The \( pknH \) gene restrictively expressed in heterocysts is required for diazotrophic growth in the cyanobacterium \( \textit{Anabaena} \) sp. strain PCC 7120

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\( \textit{Anabaena} \) sp. strain PCC 7120 is a filamentous cyanobacterium in which certain vegetative cells differentiate into heterocysts, which are specialized cells for nitrogen fixation. Heterocysts are unable to carry out photosynthesis and are supplied with carbohydrate required for nitrogen fixation from neighbouring vegetative cells. Thus, filament integrity is very important for diazotrophic growth of the heterocystous cyanobacteria. The \( pknH \) gene (\( alr1336 \)), encoding a putative Ser/Thr protein kinase, was upregulated in heterocysts after nitrogen deprivation. Its expression was developmentally regulated by the \( \textit{hetR} \) gene. Expression levels of genes involved in heterocyst maturation, such as \( \textit{hepA} \), \( \textit{hglE} \) and \( \textit{nifH} \), in the \( pknH \) disruptant were similar to those of the wild-type strain. The disruptant was able to form heterocysts with nitrogenase activity, but most heterocysts were detached from filaments. Hence, the \( pknH \) disruptant showed a growth defect in the medium without combined nitrogen. It is concluded that the \( pknH \) gene is not involved in the development of heterocyst function but is involved in maintaining connections between heterocysts and vegetative cells.

INTRODUCTION

Cyanobacteria are a large group of eubacteria characterized by oxygen-evolving photosynthesis. Heterocysts are terminally differentiated cells of filamentous cyanobacteria specialized for nitrogen fixation. Upon limitation of combined nitrogen in the medium, particular vegetative cells differentiate into heterocysts with a regular spacing of 10–15 cells (Flores & Herrero, 2010; Kumar \textit{et al.}, 2010). Heterocysts provide the microoxic environment for the oxygen-labile nitrogenase complex, the enzyme responsible for nitrogen fixation (Wolk \textit{et al.}, 1994). In heterocysts, \( \text{O}_2 \)-evolving photosystem II is inactivated, respiration is enhanced, and a thick envelope consisting of layers of polysaccharide and glycolipid that limits oxygen diffusion into cells is formed outside the cell wall. Because heterocysts are unable to fix \( \text{CO}_2 \) photosynthetically, vegetative cells supply carbohydrate to heterocysts and, in return, receive nitrogen fixation products from heterocysts. Thus, vegetative cells and heterocysts depend on each other and filament integrity is important for diazotrophic growth.

In the filamentous cyanobacterium \( \textit{Anabaena} \) (\( \textit{Nostoc} \)) sp. strain PCC 7120 (hereafter \( \textit{Anabaena} \) PCC 7120), approximately 10 % of chromosomal genes are upregulated with spatiotemporal regulation in the process of heterocyst development (Ehira \textit{et al.}, 2003; Ehira & Ohmori, 2006a). Although little is known of the regulation of gene expression during heterocyst development, the regulatory network of the early stages of heterocyst development is known in outline. The developmental process is initiated by an increase in the level of 2-oxoglutarate (Laurent \textit{et al.}, 2005). 2-Oxoglutarate binds to NtcA (Zhao \textit{et al.}, 2010), a transcriptional regulator that globally controls nitrogen metabolism in cyanobacteria (Herrero \textit{et al.}, 2004). NtcA activates transcription of the \( \textit{nrrA} \) gene, whose product in turn activates \( \textit{hetR} \) expression (Ehira & Ohmori, 2006a, b; Muro-Pastor \textit{et al.}, 2006). HetR is a master regulator of heterocyst development, and plays a key role in differentiation and pattern formation (Buikema & Haselkorn, 1991; Buikema & Haselkorn, 2001). HetR is thought to activate expression of many genes involved in heterocyst development (Higa & Callahan, 2010; Huang \textit{et al.}, 2004; Saha & Golden, 2011).

In addition to transcriptional regulation, the importance of post-translational regulation in heterocyst development has

Abbreviations: qRT-PCR, real-time quantitative RT-PCR; WT, wild-type.

