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A Pair of Iron-Responsive Genes Encoding Protein Kinases with a Ser/Thr Kinase Domain and a His Kinase Domain Are Regulated by NtcA in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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The filamentous cyanobacterium *Anabaena* sp. strain PCC 7120 can fix N_2 when combined nitrogen is not available in the growth medium. It has a family of 13 genes encoding proteins with both a Ser/Thr kinase domain and a His kinase domain. The function of these enzymes is unknown. Two of them are encoded by *pkn41* (alr0709) and *pkn42* (alr0710). These two genes are separated by only 72 bp on the chromosome, and our results indicate that they are cotranscribed. The expression of *pkn41* and *pkn42* is induced by iron deprivation irrespective of the nature of the nitrogen source. Mutants inactivating either *pkn41*, *pkn42*, or both grow similarly to the wild type under normal conditions, but their growth is impaired either in the presence of an iron chelator or under conditions of nitrogen fixation and iron limitation, two situations where the demand for iron is particularly strong. Consistent with these results, these mutants display lower iron content than the wild type and a higher level of expression for *nifJ1* and *nifJ2*, which encode pyruvate:ferredoxin oxidoreductases. Both *nifJ1* and *nifJ2* are known to be induced by iron limitation. NtcA, a global regulatory factor for different metabolic pathways, binds to the putative promoter region of *pkn41*, and the induction of *pkn41* in response to iron limitation no longer occurs in an *ntcA* mutant. Our results suggest that *ntcA* not only regulates the expression of genes involved in nitrogen and carbon metabolism but also coordinates iron acquisition and nitrogen metabolism by activating the expression of *pkn41* and *pkn42*.

Protein phosphorylation catalyzed by protein kinases regulates a variety of cellular activities in both prokaryotes and eukaryotes and is essential for signal transmission. Three major families of protein kinases are usually found to be involved in signal transduction, the family of Ser/Thr- and/or Tyr-specific protein kinases, the family of Tyr-specific protein kinases, and the family of His kinases (7, 15, 39). His kinases are typically found in two-component signaling systems. Recently, a new family of protein kinases has been identified; these protein kinases, represented by HstK from the filamentous cyanobacterium *Anabaena* sp. strain PCC 7120, have both a Ser/Thr and/or Tyr kinase domain and a His kinase domain, and these two domains are separated by other domains such as a GAF (cGMP-specific and stimulated phosphodiesterase, *Anabaena* adenylate cyclose, and *Escherichia coli* FmA) domain for those in *Anabaena* strain PCC 7120 (28, 30, 45). HstK-like protein kinases are also found in organisms such as *Schizosaccharomyces pombe* and *Rhodococcus sp*. M5 (22, 30). The function of the HstK-like protein kinases is unknown. *Anabaena* strain PCC 7120 contains 53 genes encoding Ser/Thr kinases; 13 of these encode proteins similar to HstK (45, 51). All protein kinases of the HstK family in *Anabaena* strain PCC 7120 are very large, ranging from 1,777 to 2,121 amino acid residues.

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Cyanobacteria are phototrophic prokaryotes that carry out oxygenic photosynthesis (38). Most cyanobacteria are able to use nitrate or ammonium as a source of nitrogen for growth, and many strains are capable of dinitrogen fixation (10). NtcA, a transcriptional regulator of the Crp (*c*yclic AMP *r*eceptor *p*rotein) family, is a global regulator of nitrogen metabolism (18, 19). Ammonium is the preferred nitrogen source, and the use of alternative nitrogen sources in the presence of ammonium is repressed. In the absence of ammonium, NtcA activates genes required for the use of alternative nitrogen sources, such as nitrate or N_2 (11, 13, 46). In *Anabaena* strain PCC 7120, the fixation of dinitrogen takes place under aerobic conditions in heterocysts, cells differentiated from vegetative cells when a source of combined nitrogen becomes limiting (47). NtcA is necessary for the initiation of heterocyst differentiation (11, 46). The mutant strain CSE2, corresponding to an insertional mutant of *ntcA* in *Anabaena* strain PCC 7120, is unable to grow on either nitrate or dinitrogen, whereas it grows normally on ammonium (11, 46). In addition to its positive role in the expression of genes involved in nitrogen assimilation, NtcA may also act as a repressor for the expression of genes, such as those of the *rbcLS* operon, encoding the two subunits of ribulose-1,5-bisphosphate carboxylase (20, 32). Beyond its regulatory function in nitrogen and carbon metabolism, NtcA has been recently proposed as a regulator of photosynthesis according to a bioinformatic analysis (41). Therefore, NtcA is increasingly being considered as a global regulator that orchestrates different cellular activities in cyanobacteria. The binding sites of NtcA contain the con-

sensus signature $GTAN_8TAC$ in which the $GTN_{10}AC$ subset is essential for binding of NtcA (18, 19, 21). The regulation of gene expression by NtcA is also proposed to be under the control of the redox state (1, 20).

It has been noted that some aspects of iron stress symptoms in cyanobacteria are similar to those induced by nitrogen limitation (40). These shared symptoms include the reduced amount of phycobilisomes and the accumulation of glycogen (16, 17, 37). Iron is an essential nutrient for almost all living organisms, acting as a cofactor within many enzymes or as a catalyst in electron transfer pathways (9). Nitrate reduction or nitrogen fixation by nitrogenase requires iron-rich enzymes (10, 40). Considering that iron is a critical element in nitrogen metabolism raises the possibility that cellular response to iron or nitrogen limitation may share a common regulatory pathway or element. So far, little is known about the coordination of cell responses to iron limitation and nitrogen limitation. One gene, *nifJ* encoding pyruvate:flavodoxin oxidoreductase, which is necessary for nitrogen fixation when iron is limited, has been identified from *Anabaena* strain PCC 7120 (3, 35). A mutant inactivating *nifJ* grows normally and fixes N_2 when iron is present but fails to grow when the growth medium is deprived of both iron and combined nitrogen (3). A second copy of *nifJ* has been found in *Anabaena* strain PCC 7120, and the two copies of *nifJ* were named *nifJ1* and *nifJ2*, respectively (36).

