to complete differentiation between 9 and 13 h after nitrogen step-down (Yoon & Golden, 2001).

During maturation, a polysaccharide layer is deposited around the proheterocyst followed by the deposition of a glycolipid layer between the outer membrane and the polysaccharide layer. Synthesis and deposition of the heterocyst envelope polysaccharide (Hep) layer requires a cluster of genes that includes *hepA* and *hepC*, and other genes outside of the cluster including *hepB*, *alr3699* and *all4160*; expression of these genes is upregulated early during heterocyst differentiation 5–8 h after induction (Huang *et al.*, 2005; Wang *et al.*, 2007). Several genes with regulatory roles have been shown to be required for regulation of the *hep* genes, including *hepK* and *devR*, which form a two-component system: *henR* (response regulator), *hepN* (histidine kinase) and *hepS* (serine/threonine kinase) (Lechno-Yossef *et al.*, 2006).

Synthesis of the heterocyst glycolipid (Hgl) layer requires a cluster of *hgl* genes, and *devBCA* and *hglK*, which are required for transport and assembly (Awai *et al.*, 2009; Fan *et al.*, 2005; Muro-Pastor *et al.*, 2009). Mutations that compromise the integrity of the polysaccharide or the glycolipid layer cause a *Fox⁻* phenotype characterized by the inability of the heterocysts to perform nitrogen fixation in the presence of oxygen.

Fan *et al.* (2006) previously identified gene *all0187*, the subject of this paper, in a transposon mutagenesis screen for mutants with a *Fox⁻* phenotype. They named the gene *conR* for constriction regulator; however, the annotation as a transcriptional regulator was erroneous (see Results). The *conR* transposon mutant was unable to grow diazotrophically. Although the polysaccharide and glycolipid envelope layers were present in the mutant, the polar junctions connecting heterocysts to vegetative cells were incomplete or widely open, which they hypothesized would allow oxygen to enter the heterocysts and inactivate nitrogenase (Fan *et al.*, 2006).

Here, we report our independent identification of the *Anabaena* PCC 7120 *conR* gene and additional characterization of its expression and function.

METHODS

Strains and culture conditions. Strains and plasmids used in this study are listed in Table 1. *Anabaena* PCC 7120 and its derivatives were typically grown in 100 ml BG-11 or BG-11₀ (lacking sodium

nitrate) medium in 250 ml flasks at 30 °C with white-light illumination of approximately 75 µmol photons m⁻² s⁻¹ as described previously (Wu *et al.*, 2007). Plates contained 40 ml medium solidified with 1.5 % agar; for BG-11₀ plates, the agar was washed (Waterbury & Willey, 1988) to remove trace nitrogen compounds. For media containing ammonium, ammonium chloride (2.5 mM) and MOPS buffer (5 mM, pH 8.0) were added to BG-11₀. For *Anabaena* PCC 7120 strains containing shuttle or suicide plasmids grown on solid medium, antibiotics were used at the following concentrations: neomycin 25 µg ml⁻¹, or spectinomycin and streptomycin 2 µg ml⁻¹ each; for liquid medium these concentrations were reduced by half. *Escherichia coli* strains were grown in LB (Lennox L) medium containing appropriate antibiotics at 37 °C. *E. coli* strain DH10B was used for all cloning experiments.

Suicide and shuttle plasmids were transferred to *E. coli* donor strain AM1359 by electroporation and transferred to *Anabaena* PCC 7120 strains by conjugation using standard protocols (Elhai *et al.*, 1997) with minor modifications (Khudyakov & Golden, 2001). For cyanobacterial genetics experiments, the phenotype of several independent exconjugant clones was determined to check for consistency. To obtain segregated gene knockouts by single recombination with a suicide plasmid containing an internal fragment of the gene, at least six original exconjugant colonies were restreaked three times onto fresh BG-11 antibiotic plates and then grown in liquid BG-11 with antibiotic before inducing heterocyst development by nitrogen step-down.

Bioinformatics. Standard bioinformatics analyses were performed with web-based software including CyanoBase, UniProtKB and Pfam.

Plasmid constructions. Standard protocols were used for cloning and *E. coli* transformation. Total DNA from cyanobacterial strains was isolated as described previously (Khudyakov & Golden, 2001). DNA sequencing of plasmid inserts was performed by the Gene Technologies Laboratory (Texas A&M University) following the Big Dye sequencing protocol (Applied Biosystems). All plasmid constructions were verified by DNA sequence analysis.

All primers used in this study are listed in Table 2. The open reading frame *conR* was inactivated by single homologous recombination with suicide plasmid pAM3438, which contained an internal fragment of *conR* amplified by PCR using oligonucleotides AMO-903 and AMO-904. The amplified fragment was digested with *XhoI* and *SacI* and inserted into the corresponding sites of the suicide plasmid pRL277. Recombination of the suicide plasmid into the *conR* gene and the absence of wild-type copies of the gene were confirmed by PCR and Southern blot analysis for three exconjugants (data not shown).