A supplementary table, showing primers used in this study, is available with the online version of this paper.
been recognized in the last decade. The genome of *Anabaena* PCC 7120 contains a large number of genes that encode protein kinases and phosphatases (Ohmori et al., 2001; Wang et al., 2002). Some of these genes are involved in heterocyst development, mostly in heterocyst maturation and function. The *pknE* gene, encoding a Ser/Thr protein kinase, is upregulated by nitrogen deprivation (Ehira & Ohmori, 2006a). A *pknE* inactivation mutant shows a defect in diazotrophic growth (Zhang et al., 1998), and over-expression of *pknE* blocks heterocyst differentiation (Saha & Golden, 2011). A Ser/Thr protein kinase, HepS, is involved in the synthesis of heterocyst envelope polysaccharide (Fan et al., 2006). Two protein kinases, Pkn30 and Pkn44, of the HstK family, which contain a putative Ser/Thr-kinase domain at their N termini and a His-kinase domain at their C termini, are involved in the synthesis of the minor heterocyst-specific glycolipid (Shi et al., 2007). In the present study, we have found that the *pknH* gene (*alr1336*), encoding a putative Ser/Thr protein kinase, is restrictively expressed in heterocysts and is required for diazotrophic growth.

**METHODS**

**Bacterial strains and culture conditions.** *Anabaena* sp. strain PCC 7120 and its derivatives were grown in the BG-11 medium (containing NaNO₃ as nitrogen source), as described previously (Ehira & Ohmori, 2006a). Liquid cultures were bubbled with air containing 1.0 % (v/v) CO₂. For nitrogen-deprivation experiments, cells grown in BG-11 medium until they reached OD₇₅₀ 0.4–0.5 were washed with nitrogen-free medium (BG-11₀) and then resuspended in BG-1₁₀ medium. Spectinomycin was added to the medium at a final concentration of 10 μg·ml⁻¹ when required.

**Mutant and plasmid construction.** All primers listed in Table S1, available with the online version of this paper, were designed based on genome data from Cyanobase (Nakao et al., 2010). A plasmid, pRhetRS, for *hetR* gene disruption was constructed as follows. A DNA fragment containing the *hetR* gene was amplified by PCR using the primer pair hetR-uf and hetR-RB (Table S1) and cloned between the *Hin*CII and BamHI sites of pPCR-Script Amp SK+ (Agilent Technologies). A spectinomycin-resistance cassette excised by digestion with *Xba*I from plasmid pDW9 (Golden & Wiest, 1988) was inserted into the *Xba*I site within the coding region of *hetR*. The *Sac*-Xhol fragment was excised from the resultant plasmid and cloned between the *Sac*I and *Xho*I sites of pRL271 (Black et al., 1993) to construct pRhetRS. A plasmid, pR1336S, for *pknH* gene disruption was constructed as follows. DNA fragments upstream and downstream of the *pknH* gene were amplified by PCR using the primer pairs 1336-5F and 1336-5R, and 1336-3F and 1336-3R, respectively (Table S1). The upstream fragment was cloned between the *Sac*I and *Bam*HI sites of pBluescript II KS+ (Agilent Technologies), and then the downstream fragment was cloned between the *Bam*HI and *Xho*I sites. A spectinomycin-resistance cassette excised by digestion with *Bam*HI from plasmid pDW9 was inserted into the *Bam*HI site between the upstream and downstream fragments. The *Sac*-Xhol fragment was excised from the resultant plasmid and cloned between the *Sac*I and *Xho*I sites of pRL271 to construct pR1336S. pRhetRS and pR1336S were transferred by conjugation into *Anabaena* PCC 7120 according to Elhai & Wolk (1988) to construct a *hetR* mutant strain, D*RhetRS*, and a *pknH* mutant strain, DR1336S, respectively.

To complement the *pknH* mutant, the *pknH* gene and its flanking regions were amplified by PCR using the primer pair 1336-5F and 1336-3R. The amplified fragment was digested with *Xba*I and *Hind*III, and then cloned between the *Xba*I and *Hind*III sites of pBluescript II KS+. The *Sac*I–*Kpn*I fragment was excised from the resultant plasmid and cloned between the *Sac*I and *Kpn*I sites of shuttle vector pAM505 (Yoon & Golden, 1998) to construct pA1336.