In this study, we report that *pkn41* and *pkn42*, two genes closely linked on the chromosome and belonging to the gene family of *hstK*, regulate iron content within the cells and are required for growth when the demand for iron is high or when iron is severely limited by the presence of an iron chelator. The expression of these two genes is directly regulated by NtcA, suggesting that NtcA is involved in the coordination of nitrogen metabolism and iron homeostasis.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Anabaena* sp. strain PCC 7120 was grown at 30°C in BG11 medium in the presence of either nitrate or ammonium as a source of combined nitrogen (33). $BG11_0$ medium (BG11 without a source of combined nitrogen) was used to grow cells under conditions of nitrogen fixation. Six milligrams/liter of ferric ammonium citrate (Merck), corresponding to 20 μM of iron, was present in the medium as a source of iron (33); when no ferric ammonium citrate was added, the amount of iron as a contaminant from other chemicals used to prepare the medium was calculated to be 1.33 μM according to the indications of the suppliers. The mutant strains CS41 (an insertional mutant of the *pkn41* gene) and CS42 (an insertional mutant of the *pkn42* gene) were grown on BG11 supplemented with 5 μg/ml of spectinomycin (Sp) and 5 μg/ml of streptomycin (Sm) in liquid medium or 2.5 μg/ml of Sp and 2.5 μg/ml of Sm in solid medium. The *ntcA* mutant strain CSE2 and the *Escherichia coli* strain BL21(DE3) containing the plasmid pCSAM70 for expressing the recombinant NtcA were grown as described previously (11). Bacterial growth was monitored according to the optical density at 700 nm.

Construction of mutants. All PCR primers are listed in Table 1. Triparental conjugation and the selection of double recombinants were performed as described previously (5, 8). To inactivate *pkn41* (alr0709), a 1-kb fragment at the 5′ end of the *pkn41*-coding region was amplified by PCR using primers 411 and 412. The resulting PCR product was cloned into pBluescript SK^+ digested by XhoI and PstI, giving pSK41. An SmaI fragment containing the 2.0-kb Sp^r/Sm^r cassette from pHP45Ω (31) was cloned into pSK41 digested by AccI and treated with T4 DNA polymerase. The resulting plasmid was called pSK41Ω. The insert in pSK41Ω was cut out with XhoI and PstI and was cloned into the same sites of pRL271 to generate pRL41Ω. To inactivate *pkn42* (alr0710), a region of *pkn42* was amplified by PCR with oligonucleotide primers 421 and 422. The resulting PCR product was cloned into the XhoI and PstI sites of pBluescript SK^+ to produce pSK42. The other steps were similar to those used for the inactivation of *pkn41*, and the final construct was called pRL42Ω.

TABLE 1. List of oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)	Target
411	CTTCTCGTGAAACCCCCAGCTAGTGATT	*pkn41*
412	CCTCTGCAGATGGAATTTCTGAATTAA	*pkn41*
413	CTGAGTCTGCATTTCTCG	*pkn41*
414	CAACAAGAGTGCTAGTCA	*pkn41*
421	CTTCTCGAGAATATCAATTGCTGATAAT	*pkn42*
422	CTTCTGCAGATTAATTTCATGTGCTAAA	*pkn42*
DM11	CTTACTAGTCTAGGTTTGAGATGAATGAC	*pkn41*
DM12	GTCTTGTTGCTGCTGTTG	*pkn41*
DM21	GACGGATCCCTTTCCCTACGAGACGCA	*pkn42*
DM22	CTTCTCGAGAATTGAGAACGCAGTTAGAC	*pkn42*
R411	TTAAAAATTGCCCTGGCT GAAC	*pkn41*
R412	CTTAAAACTGCATTCACGAGATC	*pkn41*
R421	TACCTAAGGGTGACACAACAG	*pkn42*
R422	GGTGCGCCCATTGAGCCAG	*pkn42*
IGF1	AGGAAATTGACTTAGATTTTATGTT	Intergenic region
IGF2	GATCAA CTTCTCGATAACCTCC	Intergenic region
RnifJ11	GGTGCAAACCCTTACTACAAC	*nifJ1*
RnifJ12	ATTTTGGGGACTGGGGACT	*nifJ1*
RnifJ21	GGCGGACAAATGTCGAAAGC	*nifJ2*
RnifJ22	TAGCGTTGG TGTGCGGGAG	*nifJ2*
RisiB1	GGTAAATTGGTTGCCTACTTT	*isiB*
RisiB2	TTACAAACCAAATTCAGACTT	*isiB*
rnpB1	AAGCCGGGTTCTGTTCTCTG	*rnpB*
rnpB2	ATAGTGCCACAGAAAAATACCG	*rnpB*
glnA1	GGATTTTATGTCAAAGTTGA	*glnA*
glnA2	CGAAACAAAGTTGATGAC	*glnA*

To construct the mutant in which both *pkn41* and *pkn42* were deleted, a DNA fragment (−1,000 to −190 bp upstream of the putative translational start codon of *pkn41*) was amplified by PCR with primers DM11 and DM12 and inserted into the vector pMD-18T to generate pDM1. A BamHI fragment containing the Sp^r/Sm^r cassette from pHP45Ω (31) was cloned into the BamHI site of pMD1 to generate pDM1Ω. A DNA fragment located downstream of *pkn42* (134 to 970 bp downstream of the stop codon of *pkn42*) was amplified by PCR with primers DM21 and DM22 and cloned into the BamHI and XhoI sites of pBluescript SK^+, giving plasmid pSKDM2. An SpeI-EcoRI fragment from pDM1Ω was cloned into the same sites of pSKDM2 to give pSKDMΩ. The insert in pSKDMΩ was cut out with XhoI and SpeI and was cloned into the same sites of pRL271 to generate the final construct pRLDMΩ. The plasmid was transferred into *Anabaena* strain PCC 7120, and the resulting mutant strain was called CSDM.