Plasmid pAM3348 was constructed for overexpression of *conR* from the *Anabaena* PCC 7120 *rbcL* promoter. This plasmid contains the *conR* gene, including 37 bp upstream and 2 bp downstream of the ORF, on a PCR fragment generated with primers AMO-955, which contains an engineered *Clal* site, and AMO-956, which contains an engineered *Sall* site. The PCR product was digested with *Clal* and *Sall* and cloned into the same sites of pAM1824.

To construct a shuttle plasmid carrying a *P_{conR}-gfp* transcriptional fusion, a fragment extending from 402 bp upstream to 63 bp downstream of the *conR* translational start site was amplified by PCR with primers AMO-959 and AMO-960. The fragment was digested with *SmaI* and *XbaI* and cloned into pBluescript SK+ digested with *EcoRV* and *XbaI* to produce pAM3451. A *Sall-SacI* fragment containing the insert was excised from pAM3451 and ligated into the same sites of pAM1956, a shuttle plasmid containing the promoterless *gfpmut2* gene, producing pAM3450.

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristic	Reference/source
Anabaena strains		
PCC 7120	Wild-type <i>Anabaena</i> sp. strain PCC 7120	Robert Haselkorn, University of Chicago
AMC1369	PCC 7120 carrying suicide plasmid pAM3438 with an internal fragment of <i>conR</i> (all0187); Sm ^r Sp ^r	This study
AMC1370	PCC 7120 carrying shuttle plasmid pAM3450; Nm ^r	This study
Plasmids		
pAM504	Shuttle plasmid pAM502 without the Sp/Sm cartridge containing unique cloning sites <i>Xba</i> I, <i>Bam</i> HI, <i>Xma</i> I, <i>Kpn</i> I, <i>Sst</i> I and <i>Acc</i> I; Km ^r Nm ^r	Wei <i>et al.</i> (1994)
pRL277	Conjugal suicide plasmid; Sm ^r Sp ^r	Elhai <i>et al.</i> (1997)
pAM1011	Higher-copy-number version of pCCB110 shuttle plasmid; Km ^r Nm ^r	Wu <i>et al.</i> (2007)
pAM1824	Shuttle plasmid containing the <i>Anabaena</i> PCC 7120 <i>rbcl</i> promoter on an <i>Eco</i> RI– <i>Sal</i> I fragment from pAM496; Km ^r Nm ^r	Liu & Golden (2002)
pAM1956	Shuttle plasmid pAM505 containing the <i>gfpmut2</i> gene; Km ^r Nm ^r	Yoon & Golden (1998)
pAM2178	pARO180 with Omega Sp ^r Sm ^r cassette inserted into unique <i>Ssp</i> I site; Ap ^r Sp ^r Sm ^r	Khudyakov & Golden (2001)
pAM3438	pRL277 containing internal fragment of all0187; Sm ^r Sp ^r	This study
pAM3448	pAM1824 containing the entire all0187 ORF, including 76 bp upstream from the translational start site, under control of the <i>rbcl</i> promoter; Km ^r Nm ^r	This study
pAM3450	pAM1956 carrying a <i>Sal</i> I– <i>Sac</i> I fragment from pAM3351 containing the –472 bp to +86 region of all0187; Km ^r Nm ^r	This study
pAM3451	pBluescript KS+ containing a –472 to +86 bp region relative to the translation start site of all0187; Ap ^r	This study
pAM2981	pRL277 containing internal fragment of all3290; Sm ^r Sp ^r	This study
pAM4403	pRL277 containing internal fragment of alr0295; Sm ^r Sp ^r	This study
pAM4404	pRL277 containing internal fragment of alr4884; Sm ^r Sp ^r	This study
pAM4405	pRL277 containing internal fragment of alr0996; Sm ^r Sp ^r	This study
pAM4406	pRL277 containing internal fragment of all1738; Sm ^r Sp ^r	This study
pAM4407	pRL277 containing internal fragment of all3169; Sm ^r Sp ^r	This study
pAM4408	pRL277 containing internal fragment of alr4808; Sm ^r Sp ^r	This study
pAM4409	pRL277 containing internal fragment of alr0141; Sm ^r Sp ^r	This study
pAM4410	pRL277 containing internal fragment of alr4738; Sm ^r Sp ^r	This study
pAM4411	pRL2178 containing internal fragment of alr3871; Ap ^r Sm ^r Sp ^r	This study
pAM3986	pRL2178 containing internal fragment of all1682; Ap ^r Sm ^r Sp ^r	This study
pAM3988	pRL2178 containing internal fragment of all4160; Ap ^r Sm ^r Sp ^r	This study
pAM4062	pRL2178 containing internal fragment of all3924; Ap ^r Sm ^r Sp ^r	This study
pAM4065	pRL2178 containing internal fragment of all1688; Ap ^r Sm ^r Sp ^r	This study

