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ppGpp Metabolism Is Involved in Heterocyst Development in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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When deprived of a combined-nitrogen source in the growth medium, the filamentous cyanobacterium *Anabaena* sp. PCC 7120 (*Anabaena*) can form heterocysts capable of nitrogen fixation. The process of heterocyst differentiation takes about 20 to 24 h, during which extensive metabolic and morphological changes take place. Guanosine tetraphosphate (ppGpp) is the signal of the stringent response that ensures cell survival by adjusting major cellular activities in response to nutrient starvation in bacteria, and ppGpp accumulates at the early stage of heterocyst differentiation (J. Akinyanju, R. J. Smith, FEBS Lett. 107:173–176, 1979; J Akinyanju, R. J. Smith, New Phytol. 105:117–122, 1987). Here we show that *all1549* (here designated rel_{ana}) in *Anabaena*, homologous to *relA/spoT*, is upregulated in response to nitrogen deprivation and predominantly localized in vegetative cells. The disruption of rel_{ana} strongly affects the synthesis of ppGpp, and the resulting mutant, *all1549*Ωsp/sm, fails to form heterocysts and to grow in the absence of a combined-nitrogen source. This phenotype can be complemented by a wild-type copy of rel_{ana}. Although the upregulation of *hetR* is affected in the mutant, ectopic overexpression of *hetR* cannot rescue the phenotype. However, we found that the mutant rapidly loses its viability, within a time window of 3 to 6 h, following the deprivation of combined nitrogen. We propose that ppGpp plays a major role in rebalancing the metabolic activities of the cells in the absence of the nitrogen source supply and that this regulation is necessary for filament survival and consequently for the success of heterocyst differentiation.

The stringent response is primarily defined as inhibition of rRNA and protein synthesis in response to amino acid starvation in *Escherichia coli* (1). In addition to amino acid starvation, the stringent response is also involved in cell regulation under other stress conditions such as limitation of carbon (2), of iron (3), and of fatty acid (4), oxidative stress (5), or acid shock (6). This mechanism allows bacteria to divert cellular resources from growth toward survival and thus constitutes an important means for cellular adaptation under stress conditions. The stringent response is mediated by the signal molecules guanosine tetraphosphate (ppGpp) and guanosine pentaphosphate (pppGpp), collectively referred to as ppGpp. In *E. coli*, these signaling molecules are produced by two homologous enzymes, RelA and SpoT. RelA is involved in ppGpp synthesis, and SpoT is a bifunctional enzyme which contributes predominately to ppGpp degradation or slightly to ppGpp synthesis (1). However, by bioinformatics analysis, Mittenhuber found that most bacterial genomes encode only one dual-function enzyme which displays both synthesis and degradation activities (7). ppGpp signaling is widely present not only in bacteria but also in plants (8, 9). In *Arabidopsis*, the At-RSH1, a Rel/SpoT homolog, can complement the *rel* mutant phenotypes in *E. coli* and *Streptomyces coelicolor* A3 (2, 8). Recently, studies in metazoa provided evidence that ppGpp may also be present in animals; indeed, Mesh1 encoding ppGpp hydrolase was found in *Drosophila* and human (10). In *Drosophila*, deletion of Mesh1 led to body growth retardation and starvation resistance damage (10). These results imply that the control of cellular processes by ppGpp is evolutionarily conserved.

ppGpp acts as a global regulator in the control of a variety of developmental processes in bacteria such as biofilm formation in *Listeria monocytogenes* (11), quorum sensing in *Pseudomonas aeruginosa* (12), fruiting body development in *Myxococcus xanthus* (13), antibiotic production in *Streptomyces* (14, 15), and virulence in *Legionella pneumophila* (16). *Anabaena* sp. PCC 7120 (here *Anabaena*) is a filamentous nitrogen-fixing cyanobacterium that forms heterocysts when grown in a medium deprived of combined nitrogen. Heterocysts are specialized cells and provide a micro-oxic environment suitable for nitrogen fixation catalyzed by the oxygen-sensitive nitrogenase (17). Several hundreds of genes are involved in heterocyst differentiation (17, 18); among them, NtcA and HetR are key regulators required for the initiation of heterocyst development. NtcA is a global nitrogen-control transcription factor, and its DNA-binding activity is regulated by 2-oxoglutarate through an allosteric control mechanism (19, 20). HetR, also a DNA-binding protein, is a master regulator specifically required for the initiation of heterocyst development (21). A *hetR* mutant fails to form heterocysts, whereas overexpression of *hetR* increases heterocyst frequency (22–24). Expression of *ntcA* and *hetR* shows a mutual dependency during heterocyst development (25, 26).