For mutant complementation, the plasmid pKN2541 containing the coding region of *pkn41* and a 786-bp upstream region was constructed. The whole region was 6.6 kb in size and was obtained as a SnaBI-PacI fragment from the genomic clone pANP02796 (kindly provided by C. P. Wolk) and inserted into the SmaI site of pBluescript SK^+. The plasmid obtained was treated with XhoI and T4 DNA polymerase and then digested with BamHI. The insert was cloned into the NotI (treated with T4 DNA polymerase) and BamHI sites of the shuttle vector pRL25c to generate pKN2541. The plasmid was then transferred by conjugation into the mutant CS41.

Southern hybridization. Total DNA was extracted and digested with appropriate restriction enzymes, separated by electrophoresis, and transferred onto a Hybond-C membrane (Amersham) following the instructions of the manufacturer. The *pkn41* probe (pSA41) (Fig. 1A) was a 502-bp XbaI-PstI fragment from the plasmid pSK41 (see above), and the *pkn42* probe was an SpeI-PstI fragment from the plasmid pSK42 (see above). Probes were labeled using a random priming kit (Amersham), and DNA/DNA hybridization was performed as described previously (49).

RNA isolation, RNA dot blotting, and RT-PCR. For RNA isolation, cells growing exponentially in BG11 supplemented with NH_4Cl as a source of nitrogen were harvested, washed three times in BG11 with or without ferric ammonium citrate, resuspended in the same new medium, and grown for 5 days. The optical densities of these cultures before RNA extraction were about 0.6 to 0.8. For the analysis of the expression kinetics of *pkn41* and *pkn42*, cells growing exponentially in BG11 with NH_4Cl were transferred into fresh BG11 medium containing a 0.1 mM concentration of the iron chelator 2,2′-dipyridyl, and samples were taken at different time points for RNA isolation. After culturing, all cell samples were cooled on ice and collected by centrifugation at 4°C. Cell pellets were frozen in liquid nitrogen before RNA extraction. RNA was prepared with the acidic hot phenol method as described previously (50). The resulting RNA samples were treated with RNase-free DNase I (Takara) to eliminate contaminating DNA and controlled by PCR to make sure that contaminant DNA was not

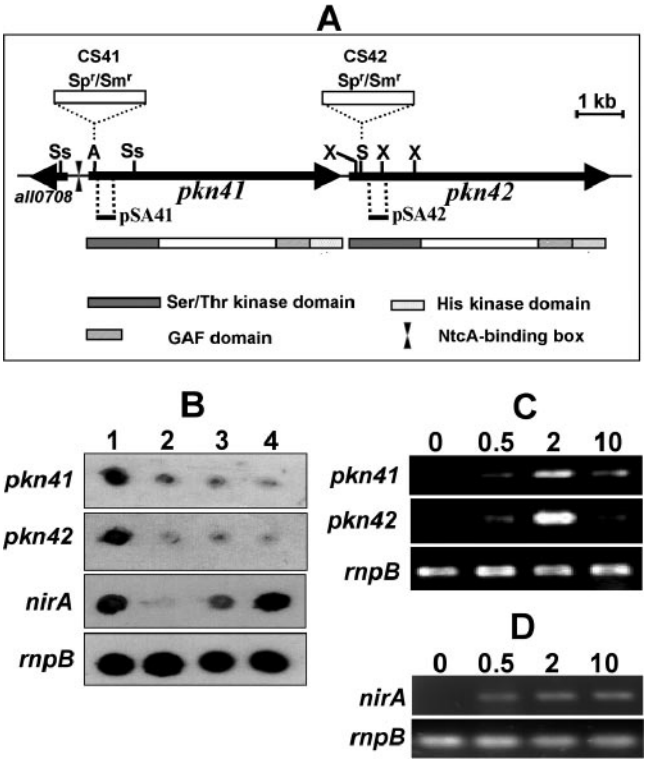

FIG. 1. The *pkn41-pkn42* gene cluster and activation of *pkn41* and *pkn42* by iron limitation. (A) Each open reading frame of the gene cluster and its transcriptional orientation are indicated by an arrow. The insertion of the Sp^r/Sm^r resistance cassette is shown above each gene. The relevant domains for the protein encoded by *pkn41* or *pkn42* are shown below each gene. The location of the NtcA-binding box upstream of *pkn41* is depicted. The two DNA probes used for DNA/DNA or DNA/RNA hybridization, pSA41 and pSA42, are indicated. Abbreviations for relevant sites of restriction endonucleases: A, AccI; Ss, SspI; S, SpeI; X, XbaI. (B) RNA dot blot analysis of gene expression. Lanes: 1, BG11 with NH_4Cl as a source of nitrogen without iron; 2, BG11 with NH_4Cl as a source of nitrogen with iron; 3, BG11 with $NaNO_3$ as a source of nitrogen with iron; 4, $BG11_0$ without a source of combined nitrogen. (C) RT-PCR analysis of expression of *pkn41* and *pkn42* following iron starvation in the presence of the iron chelator 2,2′-dipyridyl. Cells were precultured in BG11 with NH_4Cl, then 2,2′-dipyridyl at 0.1 mM was added and samples were taken at various time points (indicated in hours) for RT-PCR analysis. The *rnpB* gene served as a control for the amount of RNA template. (D) RT-PCR analysis of expression of *nirA* after addition of 2,2′-dipyridyl under the conditions described for panel C.

detected without reverse transcriptase under the reverse transcription (RT)-PCR conditions used.

For RNA dot blot, RNA samples (5 μg each) were deposited onto Hybond-C membranes (Amersham) and hybridized with the radioactively labeled probes pSA41 and pSA42 (Fig. 1A). For RT-PCR analysis (primers are listed in Table 1), 1 μg of total RNA was used as template. Different PCR cycles were tested, and those chosen in this study always corresponded to those still at the exponential phase of amplification. The number of PCR cycles was determined as follows: for *pkn41* and *pkn42*, 35 cycles; for *rnpB*, 10 cycles.