RNA isolation. Total RNA was extracted using the RiboPure-Bacteria kit (Ambion) according to the manufacturer's instructions. For synchronous induction of heterocyst development by nitrogen step-down, *Anabaena* PCC 7120 filaments from a dark-green BG-11 culture were inoculated into 100 ml BG-11₀ supplemented with 2.5 mM ammonium chloride and 5 mM MOPS (pH 8.0) to obtain a starting OD₇₅₀ of 0.025–0.035. The cells were grown overnight until OD₇₅₀ reached 0.05–0.075, after which they were collected by centrifugation, washed twice with sterile water and then transferred to 100 ml BG-11₀. At 0, 4, 8, 12, 16, 20 and 24 h after nitrogen deprivation, 100 ml cultures were poured into a conical 250 ml centrifuge tube containing 100 g ice to rapidly stop mRNA synthesis and degradation, and the filaments were collected by centrifugation at 5000 g for 10 min at 4 °C. The cell pellet was immediately frozen at –80 °C for storage until RNA extraction was performed.

Northern RNA blot analysis. Five micrograms of total RNA for each sample was denatured and run on a 1% agarose denaturing formaldehyde gel in MOPS buffer and transferred to a MAGNACHARGE nylon membrane (GE Water & Process Technologies) with 10 × SSPE (Golden *et al.*, 1991; Sambrook & Russell,

2006). Blots were hybridized with radioactively labelled DNA probes. Probe fragments were amplified by PCR using the corresponding primers (Table 2), labelled with [³²P]-dCTP by random primer labelling, and purified on Micro Bio-Spin P-30 columns (Bio-Rad). The hybridization solution contained 5 × SSPE, 50% formamide, 0.5% SDS and 5 × Denhardt's solution, and hybridization was performed overnight at 42 °C. Blots were washed twice for 5 min with 2 × SSC/0.1% SDS at room temperature, once for 10 min with 1 × SSC/0.1% SDS at 65 °C, and once for 10 min with 0.5 × SSC/0.1% SDS at 65 °C. Blots were exposed on a phosphorimager plate and scanned with a Phosphorimager BAS-5000 (Fujifilm).

Microarray experiments. Labelled *Anabaena* PCC 7120 RNA samples were hybridized to a custom microarray slide manufactured by Integrated Genomics. Each slide contained probes for 768 ORFs from the *Anabaena* PCC 7120 genome, including heterocyst-related genes, regulatory genes, photosynthesis genes, clock genes and selected additional genes. The array included six duplicates of each gene, 96 negative controls (repeated six times each) and 11 positive controls (repeated 48–196 times). In total, each slide contained 5760 spots; 50 probes were 70-base oligonucleotides and the remainder

Table 2. Primers used in this study

Gene	Forward primer		Reverse primer	
	Name	Sequence (5'→3')	Name	Sequence (5'→3')
all0187-internal	AMO-903	CTCGAGTGTTCCTCCGTAACGTGGTTG	AMO-904	GAGCTCAAACCTCACCTAGAGCGACG
all0187-ORF	AMO-955	GCTTGATCGATCATAAATTTAGGGAG	AMO-956	CCACTGTCGACTAACTATTTCCAATC
<i>P_{conR}-gfp</i>	AMO-959	GGCCCCGGGCGTGCTTCTACCCTG	AMO-960	CACCTCTAGATTGAGATCCGGTATTG
all0185	AMO-1032	CCCCAGATTTTCTTGGCAG	AMO-1033	CATGGGCTGCATTGACAC
all0186	AMO-1005	GTTATGCTGGCGTGG	AMO-1006	CAATACCCGTAACCTC
alr0188	AMO-1007	GACTGGTTAAGTTCG	AMO-1008	GACTGGTTAAGTTCG
<i>rnpB</i>	AMO-489	GTACTGCGGGTAACTAAAG	AMO-490	AATGACTATTGACTAAAAG
<i>nifH</i> (all1445)	AMO-622	TTCACGGTCAACCTTACGG	AMO-1038	CGGTAAAGGCGG
<i>hcrA</i> (alr0093)	AMO-1549	CAGTGCTGCACCCGG	AMO-1550	GTTCTGGTTGGGGT
all3290	AMO-659	AACCAACATATG GTGATTCTGCTTGTAAACC	AMO-660	AACCAACCCGGGTCATGGCTTGACTACCTCC
alr0295	AMO-886	CAAGGCTCGAGAGAACTGCTGTAG	AMO-887	GACCTGAGCTCAAAAACAGCAGAGTATC
alr4882	AMO-888	CTTCAGCTCGAGCAGGATAAAATGTCTGC	AMO-889	ACAATAGAGCTCTGAGCGATCGCTAACCC
alr0996	AMO-892	GACCTCGAGCAAAGAAATTGCTGGTGGTAATG	AMO-893	GTTGAGCTCAGAGTCTGTTTTGGGTGTAG
all1738	AMO-895	GTAACCAAGTATGG	AMO-896	GAATTCGACCACCAATTACTAC
all3169	AMO-897	AAGCTTGGGGGTGACCTGCATCC	AMO-898	TCTGAATTCATGCGCTGGCTAGAATC
alr4808	AMO-899	GACTTGTGAAACCATTCG	AMO-900	CCGAATTCAAACCAACCCACAG
alr0141	AMO-901	TAGAAGCTTCTTTGACAAGTCGGGG	AMO-902	ACAGAATTCACAGAGCGAAGAGC
alr4738	AMO-927	GTGACTCGAGGTATGAGTTATTGC	AMO-928	CACCGAGCTCTTGTGCGCCCAAGCC
alr3871	AMO-937	AGAGAGCTCCAGTTGGCACACTTG	AMO-938	ACAAGGATCCGGCTGTAGTTGAAAGG
all1682	AMO-1794	CAGAAGCTTGTACAAATACTAGTG	AMO-1795	TTCCAATCCCCAATATAAAACAAC
all4160	AMO-1800	TGAAAGCTTGAATGTCACTCCCC	AMO-1801	ACTGAGCTCCTGCCATTCACTTGC
all3924	AMO-1802	TAAGTCTAGAGCTTACGACTTGGGGC	AMO-1803	GCGAGAATTCACCAATTACTGTTAG
all1688	AMO-1820	CTCTCGTCGACCAGATTTCCCAAGAG	AMO-1821	ATACGAATTCCTGTATCTTCTACTTG