Heterocyst development is induced upon deprivation of combined nitrogen, and this process lasts about 20 to 24 h before mature heterocysts can fix nitrogen to support filament growth. During this long period, extensive metabolic modification, such as the degradation of nitrogen reserves, occurs to ensure that the cells can survive during this period of nitrogen limitation (27). Since the stringent response plays critical roles in cell adaptation under nutrient limitation conditions in various bacteria, we wondered whether ppGpp signaling could be involved in the process of het-

Received 20 June 2013 Accepted 1 August 2013
Published ahead of print 9 August 2013
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doi:10.1128/JB.00724-13

TABLE 1 Strains and plasmids

Strain or plasmid	Description[a]	Source
Strains		
Anabaena sp. PCC 7120	Wild type	Pasteur Culture Collection
*Anabaena all1549*Ωsp/sm	Disruption rel_{ana}; Sp^r Sm^r	This study
Anabaena C1549	Nm^r Sp^r Sm^r; pRL25T-C1549 introduced into *all1549*Ωsp/sm strain	This study
E. coli MG1655	Wild-type K-12 strain	37
E. coli CF1693	MG1655 Δ*relA251* Δ*spoT207*	37
Plasmids		
pGEX-6p-1	Expressing vector	GE Healthcare
pGEX-6p-1549	pGEX-6p-1 carrying rel_{ana} ORF	This study
pRL277	Sm^r Sp^r; *sacB*-bearing cloning vector	33
pRL277-HRS	Sm^r Sp^r; pRL277 carrying an internal fragment (from position 277 to 1173 relative to the translational initiation codon) of the rel_{ana} gene	This study
pRL25T	Km^r Nm^r; pDU1-based shuttle vector	32, 45
pRL25T-p69-*gfp*	Km^r Nm^r; pRL25T carrying rel_{ana} promoter and *gfp* coding sequence	This study
pRL25T-C1549	Km^r Nm^r; pRL25T containing rel_{ana} ORF and native promoter, used to complement mutant	This study
pRL25T-P_{petE}-*hetR*	Km^r Nm^r; pRL25T carrying P_{petE}-*hetR* fusion	This study
pRL25N-1549-*lgfp*	Km^r Nm^r; pRL25N-*lgfp* carrying rel_{ana} under the control of native promoter	This study
pRL25N-ACT-*lgfp*	Km^r Nm^r; pRL25N-*lgfp* carrying rel_{ana} without ACT domain coding sequence under the control of native promoter	This study

[a] Km, kanamycin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin.

erocyst development. In a previous study, Ning et al. reported that *all1549* (*ana-rsh*), a *spoT* homolog in *Anabaena*, could complement the *relA spoT* mutant in *E. coli*. They were unable to inactivate *all1549* and thus suggested that *all1549* was essential for cell growth but with no link to heterocyst differentiation (28). On the basis of the present study, we report that the *all1549* gene, here proposed as rel_{ana} following the recent nomenclature (29), is required for diazotrophic growth of *Anabaena* and that the Rel_{ana} protein is localized specifically in vegetative cells. Our results indicate that rel_{ana} is involved in heterocyst development because it is required for cell survival during nitrogen step-down.

MATERIALS AND METHODS

Bacterial strains and growth conditions. All strains and plasmids used in this study are described in Table 1. *Anabaena* and its derivatives were grown in BG11 medium (with nitrate) or $BG11_0$ medium (without combined nitrogen), as described previously (30). All cyanobacterial strains were grown at 30°C under conditions of illumination (~40 μE m^{-2} s^{-1}). Neomycin (50 μg ml^{-1}) or spectinomycin (10 μg ml^{-1}) was added to the culture medium when necessary. For the induction of amino acid starvation, serine hydroxamate (SHX; Sigma) was added to the culture at the final concentration of 1 g $liter^{-1}$ (31). *E. coli* strains were grown in LB or minimal medium (MM). Plasmids were transferred from *E. coli* into *Anabaena* strains by conjugation as described previously (32).

Plasmid construction. *E. coli* strain TG1 was used for all cloning experiments. All constructs were verified by DNA sequencing. All primers used in this study are listed in Table 2.

Plasmid pRL277-HRS for rel_{ana} gene disruption was constructed with the suicide vector pRL277 (33). Plasmid pRL277-HRS contained an internal fragment of rel_{ana} (from position 277 to 1173 starting with the corresponding ATG codon) amplified by PCR using primers HRS-1 and HRS-2. The amplified fragment was digested with BglII and XhoI and subsequently ligated into the pRL277 suicide vector digested by the same enzymes.

To complement the *all1549*Ωsp/sm mutant, the rel_{ana} gene and its promoter region were amplified by PCR using primers 1549gfp-1 and oe1549-2. The amplified fragment was digested with BamHI and XhoI and then cloned into the same sites of pRL25T. The final construct was named pRL25T-C1549. The pRL25T-p69-*gfp* plasmid was used to express the transcriptional fusion between rel_{ana} and the green fluorescent protein (GFP) gene, *gfp*. The promoter region of rel_{ana} was amplified by PCR using primers 1549gfp-1 and p69-2. The amplified fragment was digested with BamHI and PstI, cloned between the same sites of pBS-*gfp*, and then re-excised as a BamHI-XhoI fragment and inserted into the pRL25T shuttle vector between the BamHI and XhoI sites to give pRL25Tp69-*gfp*. The translational GFP fusion under the control of the rel_{ana} promoter was amplified using primers 1549gfp-1 and Del_1549-2. This fragment was digested by BamHI and XhoI and then cloned into pRL25N-*lgfp*, which was derived from pRL25T bearing the *gfp* coding sequence (unpublished data) to produce pRL25N-1549-*lgfp*. Plasmid pRL25N-ACT-*lgfp* was a