Electrophoretic mobility shift assays. The expression and purification of recombinant NtcA were performed as described previously (27). DNA fragments used in electrophoretic mobility shift assays were obtained by PCR amplification (primers are listed in Table 1). Primers 413 and 414 were used to amplify the promoter region of *pkn41*. Primers glnA1 and glnA2 were used to amplify the promoter region of *glnA*. DNA fragments were end labeled with T4 polynucleotide kinase (Promega) in the presence of [^{32}P]dATP. The DNA-binding reaction of NtcA was carried out as described previously (26) in 20 μl of binding buffer (12 mM HEPES-NaOH [pH 8], 4 mM Tris-HCl [pH 8], 60 mM KCl, 1 mM EDTA, 1 mM dithiothreitol [DTT], 12% glycerol, 25 μg/ml bovine serum albumin). After incubation for 30 min at 30°C, the mixture was subjected to electrophoresis on a native 5% polyacrylamide gel, and then the gel was dried and analyzed by autoradiography.

Measurement of total iron content in cells. The amounts of total iron content were measured using an inductively coupled plasma atomic emission spectrometer (Ultrace; model no. JY238). Cells were grown under iron repletion or iron-limiting conditions for 5 days and collected by centrifugation. The pellet was washed three times with 20 mM HEPES-NaOH (pH 8.0) containing 10 mM EDTA, dried, and used for the measurement of total iron content.

RESULTS

Analysis of the expression pattern of *pkn41* and *pkn42*. *pkn41* (alr0709) and *pkn42* (alr0710), two members of the *hstK* gene family, are located on the same DNA strand and separated by only 72 bp on the chromosome (Fig. 1A). As other members in the Hstk family, Pkn41 and Pkn42 are very large, being comprised of 1,799 and 1,802 amino acidic residues, respectively. The nucleotide sequences of *pkn41* and *pkn42* share significant identity. The identity is 92% within the 5′-terminal region encoding the Ser/Thr kinase domain and 84% within the 3′-terminal region encoding the GAF domain and the His kinase domain. The central regions of these two genes, located between the Ser/Thr kinase domain and the GAF domain, show no significant similarity at the DNA level. Interestingly, the 50-bp DNA sequences at the 5′-nonencoding regions upstream of the putative translational start codons (ATG) of *pkn41* and *pkn42*, respectively, are highly conserved, with a nucleotide sequence identity of 86% (data not shown).

We first analyzed the expression of these two genes under different nitrogen regimes using RNA/DNA hybridization on dot blot (Fig. 1B). Total RNAs were isolated from *Anabaena* strain PCC 7120 cultured in BG11 medium in the presence of ammonium or nitrate or in the absence of combined nitrogen. The levels of *rnpB* RNA varied little under our conditions and were used as controls. Another control was *nirA*, a gene encoding nitrite reductase (12, 25). As reported previously (12, 25), the expression level of *nirA* was low in the presence of ammonium, enhanced in the presence of nitrate, and strongly stimulated in the absence of combined nitrogen (6). However, for both *pkn41* and *pkn42*, the levels of expression were low and did not change according to the nature of the nitrogen source in the growth medium (Fig. 1B). However, we found that the expression of both genes was strongly enhanced when filaments were cultured in a medium deprived of iron for 5 days versus a similar culture in the presence of the iron source for 5 days. The expression of *nirA* was also induced under similar conditions (Fig. 1B).

We have determined the kinetics of the expression of *pkn41* and *pkn42* by RT-PCR analysis (Fig. 1C). Iron starvation takes a long time to achieve for cyanobacteria, as for many other organisms, since even a trace amount of iron can still be enough for cyanobacterial growth (40). Iron deprivation in cyanobacteria leads to the expression of *isiA*, a gene encoding CP43′ associated with photosystem I (PSI) (4, 14, 24, 44, 48). For *Anabaena* strain PCC 7120, the association between CP43′ and PSI is characterized by a subtle shift of the spectral absorbance from 682 nm to 672 nm when cells are transferred from iron-replete to iron-limiting conditions (data not shown). When fluorescence emission is measured at 77 K, the spectral

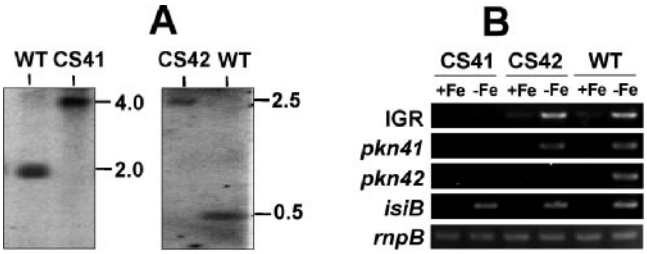

FIG. 2. Inactivation and cotranscription of *pkn41* and *pkn42*. (A) Southern hybridization showing full segregation of the CS41 and CS42 mutants. The size in kilobases of each hybridization signal is shown on the right of each autoradiogram. (B) Cotranscription of *pkn41* and *pkn42* determined by RT-PCR. Transcripts were analyzed from the wild type (WT) and the CS41 and CS42 mutants in BG11 with ammonium as nitrogen source in the presence of ferric ammonium citrate (+Fe) or in its absence (−Fe). IGR, intergenic region between *pkn41* and *pkn42*.

change occurs as a shift from 730 nm to 726 nm (48). The expression of *isiA* and the association of CP43′ with PSI, as measured by spectroscopy or fluorescence emission at low temperature, can serve as an internal marker for iron starvation. When cells of *Anabaena* strain PCC 7120 were cultured in BG11 without the iron source (ferric ammonium citrate), the presence of CP43′ and its association to PSI were detected only after 5 days or even longer, indicating that real iron starvation took a long time to achieve in this strain. We have therefore used the iron chelator 2,2′-dipyridyl to deplete iron more efficiently in order to study the kinetics of induction of *pkn41* and *pkn42* (Fig. 1C). This chelator is routinely used to deplete iron in other organisms, such as *E. coli* and *Bacillus subtilis* (for examples, see references 2 and 29). Filaments of *Anabaena* strain PCC 7120 were cultured in BG11 in the presence of ammonium, and 0.1 mM 2,2′-dipyridyl was added. Samples were taken at different time intervals and used for RNA extraction and RT-PCR analysis. Under similar conditions, no transcript of *pkn41* or *pkn42* was detectable when ferric ammonium citrate was present (Fig. 1C). Once 2,2′-dipyridyl was added, it elicited a rapid cell response, since 0.5 h after its addition transcription of *pkn41* and *pkn42* could be found, and it reached a high level 2 h after the depletion of iron, then decreased again afterwards. The addition of the iron chelator also induced the expression of *nirA* (Fig. 1D), although the expression level of *nirA* stayed at a high level 10 h after treatment with the chelator.