Plasmid pA1336G was used to express a *pknH-gfp* transcriptional fusion. An upstream fragment of the *pknH* gene was amplified by PCR using the primer pair 1336-5F and 1336-5R, and then cloned between the *Sac*I and *Bam*HI sites of pKEN2-GFPmut2 (Cormack et al., 1996). A *Sac*I–*Hind*III fragment containing the *pknH-gfp* transcriptional fusion was cloned between the *Sac*I and *Smal*I sites of pAM505 to construct pA1336G.

**RNA analysis.** Total RNA was extracted from whole filaments according to Pinto et al. (2009) and treated with DNase I (Takara Bio). Real-time quantitative RT-PCR (qRT-PCR) was carried out as described previously (Ehira & Ohmori, 2011) using a *pknH*-specific primer set (Table S1). For RNA gel blot analysis, 5 μg aliquots of total RNA were subjected to electrophoresis in 1 % agarose gels containing formaldehyde and transferred to Bioxyne Plus membranes (Pall). Hybridization and detection of hybridized probes were carried out as described previously (Ehira et al., 2003). Each digoxigenin-labelled probe was prepared by PCR with a set of specific primers (Table S1).

**Acetylene reduction assays.** Nitrogenase activity was determined by acetylene reduction assays using GC (GC-8A gas chromatograph, Shimadzu), as described previously (Ehira & Ohmori, 2011).

**RESULTS**

**Expression of *pknH* is upregulated in heterocysts**

In the previous study, changes in whole-gene expression of *Anabaena* PCC 7120 by nitrogen deprivation were investigated using a DNA microarray (Ehira & Ohmori, 2006a). The results showed induction of *alr1336*, encoding a putative Ser/Thr kinase, by nitrogen deprivation. In this study, spatiotemporal expression of *alr1336* was closely analysed by qRT-PCR and the use of a *gfp* transcriptional fusion gene. Expression of *alr1336* was induced 8 h after nitrogen deprivation, with increases in the transcript level of about ninefold after 24 h (Fig. 1). Fig. 2 shows the spatial regulation of *alr1336* expression. In *Anabaena* PCC 7120 containing plasmid pA1336G, the *gfp* gene is transcribed from a promoter of *alr1336*. No GFP fluorescence was observed when the filaments were grown with nitrate or subjected to nitrogen deprivation for 3 h (Fig. 2e, f). After 12 h of nitrogen deprivation, differentiating cells were structurally distinguishable from vegetative cells and slight GFP fluorescence was detected in those cells, while vegetative cells showed no detectable fluorescence (Fig. 2c, g). After 24 h, fluorescence levels increased only in heterocysts, though fluorescence from some heterocysts was weak (Fig. 2d, h). This indicated that expression of *alr1336* is restrictively induced in heterocysts by nitrogen deprivation.

The product of *alr1336* has a Ser/Thr protein kinase domain at its N terminus and a DUF323 domain at its C terminus (Zhang et al., 2007). Ser/Thr kinases with the DUF323 domain are found in filamentous cyanobacteria,
but not in unicellular strains (Zhang et al., 2007). The kinase domain of Alr1336 shows 42% amino acid identity with SpkC of Synechocystis sp. PCC 6803, which has been shown to phosphorylate both itself and general substrate proteins (Kamei et al., 2002). Moreover, the well-conserved 12 amino acid residues in the catalytic domains of Ser/Thr kinases are completely conserved in the deduced amino acid sequence of the alr1336 product (Hanks & Hunter, 1995). Thus, we designated alr1336 as pknH (protein kinase expressed in heterocysts).

To determine the correlation between pknH expression and heterocyst development, the transcript level of pknH in the nrrA disruptant DR4312S (Ehira & Ohmori, 2006a) and the hetR disruptant DRhetRS was determined (Fig. 1). Heterocyst development is delayed in DR4312S (Ehira & Ohmori, 2006a) and completely abolished in DRhetRS, as reported elsewhere (Buikema & Haselkorn, 1991). Induction of the pknH gene was delayed to 24 h after nitrogen deprivation in DR4312S, and abolished in DRhetRS (Fig. 1). It is concluded that the expression of pknH is developmentally regulated and that hetR is necessary for the induction of pknH.