TABLE 2 Primers used in this study

Primer	Sequence (5′ to 3′)
HRS-1	CCTCTGCAGGGATCCATGATGTAGTTGAAGATACAGA
HRS-2	CCTAGATCTACTTTGCCATTCCAATAATT
oe1549-1	CTTGAATTCATGAGCAGCATCGCTATTAGTTCCC
oe1549-2	CTTCTCGAGTCATTCATCAATTTGACCAACACG
1549gfp-1	CTTGCGGCCGCGGATCCAAGAGTTGCTTTAACCCAGC
1549gfp-2	CCTCATATGACAATAGAAGAGGTGATCGG
Del_1549–2	CTGCAGCTCGAGTTCATCAATTTGACCAACACGGCG
Del_ACT-2	CTGCAGCTCGAGGACTTCGCCATAACCCATAC
PpetE-1	CTTGGATCCTAAAGCCTGTGAAATTAACTG
petER-2	GTTACTCATATGCGTTCTCCTAACCTGTAG
oehetR-1	AGAACGCATATGAGTAACGACATCGATCTGA
oehetR-2	CTTCTCGAGGCCGAGTCATTTGTCATCAC
rnpB-1	CCAGTTCCGCTATCAGAGAG
rnpB-2	GAGGAGAGAGTTGGTGGTAAG
hetR-1	TACTCTGGCACGGTGACAAG
hetR-2	AGGGCATAGAAGGGCATTCC
RT1549-1	GATAGCCGTCGTCGTTCAG
RT1549-2	TAATTCGCCAGATCCCTAAGC
cm1549-1	CATTTGTATCTGGTGGCTTTTGTT
cm1549-2	ACGCTATGTTCTCTTGCTTTTGTC

construct derived from Rel_{ana}-GFP fusion but with the corresponding ACT domain deleted. DNA fragment was amplified by PCR using primers 1549gfp-1 and Del_ACT-2 and then digested by BamHI and XhoI and cloned into pRL25N-*lgfp* to produce pRL25N-ACT-*lgfp*. To construct plasmid pGEX-6p-1549, we amplified the rel_{ana} gene by PCR using primers oe1549-1 and oe154-2. The 2,256-bp PCR product was digested by EcoRI and XhoI and cloned into vector pGEX-6p-1. The pRL25T-P_{petE}-*hetR* plasmid was made by overlapping extension PCR using primers PpetE-1 and petER-2 and primers oehetR-1 and oehetR-2. Subsequently, the P_{petE}-*hetR* fragment was digested by BamHI and XhoI and inserted into pRL25T at the same sites.

Construction of mutant. For construction of the *all1549*Ωsp/sm mutant, plasmid pRL277-HRS was introduced into wild-type *Anabaena* by triparental mating. The mutant was selected by positive selection with spectinomycin. The single-crossover mutant, here called *all1549*Ωsp/sm, was confirmed by PCR using wild-type DNA as a positive control. For complementation, plasmid pRL25T-C1549 was introduced into the *all1549*Ωsp/sm mutant by conjugation, and the complemented strain was named C1549.

Measurement of ppGpp by high-performance liquid chromatography (HPLC). The procedure for extraction of nucleotides with formic acid was adapted from those described previously (34, 35). For *E. coli*, 10 ml cells was collected from LB liquid culture by centrifugation. These cells were washed twice with MM and resuspended in an equal volume of MM (optical density at 600 nm [OD_{600}] at 0.5). A 1-ml volume of this suspension was immediately transferred into a new tube containing 0.1 ml of 11 M formic acid and then vigorously mixed and incubated on ice for 45 min. In parallel, a 1-ml suspension was cultured for the indicated times at 37°C in the presence of serine hydroxamate (SHX; Sigma) at a final concentration of 1 g $liter^{-1}$ followed by extraction with formic acid as described above. For *Anabaena*, 1.5 liters of cells (OD_{750} at 0.5) was collected by membrane filtration and then divided into three aliquots. One aliquot was resuspended in 100 ml BG11 medium and immediately extracted with 11 M formic acid. Another two parts were cultured for the indicated times in 100 ml BG11 medium plus 1 g $liter^{-1}$ SHX followed by collection with filtration and extraction with 11 M formic acid. After extraction, all samples were centrifuged at 12,000 rpm for 10 min at 4°C. The supernatant was filtered through 0.22-μm-pore-size filters, adjusted to pH 4.0 with KOH, and stored at −20°C.

For the assay of ppGpp, a sample of 90 μl was subjected to processing using an anion-exchange column (Partisil SAX 10; Whatman) (4.6 by 250 mm) at a flow rate of 1 ml min^{-1}. Nucleotides were separated by a gradient made of buffer A (7 mM KH_2PO_4; adjusted to pH 4.0 with H_3PO_4) and buffer B (0.5 M KH_2PO_4, 0.5 M Na_2SO_4; adjusted to pH 5.0 with KOH). The proportion of buffer B increased during 20 min from 0% (vol/vol) to 100% (vol/vol) and was then maintained for 20 min at 100% (vol/vol). Signals were detected by UV absorbance at 260 nm. The ppGpp standard was purchased from TriLink Biosciences. Standard curves were established using different concentrations of ppGpp, and the detection limit was approximately 100 nM.