Inactivation of *pkn41* and *pkn42* and determination of their cotranscription. *pkn41* and *pkn42* were inactivated by the insertion of 2.0-kb Ω cassettes conferring resistance to spectinomycin and streptomycin, respectively. The Ω cassette was derived from pHP45Ω and carried a transcriptional terminator on each side (31). After conjugation, double recombinants were obtained and confirmed by Southern hybridization using pSA41 and pSA42, respectively, as probes (Fig. 1A and 2A). The mutant inactivating *pkn41* was called CS41, and the mutant inactivating *pkn42* was called CS42 (Fig. 1A and 2A). In CS41, the 2-kb wild-type fragment obtained by digestion with SspI was replaced by a 4-kb fragment, while in CS42, the 0.5-kb XbaI fragment in the wild type was replaced by a 2.5-kb fragment (Fig. 2A).

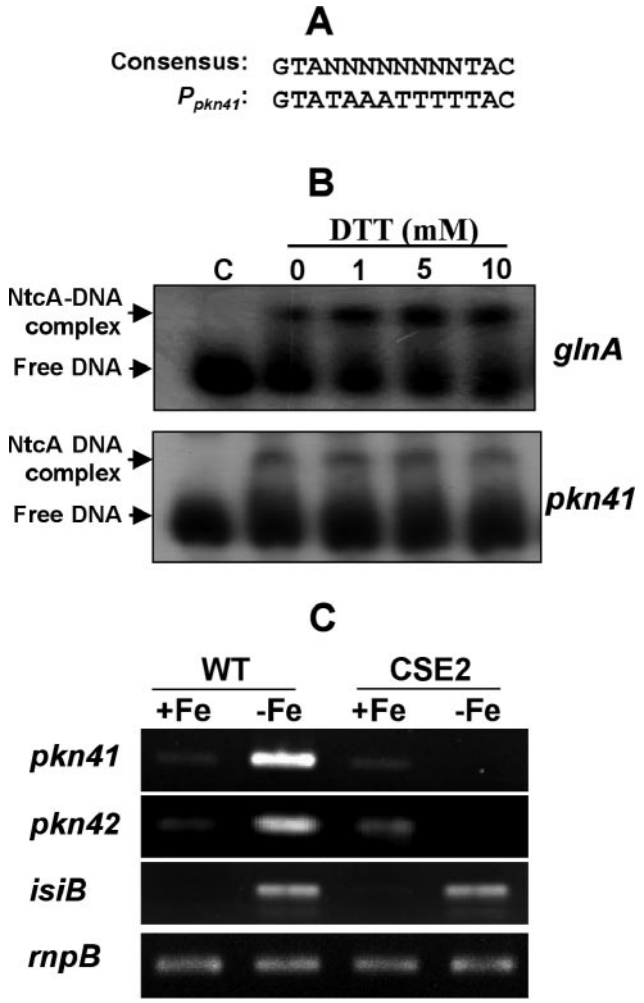

FIG. 3. Regulation of expression of *pkn41* and *pkn42* by NtcA. (A) Comparison between the consensus NtcA-binding site and the putative NtcA-binding site 80 bp upstream of the translational start point of *pkn41*. N, any of the four nucleotides. (B) Interaction between NtcA and the promoter region of *pkn41* determined by DNA motility shift. A DNA fragment (170 bp for *glnA* and 220 bp for *pkn41*) corresponding to the promoter region of *glnA* or *pkn41* was radioactively labeled and used to determine the interaction with NtcA with or without DTT. C, control without NtcA. (C) Expression of *pkn41* and *pkn42* in the wild type (WT) or the *ntcA* mutant CSE2 determined by RT-PCR. Cells were cultured in BG11 with ammonium as nitrogen source. The relative amount of total RNA was controlled by *rnpB*. Total RNA was prepared from cells cultured in the presence of iron (+Fe) or in the absence of iron (−Fe).

Since *pkn41* and *pkn42* are closely linked on the chromosome, we sought to determine whether they could be cotranscribed (Fig. 2B). Because these two genes were induced under conditions of iron limitation, total RNAs were prepared from the mutants and the wild type under both normal and iron-limiting conditions. As shown in Fig. 2B, a 775-bp cDNA fragment corresponding to the intergenic region between *pkn41* and *pkn42* could be amplified by RT-PCR from the wild-type strain. Using similar RT-PCR conditions, the amount of this cDNA fragment obtained was higher with cells cultured in the absence of iron than with cells cultured in the presence of iron. These results suggested that these two genes could be cotrans-