were PCR products. Oligonucleotides and PCR products were UV cross-linked onto Corning GAPS II coated slides (Invitrogen). Each spot had a diameter of approximately 150 µm, and spots were aligned in an area of 1.5 × 2.8 cm.

For hybridization, total RNA was converted to an amino-allyl single-stranded cDNA and post-labelled with Cy5 or Cy3 fluorescent dyes (Amersham Biosciences) using the Superscript-II reverse transcriptase system and random primers (Invitrogen). Slides were pre-hybridized in 25 % formamide, 5 × SSC, 0.1 % SDS and 2 % BSA for 45 min at 42 °C, then rinsed twice in distilled water and dried by centrifugation at 500 g for 3 min. Hybridization was performed in a dark humid chamber at 42 °C for 16 h using a 4 × hybridization solution (Amersham Biosciences). Slides were then washed with a pre-warmed solution of 2 × SSC/0.2 % SDS at 55 °C followed by 1 × SSC/0.2 % SDS at 37 °C and finally with 0.1 × SSC at room temperature for 10 min. Slides were then air-dried and subsequently scanned using an Affymetrix 428 Array scanner. Array images were generated in GPX and TIFF formats using GenePix-Pro software.

Normalization and statistical analysis of the raw microarray data were performed with GeneSpring Analysis Platform version 3.0 (Silicon Genetics) software. The median for each gene was determined from the median value for each replica spot, and each dataset for different RNA samples were normalized against the *rnpB* gene from cells grown on ammonium. Genes were clustered into groups that were upregulated early (0–4 h), middle (8–12 h) and late (12–24 h) during heterocyst development. The middle genes were further narrowed to those that clustered with 90 % correlation to *hetC* values to identify a set of genes that we would further analyse genetically.

Electron microscopy. Cultures were prepared for electron microscopy by collecting cyanobacterial filaments by centrifugation and resuspending the pellet in 2 × BG-11, 2.5 % acrolin, 2.5 % glutaraldehyde and

0.1 % DMSO. Cells were fixed in a microwave for 2 min at 21 °C and 23 mmHg. The cells were washed three times in BG-11 medium and post-fixed in 3 % K₃F(CN)₆ overnight at 4 °C. Fixed material was washed with distilled water four times at room temperature. Samples were embedded in 1 % SeaKem low-gelling-temperature agarose. The samples were then dehydrated through a graded methanol series (two changes of 5 min each in 10, 20, 30, 40, 50, 60, 70, 80 and 90 % methanol, and three changes of 10 min each in 100 % methanol) and two 10 min incubations in propylene oxide. The samples were infiltrated with a 1:1 mixture of propylene oxide-Epon 812 for 4 h and then with a 1:3 mixture of propylene oxide-Epon 812 overnight with gentle agitation. The samples were incubated in Epon 812 under a vacuum at room temperature for 4 h and then embedded in BEEM capsules with fresh degassed Epon 812 overnight at 55 °C. Thin sections were collected on Parlodion-carbon-coated copper grids and stained with 3 % uranyl acetate for 30 min in a moist chamber. The sections were examined with a Siemens 101 electron microscope operated at 80 kV.