RNA analysis. To isolate RNA for real-time quantitative reverse transcription-PCR (qPCR) analysis, 30 ml of cells was filtered quickly on a membrane (5 μm pore size). Total RNA was extracted using a hot phenol method (36). RNA samples were treated with RNase-free DNase I (Invitrogen). Quantitative PCR (qPCR) was carried out as described previously (36) using specific primers (Table 2).

Cell viability test. Cells were grown at the exponential phase and then collected and washed three times with $BG11_0$ medium. Cells were then adjusted to the initial optical density (OD_{750} = 0.12) and cultured into a medium deprived of combined nitrogen ($BG11_0$) at 30°C with shaking under conditions of illumination. To measure cell viability under such conditions over time, ammonium was added back to an aliquot at a different time point, and the aliquot was cultured to assess the ability to grow. Both optical density and chlorophyll *a* contents were monitored at various time points.

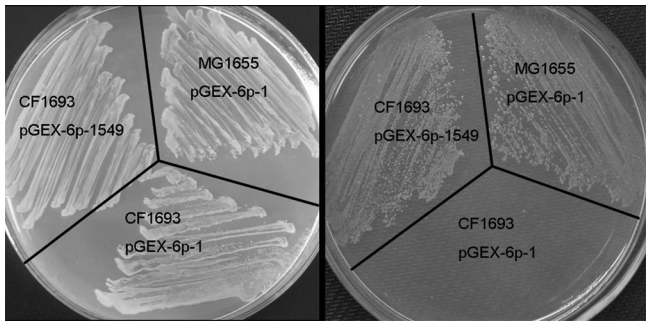

FIG 1 Complementation test of *E. coli rel spoT* mutant by rel_{ana} of *Anabaena*. The images show the results of complementation of CF1693, a strain with mutations in the *relA* and *spoT* genes, on LB (left) and MM (right) plates. MG1655, a strain of wild-type *E. coli*, was transformed with empty vector. CF1693 was transformed with the plasmids as indicated.

RESULTS

Rel_{ana} is capable of ppGpp synthesis. The Rel_{ana} protein of *Anabaena* is a protein homologous to RelA/SpoT in *E. coli*. It was previously called Ana-Rsh (28), and we propose here to use the designation Rel_{ana}, following the nomenclature of a recent review (29).To further confirm that rel_{ana} is functionally related to Rel/SpoT as proposed by Ning et al. (28), plasmid pGEX-6p-1549, which carries the entire coding region of rel_{ana}, was transformed into *E. coli* strain CF1693, a *relA/spoT* double mutant which cannot grow on minimal medium unless supplemented with multiple amino acids (37). The pGEX-6p-1 empty vector was used as a control. For growth analysis on LB and MM plates, the procedure was carried out as reported previously (31, 37). The CF1693 strain containing pGEX-6p-1 was unable to grow on a MM plate, while that containing pGEX-6p-1549 could grow under similar conditions, just as in the case of the wild-type MG1655 transformed with pGEX-6p-1 (Fig. 1). These results indicate that rel_{ana} was expressed in *E. coli* and produced a functional protein capable of ppGpp synthesis.

The rel_{ana} gene is required for diazotrophic growth and heterocyst differentiation. Rel_{ana} is composed of four domains: HD (38), RelA_SpoT, TGS (39), and ACT (40–42). The structural conservation of Rel_{ana} suggests that the corresponding gene plays an important role in the process of cell growth that is similar to that of SpoT as known in many other bacteria. In order to investigate the function of the rel_{ana} gene, we attempted to inactivate this gene. Because rel_{ana} forms a monocistronic unit (Fig. 2A), no polar effect on the transcription of its flanking genes was expected when the rel_{ana} gene was disrupted. We first attempted to inactivate the coding region by double homologous recombination with insertion of an antibiotic resistance cassette but failed despite many attempts. The reason for this failure remains unknown, but the failure is consistent with the negative result reported by Ning et al. (28). We thus tried a second approach using an internal gene fragment borne on a suicide vector for gene inactivation. Plasmid pRL277-HRS, containing an internal 897-bp fragment (from position 277 to 1173 relative to the translational initiation codon) of the rel_{ana} gene, was transferred into *Anabaena* by conjugation (43, 44). After selection, the insertion of the internal fragment on this suicide vector through homologous recombination would generate two partial alleles; neither of the alleles would be full, and thus, neither would be functional. After verification by PCR, the rel_{ana}