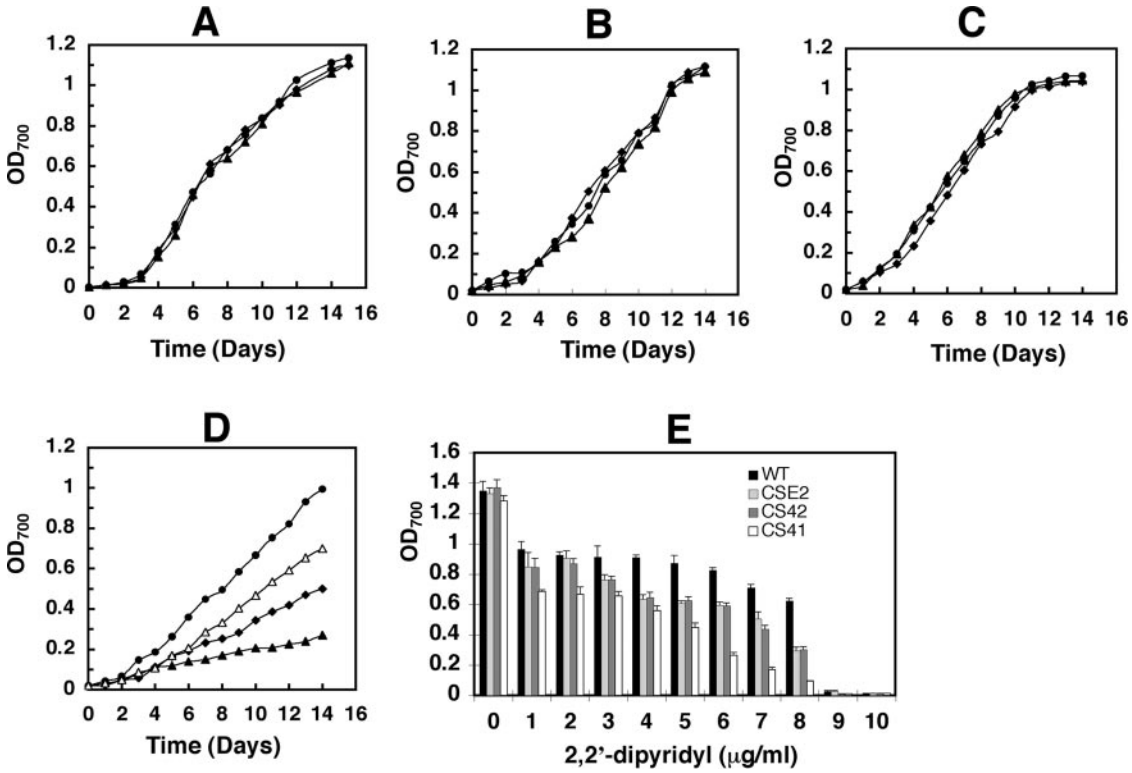

FIG. 4. Growth of the wild type (WT) and various mutants under different conditions. The optical density at 700 nm (OD_{700}) for all strains was adjusted to 0.02 at the beginning of the growth measurement. Strains were grown in BG11 in the presence of NH_4Cl as a source of nitrogen (A), in $BG11_0$ under conditions of nitrogen fixation (B), in BG11 with NH_4Cl without addition of ferric ammonium citrate (C), or in $BG11_0$ under conditions of nitrogen fixation without ferric ammonium citrate (D). Symbols: filled circle, wild type strain; filled triangle, CS41; diamond, CS42; open triangle, CS41 complemented by pKN2541 bearing *pkn41*. (E) Growth ability of different strains in BG11 (with ammonium as nitrogen source) in the presence of increasing amounts of the iron chelator 2,2′-dipyridyl. Cells were grown for 8 days, and the OD_{700} was determined. Error bars indicate standard deviations from triplicate cultures.

cribed. This conclusion was further supported by the fact that a transcript corresponding to that of *pkn42* was not found in the mutant strain CS41, in which *pkn41* was inactivated. In the mutant strain CS42, a transcript of *pkn41* could still be detected, although that of *pkn42* itself was absent as expected. In these RT-PCR experiments, we used as a control the *isiB* gene encoding flavodoxin, which is known to be inducible by iron limitation (14, 24, 40, 44, 48). Another gene, *rnpB*, was used as a control to ascertain that a similar amount of RNA was used in these RT-PCR experiments (Fig. 2B).

Expression of *pkn41* is regulated by NtcA. A putative binding site of NtcA 80 bp upstream of the translational start codon of *pkn41* could be predicted (Fig. 3A). In order to test whether NtcA could bind to this site, we performed electrophoretic mobility shift assays (Fig. 3B) with NtcA (27). The DNA fragment containing the promoter region was labeled with ^{32}P, incubated with or without NtcA, and separated by native gel electrophoresis. As shown in Fig. 3B, the promoter region of *pkn41*, when incubated with NtcA, formed a retarded band which could correspond to the DNA-NtcA complex. The promoter region of *glnA*, a gene which encodes the glutamine synthetase and is a well-known target of NtcA, was also retarded as previously reported (23, 42). The formation of the DNA-protein complex was unaffected by the presence of an excessive amount of an unrelated DNA fragment (the putative promoter region of alr7219) (data not shown). In some reported cases, the DNA-binding activity of NtcA is affected by the presence of DTT (1, 20). Since iron content may affect the electron flow, and thus the redox state, we have tested the effect of DTT on the binding of NtcA to the promoter region of *pkn41*. While the presence of DTT stimulated the binding activity of NtcA to the promoter region of *glnA* (Fig. 3B) as previously described (20), no effect of DTT on the binding of NtcA to the promoter region of *pkn41* was found (Fig. 3B).

We have further compared the levels of expression of *pkn41* and *pkn42* under conditions of iron stress between the wild-type strain and strain CSE2 in which the *ntcA* gene was inactivated (11). RNAs were isolated from cells cultured in the absence of iron for 5 days and used for the analysis of expression levels of *pkn41* and *pkn42* by RT-PCR (Fig. 3C). Iron-replete and iron-limiting conditions were compared, and the wild type displayed an induction in the expression of both *pkn41* and *pkn42* cultured in a medium without iron. In the mutant strain CSE2, the induction of the expression of *pkn41* and *pkn42* was no longer observed under similar conditions (Fig. 3C). This result suggested that NtcA was required for the inducible expression of *pkn41* and *pkn42* under iron stress. As expected, in these experiments the expression of the *isiB* gene was induced in samples cultured under conditions of iron lim-

TABLE 2. Total iron content in wild-type and mutant strains under different culture conditions

Strain	Iron content (μg/g dried cells) under culture condition:			
	BG11	BG11 without iron[a]	$BG11_0$	$BG11_0$ without iron[a]
WT[b]	5,827.6 ± 705.2	758.9 ± 98.7	5,720 ± 114.5	787.4 ± 91.0
CS41	2,772.7 ± 98.8	245.7 ± 57.5	2,453.4 ± 178.8	240.6 ± 23.7
CS42	2,974.9 ± 338.4	364.7 ± 21.8	3,717.5 ± 174.5	374.1 ± 30.2
CSDM	2,027.9 ± 184.9	122.3 ± 9.2	2,750.4 ± 227.1	131.3 ± 12.1

[a] Ferric ammonium citrate was omitted from the medium.
[b] WT, wild type.

itation (48). These results indicated that these cells were indeed starved of iron under the experimental conditions.