Light and fluorescence microscopy. Differential interference contrast (DIC) and fluorescence microscopy were performed on two microscope systems: a Zeiss Axioplan II microscope with a 40 × objective and GFP-specific emission (518 ± 13 nm) filter sets and a Hamamatsu 3 charge-coupled device (CCD) camera (C5810); and an Olympus IX70 inverted microscope with a 100 × objective and Hamamatsu OrcaER C4742-95 CCD camera and Simple PCI software version 6.0 (Compix). A Piston GFP band-pass filter set (no. 41025; Chroma Technology) was used for fluorescence images with the IX70 to minimize background from autofluorescence. The images were minimally processed to improve contrast and brightness with Adobe Photoshop CS version 8.0.

Acetylene reduction assay. Wild-type *Anabaena* PCC 7120 and the *conR* mutant strain AMC1369 were first grown in 100 ml BG-11 in

250 ml flasks under standard conditions to OD₇₅₀ 0.4. The cells were collected by centrifugation in 250 ml conical bottles at room temperature, washed twice with 50 ml water, resuspended in 3 ml BG-11₀ and transferred to 10 ml tubes that were sealed with a rubber serum stopper. One millilitre of air was replaced with acetylene, and the tube was mixed by inversion for 1 min. The cells were incubated at 30 °C with standard illumination, and 0.1 ml samples of gas were removed at 0, 8, 12, 16 and 24 h for analysis by gas chromatography with a 6-ft Porapak N column at 50 °C.

RESULTS

Identification and bioinformatics analysis of *conR*

To identify genes that are potentially involved in the early stages of heterocyst development in *Anabaena* PCC 7120, we performed DNA microarray transcription profiling experiments of the wild-type to examine gene expression in the presence of ammonium and nitrate, and at different times after nitrogen step-down. From these data, we identified a set of genes that showed a pattern of expression similar to *hetC* (data not shown). The *hetC* gene is predicted to encode a bacterial ABC protein exporter and is upregulated 5–9 h after the onset of heterocyst differentiation (Khudiyakov & Wolk, 1997).

Fifteen candidate genes that were regulated similarly to *hetC* were inactivated to assess their role in heterocyst development. The following loci were inactivated by single recombination with suicide plasmids that each contained an internal fragment of the target gene: *alr0141*, *all0187*, *alr0295*, *alr0996*, *all1682*, *all1688*, *all1738*, *all3169*, *all3290*, *alr3871*, *all3924*, *all4160*, *alr4738*, *alr4808* and *alr4882*. For the *alr0141* and *all3169* loci, no exconjugants were obtained. For the remaining loci, exconjugants were obtained and restreaked to allow segregation of mutant chromosomes, which is required because cyanobacteria contain multiple chromosome copies per cell (Hu *et al.*, 2007). With the exception of *all0187*, none of the other mutants exhibited a diazotrophic growth defect or obvious defects in heterocyst differentiation or pattern formation.

Inactivation of *all0187* (Fig. 1) caused defective diazotrophic growth, among other defects described below. Concurrent with our work, a transposon mutant of *all0187* was briefly described by Fan *et al.* (2006) who named the gene *conR*.

The *conR* gene product is predicted to contain an N-terminal transmembrane region (residues 83–105) but lacks a predicted signal sequence. ConR is predicted to contain a LytR-CpsA-Psr domain (residues 174–323), which is found in a group of proteins that are involved in septation and cell-wall maintenance (Chatfield *et al.*, 2005; Hübscher *et al.*, 2008; Johnsborg & Havarstein, 2009). These proteins have a short N-terminal cytoplasmic domain and a transmembrane domain that together form a signal anchor which can be inserted into the cell membrane (Koskiniemi *et al.*, 1998). The biochemical function of the LytR-CpsA-Psr domain is not yet known. The

Fig. 1. Map of *conR* (*all0187*) on the *Anabaena* PCC 7120 chromosome and the DNA fragments used in this study for genetic experiments. Suicide plasmid pAM3438 contains an internal fragment of *conR* (knockout) and was used to inactivate *conR* by single homologous recombination into the chromosome. Plasmid pAM3448 contains the entire *conR* ORF (overexpression) under control of the *rbcL* promoter and was used to overexpress *conR*. Plasmid pAM3450 contains the upstream region of *conR* (GFP-fusion) fused to the *gfpmut2* reporter gene. Numbering is relative to the first nucleotide of the *conR* ORF.

LytR-CpsA-Psr domain has been confused with the LytTR domain (previously also named LytR) that is found in a group of transcriptional regulators predicted to have DNA-binding properties (Nikolskaya & Galperin, 2002). This confusion in gene names has led to the misannotation of the *conR* gene and its homologues in genomics databases, and, consequently by Fan *et al.* (2006), as being a transcriptional regulator. Our bioinformatics analysis of the *Anabaena* PCC 7120 genome identified two genes predicted to contain a LytR-CpsA-Psr domain, *conR* (*all0187*) and *alr2479*, and both genes are incorrectly annotated in public databases as transcriptional regulators.