The pknH gene is required for diazotrophic growth

The pknH gene-deficient mutant DR1336S was constructed by homologous recombination. DR1336S grew normally in nitrate-containing medium (Figs 3a and 4d), while the rate of growth of DR1336S was much slower than that of the wild-type (WT) strain in medium without combined nitrogen (Fig. 3b). After 18 h of nitrogen deprivation, when the WT starts nitrogen fixation (Ehira & Ohmori, 2011), heterocysts were stained with Alcian blue, which specifically stains the polysaccharide layer of the heterocyst envelope (Fig. 4b). In DR1336S, heterocysts were visually detectable and stained with Alcian blue, indicating that the polysaccharide layer of the heterocyst had formed (Fig. 4e). However, the filament length of DR1336S was reduced and many heterocysts were detached from filaments (Fig. 4e). These phenotypes were emphasized by prolonged cultivation in nitrogen-free medium (Fig. 4f), and even in a static culture, fragmentation was

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**Fig. 1.** Changes in the transcript level of the pknH gene after nitrogen deprivation. The relative transcript levels of pknH at the indicated times were determined by qRT-PCR in the WT strain (○), the nrrA disruptant DR4312S (△) and the hetR disruptant DRhetRS (●). The transcript levels were determined in duplicate using three independently grown cultures. The transcript level of the WT strain at 0 h was taken as 1.

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**Fig. 2.** Spatiotemporal expression of the pknH-gfp transcriptional fusion gene after nitrogen deprivation. Filaments of Anabaena PCC 7120 containing plasmid pA1336G grown in the presence of nitrate (a, e) were subjected to nitrogen deficiency for 3 h (b, f), 12 h (c, g) and 24 h (d, h). The upper panels (a–d) show light micrographs and the lower panels (e–h) show corresponding fluorescence micrographs. Arrowheads indicate differentiating heterocysts.
observed (data not shown). Shuttle vector pA1336, in which the *pknH* gene is the only coding region, was transferred into DR1336S, resulting in a complemented strain that showed elongated filaments with heterocysts similar to those of the WT (data not shown). These results indicate that *pknH* is required for the stability of the connection between heterocysts and vegetative cells.

In fragmentation mutants of *Anabaena* PCC 7120, heterocyst differentiation is impaired (Flores *et al.*, 2007; Jang *et al.*, 2007; Merino-Puerto *et al.*, 2010; Nayar *et al.*, 2007). Fig. 5 shows developmental regulation of genes involved in heterocyst differentiation, including *hetR*, *hepA*, *hglE* and *nifH*, in DR1336S. Expression of *hetR* was upregulated within 3 h after nitrogen deprivation, and the *hetR* transcript level reached the maximum level at 8 h, and then decreased (Fig. 5a). In DR1336S, the increase in the *hetR* transcript was delayed to 8 h, and the transcript level remained high even after 24 h (Fig. 5a). Expression of *hepA*, which is essential for the formation of the polysaccharide layer of heterocysts (Holland & Wolk, 1990), was induced at 8 h. The induction of *hepA* was also detected in DR1336S, although the transcript level was lower than in the WT (Fig. 5b). Expression of *hglE*, which is required for the synthesis of the glycolipid layer of heterocysts (Campbell *et al.*, 1997), and *nifH*, encoding a subunit of nitrogenase, was induced at 24 h in both the WT and DR1336S (Fig. 5c, d). Thus, heterocyst maturation is likely to be unaffected by disruption of the *pknH* gene. Indeed, DR1336S developed appreciable nitrogenase activity 24 h after nitrogen deprivation, although the activity in DR1336S [1.3 ± 0.5 μmol ethylene (mg Chl⁻¹) h⁻¹] was very low compared with the WT [22.4 ± 1.7 μmol ethylene (mg Chl⁻¹) h⁻¹]. The nitrogenase activity of DR1336S was increased to 2.5-fold during the following 24 h of incubation [3.2 ± 0.4 μmol ethylene (mg Chl⁻¹) h⁻¹] and reached one quarter of the WT level [12.9 ± 3.3 μmol ethylene (mg Chl⁻¹) h⁻¹]. It is concluded that DR1336S is able to form mature heterocysts with nitrogenase activity.

**Fig. 3.** Growth of *Anabaena* PCC 7120 (●) and DR1336S (○) on different nitrogen sources. Growth was measured as OD₇₅₀.
(a) Growth on nitrate as the nitrogen source.
(b) Growth on dinitrogen as the nitrogen source. Nitrate-grown filaments were transferred to nitrogen-free medium at time zero. Data are expressed as averages of the results of two independent cultures.