Growth of mutants in which *pkn41* and *pkn42* were inactivated was impaired under conditions of iron stress. The growth ability of the mutants CS41 and CS42 under different conditions was examined (Fig. 4). When either ammonium (Fig. 4A) or nitrate (data not shown) was used as a source of combined nitrogen, both mutants grew similarly to the wild type. The same was true when they were grown in $BG11_0$ medium without a source of combined nitrogen (Fig. 4B), suggesting that these two mutants were able to fix N_2 like the wild type; heterocysts were formed normally in both mutants as observed under the microscope (data not shown). Therefore, the nature of the nitrogen source had no effect on the growth capacity of these two mutants versus the wild type. When the iron source, ferric ammonium citrate, was removed from the BG11 culture medium, both mutants could also grow like the wild type, indicating that the presence of a trace amount of iron in the growth medium (1.33 μM contaminating iron from other chemicals used for the preparation of BG11 medium) was sufficient to support the growth of *Anabaena* strain PCC 7120, albeit at a growth rate slower than that observed in the presence of ferric ammonium (Fig. 4C).

When both ferric ammonium citrate and the source of combined nitrogen were removed from the growth medium, both CS41 and CS42 grew very slowly compared to the wild type (Fig. 4D). The mutant CS41 was more severely affected than CS42, consistent with the findings that these two genes were cotranscribed and the inactivation of *pkn41* abolished the transcription of *pkn42*, while the inactivation of *pkn42* left *pkn41* still functional (Fig. 2B). When a large chromosomal region including both *pkn41* and *pkn42* was deleted, the resulting mutant strain CSDM grew similarly to CS41 (data not shown), further supporting the observation that the insertion of an antibiotic resistance cassette into the coding region of *pkn41* abolished the transcription of both *pkn41* and *pkn42*. When the mutant CS41 was complemented with a replicative plasmid (pKN2541, pRL25::*pkn41*) bearing both the promoter region and the entire coding region of *pkn41*, the complemented strain recovered its growth to the level between those of the wild-type strain and the CS42 mutant when both iron and combined nitrogen were limited in the growth medium (Fig. 4D). The fact that the growth rate of this complemented strain did not recover to the level of the wild type was consistent with the results indicating that *pkn42* was not transcribed in the *pkn41* mutant CS41.

We have also tested the growth of CS41 and CS42 when iron was severely limited by the presence of the iron chelator 2,2′-dipyridyl (Fig. 4E). As the concentration of the iron chelator increased, the optical densities reached within the same period in the two mutants were lower and lower than that of the wild-type strain. The optical density of all cultures was adjusted to 0.02 before the addition of the iron chelator, but after 8 days in the presence of 8 μg/ml of iron chelator, CS41 and CS42 reached optical densities that were only 15.5% and 48.2%, respectively, of that of the wild type under similar conditions (Fig. 4E). We have also checked the growth rate of the *ntcA* mutant CSE2, and the results indicated that it grew less well than the wild type when iron became more and more sequestered by the presence of the iron chelator (Fig. 4E). As reported previously (11), CSE2 did not grow in the presence of nitrate or under conditions of nitrogen fixation but grew similarly to the wild type and CS41 and CS42 when NH_4Cl was used as a source of combined nitrogen (data not shown).

The CS41 and CS42 mutants contained less iron than the wild type. Since *pkn41* and *pkn42* were induced by iron stress and required for growth when the demand for iron was high, they could be involved in the regulation of iron acquisition or storage. The iron content in the wild type and the two mutants was measured under different growth conditions. The results are shown in Table 2. The CS41 mutant displayed the lowest iron content under all culture conditions tested. Its iron contents represented 47%, 32%, and 42% relative to those in the wild type when filaments were cultured in normal BG11 medium, BG11 without the iron source ferric ammonium citrate, and $BG11_0$, respectively. The iron content in the CS42 mutant was also lower than that found in the wild type but higher than that found in the CS41 mutant. These results indicated that *pkn41* and *pkn42* were involved in the regulation of iron homeostasis in *Anabaena* strain PCC 7120, and their inactivation led to lower iron content in the cells. We have also measured the iron content in the mutant CSDM strain in which both *pkn41* and *pkn42* were deleted, and the iron contents in this mutant were also lower than in the wild type and the CS42 mutant (Table 2).

Consistent with the lower iron content in the CS41 and CS42 mutants, we found that the expression of both *nifJ1* (alr2803) and *nifJ2* (alr1911) was highly activated in these mutants even under iron-replete conditions (Fig. 5). The *nifJ* gene was shown

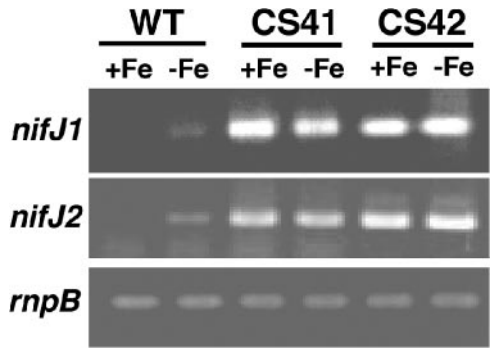

FIG. 5. Analysis by RT-PCR of expression of *nifJ1*, *nifJ2*, and *rnpB* in the wild type (WT) and mutant strains CS41 and CS42. Different strains were cultured in BG11 (with ammonium as nitrogen source) with (+Fe) or without ferric ammonium citrate (−Fe).

to be induced when iron was limited in *Anabaena* strain PCC 7120 (3). Consistent with the published results, no transcripts of *nifJ1* and *nifJ2* were detected in the wild type when iron was present in the growth medium, but the expression of both genes was induced when the iron source was removed from the culture medium. In both the CS41 and CS42 mutants, the levels of transcripts for *nifJ1* and *nifJ2* were highly abundant compared to those found in the wild type, even when iron was present in the growth medium. These results suggested that the two mutants experienced iron-limiting stress, consistent with their lower iron contents being measured under different conditions (Table 2).