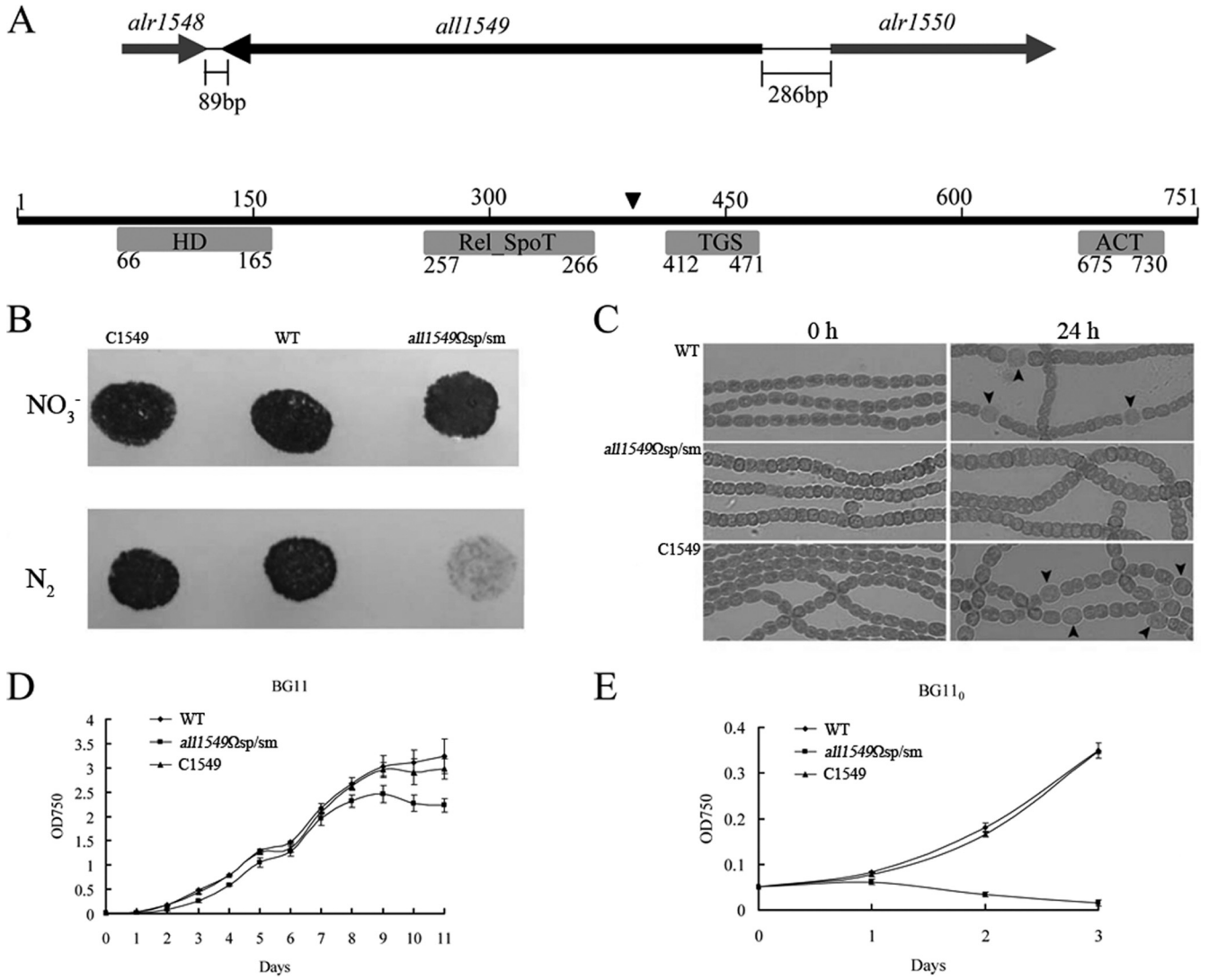

FIG 2 Phenotype of the *all1549*Ωsp/sm mutant. (A) Genomic context of rel_{ana} from *Anabaena*. The black triangle indicates the position of disruption following single-crossover recombination. (B) Growth on solid agar plates with nitrogen (NO_3^-) or without nitrogen (N_2). (C) Micrographs of the wild type (WT), the *all1549*Ωsp/sm mutant, and the complemented strain (C1549) after nitrogen deprivation. The arrowheads point to heterocysts. (D and E) Measurement of growth rate. Strains were grown in BG11 (+N) (D) or in $BG11_0$ (−N) (E). Error bars indicate standard deviations of the results determined from triplicate cultures.

gene was found to be disrupted between the RelA_SpoT domain and the TGS domain (Fig. 2A). The resultant mutant, *all1549*Ωsp/sm, was fully segregated as confirmed by PCR using gene- and vector-specific primers 1549gfp-1/oe1549-2 and cm1549-1/cm1549-2 (data not shown).

We checked the growth of the *all1549*Ωsp/sm mutant on either nitrogen-containing (BG11) or nitrogen-free ($BG11_0$) medium. The *all1549*Ωsp/sm mutant grew slightly more slowly than the wild-type strain when nitrate was used as a nitrogen source but completely failed to grow in the absence of a combined-nitrogen source (Fig. 2D and E). Furthermore, microscopic observation showed that the *all1549*Ωsp/sm mutant did not form heterocysts after induction by deprivation of combined nitrogen. In comparison, the wild-type strain formed heterocysts, as expected (Fig. 2C). To check if the phenotype of the *all1549*Ωsp/sm mutant was due to the disruption of the rel_{ana} gene, a complementation test was carried out. Plasmid pRL25T-C1549, constructed using a pDU1-based shuttle vector (32, 45) harboring the entire open reading frame (ORF) of rel_{ana} as well as its promoter region, was transferred into mutant strain *all1549*Ωsp/sm. The complemented strain, called C1549, grows at a rate slightly slower than that of the wild type when nitrate is present in the growth medium. When cultured in the absence of a combined nitrogen source, C1549 recovered the ability to grow as well as to form heterocysts just like the wild type (Fig. 2). We therefore conclude that rel_{ana} is required for heterocyst development.