Characterization of a *conR* knockout mutant

The *conR* gene was insertionally inactivated by single homologous recombination with a suicide plasmid that contained an internal fragment of the gene (Fig. 1). Southern blot and PCR analysis (data not shown) showed that no copies of the wild-type gene could be detected in the mutant strain grown under antibiotic selection. The phenotype of the resulting mutant strain, AMC1369, was analysed in medium with or without a source of combined nitrogen. In medium containing nitrate, the mutant cells grew noticeably slower than the wild-type grown under the same conditions.

Vegetative cells of the mutant strain appeared smaller and highly granular, and were partially defective for septum formation and cell division and, consequently, some cells appeared longer than normal (Fig. 2e and i). In medium without a source of combined nitrogen, the AMC1369 mutant filaments were able to form heterocysts with a fairly normal pattern along the filament, but were unable to grow diazotrophically (Fig. 2f). AMC1369 filaments transferred

from BG-11 to BG-11₀ liquid media yellowed and died after 2–3 days. The vegetative cells had a somewhat more normal appearance after nitrogen step-down but still showed partially defective septum formation (Fig. 2j).

Time-lapse microscopy of mutant filaments after nitrogen step-down (see Supplementary Movie S1, available with the online version of this paper) revealed that differentiating cells elongated and often appeared to have defective septum formation with the neighbouring vegetative cell(s), resulting in abnormally long heterocysts that appeared partially open at their ends (Fig. 2d and h). Differentiating cells exceeded normal length (and continued to elongate) at around 9 h after nitrogen step-down, a time when proheterocysts are not yet clearly morphologically distinguishable in the wild-type strain. After nitrogen step-down, if vegetative cells did not undergo division prior to differentiating into a heterocyst, then the septum formation defect was not obvious. After 24 h, only a few regions of the filaments continued to grow; presumably, those regions had heterocysts that had produced functional junctions with their neighbouring vegetative cells.

The mutant phenotype described above was observed in several independent exconjugant clones. The *conR* mutant strain AMC1369 was complemented with shuttle plasmid pAM3448, which carries the *conR* gene expressed from the

rbcl promoter. The complemented strain had a wild-type phenotype, which shows that the inactivation of *conR* was responsible for all of the observed phenotypes. Overexpression of *conR* from pAM3448 in a wild-type background did not produce an abnormal phenotype in either nitrogen-containing or nitrogen-free medium (data not shown).

The *conR* mutant strain was unable to grow diazotrophically even though heterocysts were present. Therefore, we used electron microscopy to determine whether the heterocyst envelope layers were absent or altered (Fig. 2d and g). The electron micrographs showed that the glycolipid, polysaccharide, peptidoglycan and outer membrane layers of the heterocyst envelope in AMC1369 were all present, but that both vegetative cells and heterocysts often contained round inclusions of low electron density in the cytoplasm that were not present in the wild-type. These inclusions are presumably poly- β -hydroxybutyrate granules that accumulate because the filaments are limited for fixed nitrogen (Wu *et al.*, 2001). Thin layer chromatography experiments showed that heterocyst glycolipids were present in the mutant strain (data not shown). Fan *et al.* (2006) had previously observed that a mutant strain with a transposon insertion in the *all0187* locus could not grow diazotrophically but contained heterocyst glycolipids similar to the wild-type. They also showed that the junctions between vegetative cells and between vegetative

Fig. 2. *conR* mutant phenotype. The strain AMC1369 is defective for both normal vegetative cell division and heterocyst morphogenesis. *Anabaena* PCC 7120 wild-type (a–c) and *conR* mutant AMC1369 (d–j) strains grown in nitrate-containing BG-11 medium (a, e) or nitrate-free BG-11₀ medium (b–d, f–j). Bars, 10 μ m in the DIC micrograph (a) and 500 nm in the electron micrograph (c). Light micrographs (a, b, e and f) are all the same magnification; and electron micrographs (c, d and g) are all the same magnification. (h, j) Boxed regions in (f). (i) Boxed region in (e). Arrows indicate abnormal cell poles that appear open.

cells and heterocysts were often partially defective (Fan *et al.*, 2006).

Expression of *conR*

To validate the microarray data (not shown) for *conR* and confirm that expression of *conR* increases after nitrogen step-down, we performed a Northern RNA blot analysis on independently isolated RNA samples. Total RNA was extracted from wild-type *Anabaena* PCC 7120 and from the *conR* mutant strain AMC1369 at 0, 4, 8, 12 and 24 h after nitrogen step-down. Fig. 3 shows that *conR* transcripts could be weakly detected in uninduced filaments and increased from this basal level by 8 h after nitrogen step-down, and continued to increase until 12 h, but then returned to lower levels by 24 h. The Northern blot showed

two relatively stable transcripts at 1.5 and 3.8 kb, which would correspond to, respectively, *conR* alone and to transcripts containing *conR*, all0186 and all0185. RNA-seq data from total RNA samples obtained at 0, 6, 12 and 21 h after nitrogen step-down were consistent with the microarray and Northern blot data, with RPKM [reads per kilobase of CDS (coding sequence) model per million mapped reads] values of 11.19, 9.64, 64.29, and 32.11, respectively (B. Flaherty, unpublished data). The RNA-seq data indicated a major 5' end for *conR* transcripts at −103 nt upstream of the first base of the ORF.