DISCUSSION

In this study, we showed that two members of the *hstK* gene family, adjacent on the chromosome, were cotranscribed in response to iron limitation. This conclusion was supported by several experimental data. First, both genes showed similar expression patterns under the conditions tested. Second, an intergenic region between *pkn41* and *pkn42* could be detected, and a transcript corresponding to that of *pkn42* could no longer be detected in the CS41 mutant. Third, the phenotypes of the CS42 mutant were in general weaker than the CS41 mutant, which in turn resembled those observed for the mutant inactivating both *pkn41* and *pkn42*. The expression of *pkn41* and *pkn42* was induced by iron limitation but unaffected by the nature of the nitrogen source. Alternatively, it is possible that the expression of *nifJ1* and *nifJ2* was negatively regulated, directly or indirectly, by *pkn41* and *pkn42*. In this case, the high-level induction of *nifJ1* and *nifJ2* in the CS41 and CS42 mutants could be the result of the inactivation of *pkn41* or *pkn42* rather than a response to lower iron content in these mutants than in the wild type.

Wild-type *Anabaena* strain PCC 7120 can grow by relying on the presence of a trace amount of iron (estimated to be at least 1.33 μM as contaminant from other chemicals) in the growth medium without addition of the iron source ferric ammonium citrate. This observation suggests that this cyanobacterium has highly efficient systems to acquire iron for growth. Consistently, filaments cultured in the absence of ferric ammonium citrate took several days to display a spectral shift characteristic of the presence of CP43′ encoded by *isiA*, a gene known to be induced by iron starvation (4, 14, 24, 40, 44, 48). For the mutants CS41 and CS42, we found two conditions which affected their growth ability; the first was when they were grown under conditions of nitrogen fixation while iron was limiting, and the second was when iron was severely limited due to the presence of the iron chelator 2,2′-dipyridyl. As suggested previously (3), the appearance of an abundance of the iron-rich nitrogenase necessary for nitrogen fixation may create a demand for iron that is higher than normal. Since in both CS41 and CS42, the content of iron was low compared to that in the wild type, the iron availability within these cells could be highly limited under conditions of nitrogen fixation. Similarly, the presence of increasing amounts of the iron chelator increasingly sequestered the already limited supply of iron available in these mutants, thus negatively affecting their growth more severely than that of the wild type. The regulation of *pkn41* and *pkn42*, the iron content, and the growth defect in CS41 and CS42 all suggest that these two genes regulate iron acquisition or storage under different conditions, and this regulation could be coupled to nitrogen metabolism through the action of NtcA. The high-level induction of *nifJ1* and *nifJ2* in the two mutants was a good indication that these cells underwent iron limitation stress within the cells. Curiously, the *isiB* gene is not induced in these mutants although it is also known to respond to iron limitation (14, 24, 40, 44, 48). The *isiB* gene is unlikely to be in the same regulatory pathway as *nifJ1* and *nifJ2*, and these genes may respond to different levels of iron limitation. The lower level of iron in the *pkn41* and *pkn42* mutants may be sufficient to induce the expression of *nifJ1* and *nifJ2* but not sufficient to induce the expression of *isiB*.

NtcA regulates a number of genes involved in nitrogen and carbon metabolism in cyanobacteria (18, 19). It is becoming increasingly evident that NtcA not only controls the expression of genes related to nitrogen metabolism but also coordinates different processes (41). In the present study, we provide evidence that NtcA regulates the expression of *pkn41* and *pkn42* according to the availability of iron. A conserved binding site exactly matching the consensus binding site of NtcA was identified upstream of the coding region of *pkn41* and confirmed by DNA-binding assay. Furthermore, in the *ntcA* mutant CSE2, the induction of the expression of *pkn41* and *pkn42* was no longer observed. The *ntcA* mutant CSE2 itself was affected in its ability to adapt to iron limitation in the presence of the iron chelator 2,2′-dipyridyl. Therefore, we conclude that NtcA directly controls the expression of *pkn41* and that NtcA is also involved in the control of iron homeostasis in *Anabaena* strain PCC 7120. NtcA may not be the only regulator for the expression of *pkn41* and *pkn42*. When cells were grown with nitrate or N_2, NtcA activated the expression of genes involved in nitrogen assimilation (6, 11, 13, 46), but *pkn41* and *pkn42* were not induced under similar conditions. In addition to NtcA, there could be either another activator involved in the expression of *pkn41* and *pkn42* or a repressor that prevented their expression when iron was present. The existence of an additional regulator may also explain the disappearance of the basal-level expression of *pkn41* and *pkn42* in an *ntcA* mutant in the absence of iron (Fig. 3C). Further studies will be needed to test these possibilities.

NtcA of cyanobacteria and Crp of *Escherichia coli* belong to the same family of transcription factors (18, 19, 43). Crp regulates carbon metabolism in response to carbon availability (34). Similarly, NtcA regulates mainly nitrogen metabolism according to nitrogen availability. In *E. coli*, it has been reported that Crp and the iron regulon repressor Fur interact to coordinate carbon and iron utilization by regulating the expression of *sdhA* to *-D*, *sucA* to *-D*, and *funA*, genes encoding enzymes of the Krebs cycles (52). Similarly, NtcA in cyanobacteria orchestrates different cellular processes, such as nitrogen metabolism and iron content, as suggested in this study.

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