Cellular and subcellular localization of Rel_{ana} protein. It was previously shown that rel_{ana} was downregulated in heterocysts by using a transcriptional fusion to *gfp* (28). We could confirm this result using a similar fusion, the pRL25T-p69-*gfp* plasmid (data not shown). We further tested the expression of rel_{ana} using qPCR and primers RT1549-1 and RT1549-2, following the deprivation of combined nitrogen. *hetR*, upregulated in response to nitrogen

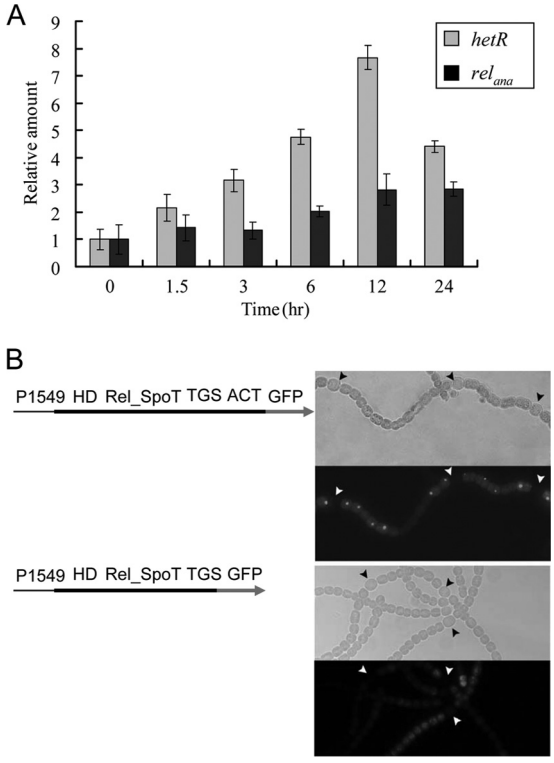

FIG 3 Spatiotemporal expression of rel_{ana}. (A) Transcriptional levels of rel_{ana} gene in wild type (WT). The result determined with the *hetR* gene was used as the positive control. The *rnpB* gene was selected as the reference gene for normalizing the data obtained by qPCR. (B) Localization of full-length and truncated Rel_{ana} in the wild-type strain bearing plasmids pRL25N-1549-*lgfp* and pRL25N-ACT-*lgfp*, respectively. P_{1549} represents the native promoter. The results were visualized under bright-field and fluorescence microscopy at 24 h after nitrogen starvation. Arrowheads indicate heterocysts.

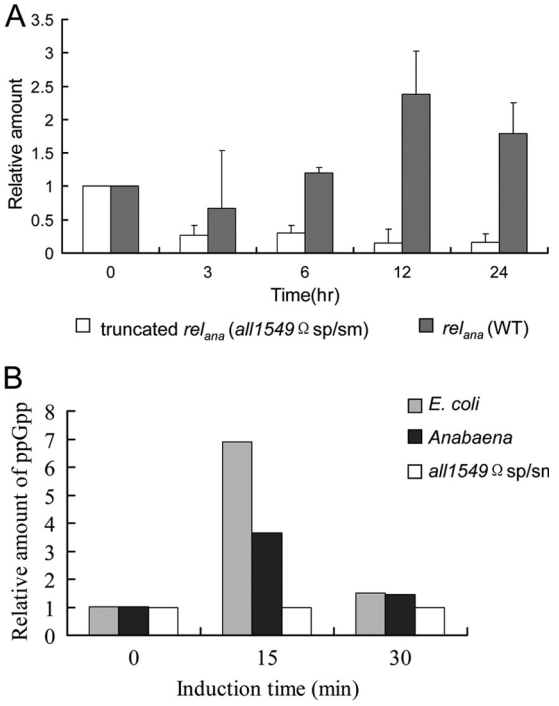

FIG 4 Determination of ppGpp level. (A) Transcriptional levels of the truncated form of rel_{ana} in the mutant *all1549*Ωsp/sm compared to that in the wild type were analyzed at the indicated times after nitrogen step-down. (B) ppGpp level in *Anabaena* induced by amino acid starvation. SHX was used at the final concentration 1 g $liter^{-1}$. Data shown are representative of the results of two independent experiments.

depletion in the wild-type strain (22), was used as a positive control. As shown in Fig. 3A, the transcriptional level of rel_{ana} was enhanced approximately 2-fold at 24 h after the culture was moved from nitrogen-replete to nitrogen-depleted medium. This result was similar to the data obtained by transcriptome analysis (46).