RNA-seq RPKM data for the other *Anabaena* PCC 7120 *LytR-CpsA-Psr-domain* gene, *alr2479*, showed RPKM values of 5.15, 4.22, 40.15 and 14.52, respectively. This expression pattern is similar to that of *conR* and suggests that *conR* and *alr2479* might be involved in similar processes.

To determine if the increase in expression of *conR* takes place in differentiating cells, we constructed a P_{conR} -*gfp* reporter (Fig. 4). Time-lapse microscopy of the wild-type carrying the P_{conR} -*gfp* reporter on shuttle plasmid pAM3450 showed that after nitrogen step-down, GFP fluorescence increased in differentiating cells, but only after about 18 h, and there was only a modest difference between vegetative cells and heterocysts. GFP fluorescence began to decrease in heterocysts after about 28 h. GFP fluorescence from vegetative cells also increased following nitrogen step-down but remained at a lower level compared with heterocysts (Fig. 4).

Nitrogen fixation in the *conR* mutant

Because the *conR* mutant strain failed to grow diazotrophically, even though it had an apparently normal heterocyst envelope, we determined if the *nifH* gene was expressed at normal levels. Previous studies have shown that the *nifHDK* operon is strongly upregulated 12–24 h after heterocyst induction in the wild-type, depending on incubation conditions (Borthakur *et al.*, 1990; Golden *et al.*, 1991; Wei *et al.*, 1994). Under our conditions, *nifH* expression in the wild-type began to increase by 12 h after nitrogen step-down (Fig. 3). In the *conR* mutant strain, *nifH* expression was nearly equivalent to the wild-type at 24 h after nitrogen step-down, but the expression was delayed such that no expression was evident at 12 h. In these experiments, the timing of heterocyst morphogenesis assessed by light microscopy was similar to the wild-type and did not show any delay.

Because we observed significant expression of the *nifH* gene, acetylene reduction assays were used to determine if nitrogenase was active in the *conR* mutant strain. Even though the *conR* mutant cannot grow diazotrophically, nitrogenase activity in a 24 h period after nitrogen step-down was 70 % of the level produced by the wild-type. These results suggest that the diazotrophic growth defect is not due to the inability of the heterocysts to express the

Fig. 3. Northern RNA blot analysis of *conR* and *nifH* genes in wild-type PCC 7120 and the *conR* mutant strain AMC1369 during heterocyst development. Lanes are labelled with the time after nitrogen step-down (h). The lower panel is the ethidium bromide-stained gel to show sample loading. The upper and middle panels show Northern RNA blots probed with radioactive probes for *conR* or *nifH* genes, respectively. For *conR*, 3.8 and 1.5 kb transcripts were detected, and for *nifH*, three transcripts were detected at 4.7, 2.8 and 1.1 kb.

Fig. 4. A P_{conR} -*gfp* reporter strain (AMC1370) showed increasing GFP fluorescence in differentiating cells after nitrogen step-down. DIC images of AMC1370 filaments (left) and corresponding GFP fluorescence images (right) of filaments 0, 12 and 22 h after nitrogen step-down. Bar, 10 μ m.

nitrogen fixation genes or to produce functional nitrogenase, but is instead due to an inability to supply fixed nitrogen to the neighbouring vegetative cells.

DISCUSSION

DNA microarray transcript profiling data showed that the *Anabaena* PCC 7120 *conR* (all0187) gene was expressed similarly to genes involved in the early stages of heterocyst development. In work done concurrently with our initial identification of *conR*, a *conR* mutant was characterized by Fan and colleagues who had identified it in a transposon mutagenesis screen for mutants defective for diazotrophic growth (Fan *et al.*, 2006). Our microarray experiments showed that the expression of *conR* was upregulated 8 h after nitrogen step-down. A heterocyst induction time-course with a P_{conR} -*gfp* reporter strain showed upregulated expression at this same time and showed moderately higher expression in differentiating cells. The *conR* expression data suggest that the ConR product is produced in higher amounts after nitrogen step-down in both vegetative cells and differentiating heterocysts.

The *conR* gene was inactivated by single homologous recombination with an internal fragment carried on a suicide vector, and similar to the transposon mutant identified by Fan *et al.* (2006), the mutant could not grow diazotrophically and produced heterocysts with abnormal

morphology and defective junctions with vegetative cells. Filaments of the *conR* mutant strain AMC1369 grown in nitrate-containing media also showed defects in cell division and septum formation of vegetative cells. After nitrogen step-down, the defect in septum formation between vegetative cells seemed less severe than for filaments grown with nitrate, which could be an indirect consequence of a general stress response or due to the slightly slowed growth caused by the transition to diazotrophic growth.