**Fig. 4.** Heterocyst development after nitrogen deprivation in the wild-type strain (a–c) and the *pknH* disruptant DR1336S (d–f). Nitrate-grown filaments were transferred to nitrogen-free medium. Micrographs were taken before (a, d) or 18 h (b, e) and 44 h (c, f) after nitrogen deprivation. The polysaccharide layer of heterocysts was stained with Alcian blue.
Expression of the pknH between heterocysts and vegetative cells.
PCC 7120, encoding a Ser/Thr protein kinase.

Bromide is shown as a loading control (e).

(d) were used for hybridization. rRNA stained with ethidium bromide is shown as a loading control (e).

**DISCUSSION**

In the present study, we characterized the pknH gene of Anabaena PCC 7120, encoding a Ser/Thr protein kinase. Expression of pknH was upregulated in heterocysts by nitrogen deprivation (Figs 1 and 2). The pknH disruptant was capable of differentiating heterocysts with nitrogenase activity (Fig. 4), but it showed a defect in diazotrophic growth (Fig. 3). After nitrogen deprivation, filaments of the pknH disruptant were fragmented, and most heterocysts were detached from filaments (Fig. 4). This indicates that the pknH gene is required for the stability of the connection between heterocysts and vegetative cells.

Expression of the pknH gene was developmentally regulated (Fig. 1). Disruption of the hetR gene abolished the induction of pknH. The pknE (alr3732) gene, encoding another Ser/Thr protein kinase, is also upregulated in heterocysts (Saha & Golden, 2011). HetR is known to interact with the pknE promoter. The inverted repeat sequence that is recognized by HetR has been determined for the promoter region of hetP (Higa & Callahan, 2010). Since the HetR-recognition sequence is not found within the upstream region of the pknH gene, transcription of the pknH gene could be regulated by a transcriptional regulator that is under the control of HetR.

In the pknH disruptant, heterocysts were formed 18 h after nitrogen deprivation and had normal morphology and Alcian blue staining (Fig. 4). Expression of genes involved in heterocyst maturation, such as hepA, hglE and nifH, was similar to that of the WT (Fig. 5). The pknH disruptant showed nitrogenase activity even in the presence of oxygen, although the levels were lower than those of the WT, indicating that the pknH disruptant differentiated mature heterocysts. However, most heterocysts of the pknH disruptant were detached from filaments (Fig. 4). These results support the conclusion that the pknH gene is required for the connections between heterocysts and vegetative cells rather than for heterocyst maturation. Heterocysts have a thick envelop outside the cell wall, which limits oxygen diffusion, but they are connected to the adjacent vegetative cells at the polar region through a special structure to allow the exchange of metabolites between heterocysts and vegetative cells (Walsby, 2007).

Proteins constituting the cell–cell joining structure have been identified over the past few years (Flores et al., 2007; Mariscal et al., 2011; Merino-Puerto et al., 2011). Since PknH is predicted to be a cytosolic protein by the secondary structure prediction system SOSUI (Hirokawa et al., 1998), PknH itself is unlikely to constitute the cell–cell joining structure. PknH could regulate activities and/or localization of proteins involved in cell–cell connections by phosphorylation.

Two Ser/Thr protein kinases, encoded by pknE and all4668, are expressed in differentiating cells (Saha & Golden, 2011), as well as the pknH gene (Fig. 2), and a Ser/Thr protein phosphatase DevT accumulates in mature heterocysts (Espinosa et al., 2010). The pknH mutant strain can grow in nitrogen-free medium, although its growth rate is slightly slower than that of the WT, and overexpression of pknE blocks heterocyst differentiation (Saha & Golden, 2011). The devT mutant strain can form morphologically mature heterocysts, but it cannot fix nitrogen (Espinosa et al., 2010). Thus, the phosphorylation of Ser/Thr residues of certain proteins in heterocysts plays important roles in heterocyst differentiation and function, and a phosphorylation network might regulate various aspects of heterocyst development. In order to understand the role of these protein kinases and phosphatases, the signals activating them as well as their target proteins need to be addressed.

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