To test the localization of Rel_{ana} in the filament in the wild-type background, we constructed a Rel_{ana}-GFP translational fusion in which the *gfp* gene was fused at the 3′ terminus of rel_{ana} with its native promoter. Fluorescence foci, often one per cell, were observed in the vegetative cells under the nitrogen-deprivation condition, but such foci were absent from heterocysts (Fig. 3B). Previous reports suggested that SpoT homologous protein is aggregated in *E. coli* (47) and forms trimers in *Mycobacterium tuberculosis* (48), but the physiological significance remains unclear. When the ACT domain at the C-terminal end of Rel_{ana} was removed from the translational fusion, the fusion product was still localized in vegetative cells but the GFP-dependent fluorescence became diffuse compared to that from the full-length Rel_{ana}-GFP fusion (Fig. 3B), although both the *gfp* fusion constructs could complement the *all1549*Ωsp/sm mutant.

Disruption of rel_{ana} gene prevents accumulation of ppGpp. As the rel_{ana} gene was interrupted at the position corresponding to the region just downstream of the RelA_SpoT domain of the whole protein (Fig. 2A), we wondered what would be the effect on the level of ppGpp produced in the cells. We first examined if the disrupted rel_{ana} gene was still transcribed and would thus produce a truncated form of mRNA. Using qPCR analysis with primers RT1549-1 and RT1549-2, the transcriptional level of rel_{ana} was determined in both the wild type and the mutant. While expression of rel_{ana} in the wild type increased after the deprivation of combined nitrogen, expression of the truncated form of rel_{ana} in the *all1549*Ωsp/sm mutant was not upregulated and was detected at a much lower level than in the wild-type strain (Fig. 4A). This result further confirmed the disruption of rel_{ana} in the *all1549*Ωsp/sm mutant and demonstrated that the truncated form of this gene was expressed only weakly.

Since the truncated form of rel_{ana} may still contain a functional catalytic domain, we thus checked the level of ppGpp in both the wild type and the mutant by using the HPLC method (34). An *E. coli* strain was included as a positive control. Serine hydroxamate (SHX), which has been shown to elicit a strong amino acid starvation response in various organisms, was used to trigger the accumulation of ppGpp (31, 49, 50). For *E. coli*, we could show a pattern of ppGpp accumulation after the treatment with SHX, as previously reported (51). As shown in Fig. 4B, ppGpp accumulation reaches the level of 32 pmol ml^{-1} OD^{-1} in the wild-type *Anabaena* strain at 15 min after treatment by SHX, followed by a drop after 30 min of treatment, but remained higher than the basal level found at time zero. In contrast, ppGpp accumulation was not observed under similar conditions in the *all1549*Ωsp/sm mutant and remained at the basal level with or without treatment by SHX (Fig. 4B). Taken together, these results indicated that the

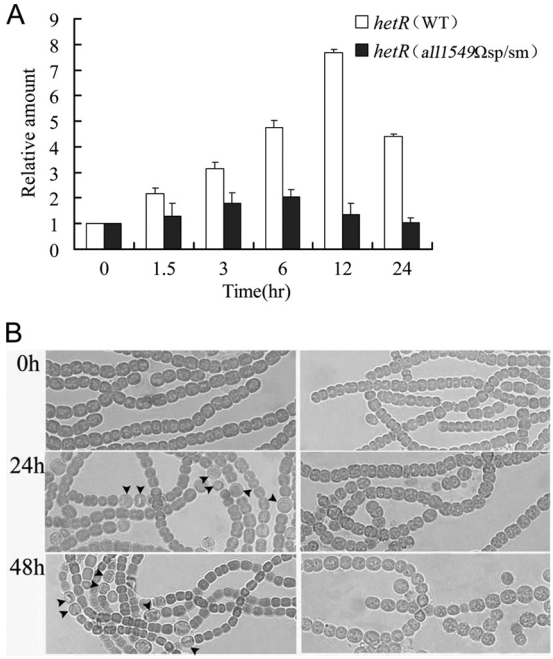

FIG 5 Ectopic expression of *hetR* in wild type (WT) and mutant. (A) Transcriptional levels of *hetR* were analyzed at the indicated times after nitrogen step-down. (B) Phenotypes after ectopic expression of *hetR* in the WT and *all1549*Ωsp/sm. Left panel, wild-type strain; right panel, *all1549*Ωsp/sm mutant strain. Arrowheads indicate the positions of heterocysts.

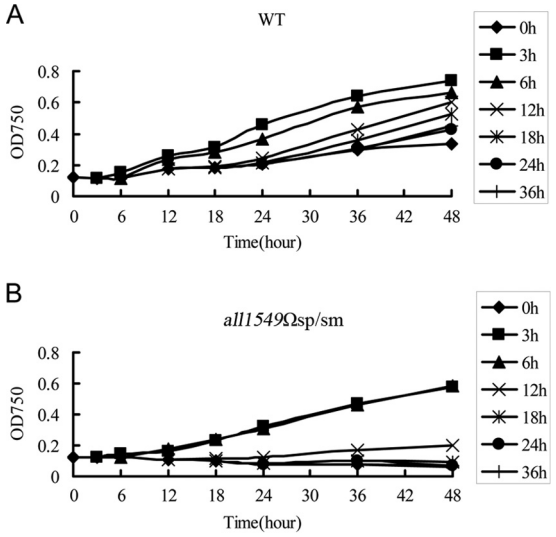

FIG 6 Cell viability test. (A) Wild-type strain. (B) *all1549*Ωsp/sm mutant. Data shown are representative of the results of two independent experiments. After nitrogen starvation, samples were taken and ammonium was added back at different time points as indicated by each curve: 0 h, 3 h, 6 h, 12 h, 18 h, 24 h, and 36 h.