A bioinformatics screen for potential NtcA-regulated genes identified a putative NtcA binding site at position -60 relative to the start codon of *conR* (Su *et al.*, 2005), which could be consistent with the upregulation of *conR* that occurs in differentiating heterocysts. However, RNA-seq data showed a major stable 5' end at -103 ; if this is a transcription start site, then that would place the *conR* promoter within the upstream divergent ORF *alr0188* and would indicate that the computationally identified NtcA binding site is not involved in the regulation of *conR*.

The *conR* gene is predicted to encode a protein containing an N-terminal transmembrane domain and a periplasmic LytR-CpsA-Psr domain. In *Bacillus subtilis*, a *lytR* mutant shows upregulation of the *lytABC* operon, which is involved in synthesis and maintenance of the cell wall; LytC is an *N*-acetyl-muramoyl-L-alanine amidase (autolysin) involved in cleaving peptidoglycan (Lazarevic *et al.*, 1992). Disruption of the *lytR* gene in *Streptococcus pneumoniae* results in misplacement of the septum (Johnsborg & Havarstein, 2009). In many bacteria, the peptidoglycan cell wall is cleaved by multiple hydrolases, including amidases, which remove peptide side chains from the carbohydrate polymer. Peptidoglycan hydrolases, autolysins, are thought to be essential for separating daughter cells after cell division is concluded, and their absence causes incomplete septal invagination (Priyadarshini *et al.*, 2007). Based on the *conR* mutant phenotype, it is possible that the set of peptidoglycan hydrolases involved in septation between vegetative cells, or their regulation, are at least partially different from those involved in septation between differentiating heterocysts and vegetative cells. Autolysins may also be required for modification of the heterocyst peptidoglycan to facilitate transport and assembly of the outer envelope layers, and autolysin activity and hydrostatic pressure may explain the often larger and rounder shape of heterocysts.

In *Anabaena* PCC 7120, the autolysin gene *hcwA* is required for normal heterocyst maturation (Zhu *et al.*, 2001). We hypothesized that the abnormal cell division of the *conR* mutant might be caused by altered expression of *hcwA*. However, we did not detect a change in *hcwA* mRNA levels in the *conR* mutant (data not shown). Alternatively, the enzymic activity or localization of HcwA or other autolysins may be altered in the *conR* mutant.

In many cases, the inability of an *Anabaena* PCC 7120 mutant to grow diazotrophically may be caused by a defect

that compromises the integrity of the envelope layers, allowing more oxygen to enter the heterocysts and inactivate nitrogenase. However, the *conR* mutant formed heterocysts with apparently normal polysaccharide and glycolipid layers. We asked if the incomplete constriction of the septum between heterocyst and vegetative cells might expose the intracellular environment of the heterocysts to oxygen and, thus, cause a decrease in the expression or activity of nitrogenase. Northern blot analysis showed that *nifH* expression in the *conR* mutant was upregulated after nitrogen step-down, but with a substantial delay compared with the wild-type. Although morphological heterocyst development observed by light microscopy was not delayed in the mutant strain, the delay in *nifH* expression may have been caused by delayed establishment of a micro-oxic environment due to delayed or partially defective septum formation or deposition of the envelope layers.

Unexpectedly, the *conR* mutant retained nitrogenase activity at 70 % of wild-type levels, and this amount of activity should be sufficient to sustain diazotrophic growth (Valladares *et al.*, 2007), although possibly at a slower rate. Inactivation of the heterocyst-specific gene *fdxH* reduced nitrogenase activity to less than 50 %, but still allowed slow diazotrophic growth (Masepohl *et al.*, 1997). However, *conR* mutant filaments completely stopped growing after a few days in medium without a source of combined nitrogen, and the cells yellowed and died. Therefore, the vegetative cells must not be receiving nitrogen even though at least some of the heterocysts of the *conR* mutant are capable of creating a micro-oxic environment that supports significant nitrogenase activity. These data show that the diazotrophic growth defect is not caused simply by defective nitrogen fixation, but may involve defective delivery of fixed nitrogen from heterocysts to neighbouring vegetative cells. Recent findings show that there is cytoplasm-to-cytoplasm movement of the small fluorophore calcein in *Anabaena* PCC 7120, which indicates the existence of non-specific intercellular channels that allow metabolite exchange between cells of the filament (Mullineaux *et al.*, 2008). In the *conR* mutant, defective septum formation and closure could interfere with the formation of these channels at the junction between heterocysts and vegetative cells and, thus, prevent normal metabolite exchange.

ACKNOWLEDGEMENTS

We thank Britt Flaherty for her unpublished RNA-seq data. This work was supported by National Science Foundation Grant 0925126.

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Edited by: C.-C. Zhang