*all1549*Ωsp/sm mutant produced a truncated form of mRNA, expressed at a very low level, and had a strong defect in ppGpp synthesis.

***hetR* induction is affected in the *all1549*Ωsp/sm mutant.** In *Anabaena*, the *hetR* gene is essential for the initiation of heterocyst development and is upregulated during the developmental process (22). To understand the reason why the *all1549*Ωsp/sm mutant fails to form heterocysts, we checked the transcript level of *hetR* by qPCR in the *all1549*Ωsp/sm mutant as well as in the wild type. In the wild-type strain, the expression of *hetR* increased about 7-fold at 12 h after deprivation of combined nitrogen. However, in the *all1549*Ωsp/sm mutant, the transcript level of *hetR* was increased only slightly under similar conditions (Fig. 5A). We also used transcriptional fusion to *gfp* as a reporter to examine the expression of *hetR*. In the wild-type strain, GFP fluorescence derived from the transcriptional fusion of *hetR-gfp* was detected after nitrogen step-down and mainly focused in the heterocyst after 24 h. In contrast, no GFP fluorescence was observed in the *all1549*Ωsp/sm mutant under similar conditions (data not shown). These results indicated that the inactivation of rel_{ana} impaired the regulation of *hetR* during heterocyst differentiation.

Since the upregulation of *hetR* was affected and heterocysts are not formed, we examined whether an ectopic expression of *hetR* could restore the capacity of *all1549*Ωsp/sm to form heterocysts. Plasmid pRL25T-P_{petE}-*hetR* containing *hetR* under the control of the copper-inducible promoter P_{petE} was transferred into both the wild type and the mutant. In the wild-type strain, overexpression of *hetR* increased the frequency of heterocysts and produced contiguous heterocysts as reported previously (Fig. 5B) (24). In contrast, no heterocyst was observed under similar conditions in the *all1549*Ωsp/sm mutant (Fig. 5B), even though the plasmid was well present and the overexpression of *hetR* occurred well in this strain as examined by qPCR (data not shown). Therefore, ectopic expression of *hetR* cannot bypass the need for ppGpp in heterocyst differentiation. The inactivation of rel_{ana}, and the drop in ppGpp levels may affect the cellular activities of which the upregulation of *hetR* is only part, suggesting that this mutation has a much broader effect (see below).

Recent studies have shown that both *hetP* and *hetZ* genes, acting at the early stages of heterocyst differentiation, are directly regulated by HetR and were able bypass the requirement of *hetR* when ectopically expressed (52, 53). Since overexpression of *hetR* failed to rescue the failure of heterocyst differentiation in the *all1549*Ωsp/sm mutant, these two genes were in turn overexpressed in the *all1549*Ωsp/sm mutant. Again, none of them could rescue the defect of heterocyst differentiation of the *all1549*Ωsp/sm mutant (data not shown).

Loss of ppGpp affects cell viability under conditions of nitrogen deprivation. Heterocyst differentiation takes about 20 h, during which no external nitrogen source is available to sustain cell growth. Heterocysts become mature enough to fix N_2 to ensure the growth of the filament only at the end of the differentiation process. The low expression level of the master regulator of heterocyst development, *hetR* alone, cannot provide a rational explanation for the phenotypes of the *all1549*Ωsp/sm mutant, since ectopic expression of *hetR* failed to rescue the phenotypes. We thus explored whether the viability of the filaments could be affected early enough so that heterocyst differentiation might not be initiated or sustained. For this experiment, filaments were cultured first under conditions of combined-nitrogen sufficiency and then deprived of the combined-nitrogen source. During different times following the step-down of combined nitrogen, ammonium was added back as a nitrogen source, and cell viability was tested by following the optical density or chlorophyll content (Fig. 6). Similar results were obtained with measurement of either the optical density or the chlorophyll content; thus, only the data corre-

sponding to the optical density measurement are shown here. For the wild type, the growth of the cells became faster when ammonium was added back, as expected, and this was true during the whole process of this experiment till 36 h after the combined-nitrogen step-down (Fig. 6A). In the *all1549*Ωsp/sm mutant, in contrast, cells could not grow under conditions of combined-nitrogen deprivation, as demonstrated earlier. When ammonium was added back 3 h after the step-down, cell growth resumed just as in the wild type. However, when ammonium was added back at or after 6 h, the filaments failed to recover their growth (Fig. 6B). Taken together, these results demonstrated that the inactivation of rel_{ana} affected the ability of the filaments to sustain their viability between 3 and 6 h after the induction of heterocyst differentiation.

DISCUSSION

ppGpp functions as a second messenger and is involved in various processes of cell growth and development. When the *rel* gene in *Rhizobium etli* is disrupted, both the morphology and the size of bacteroid are altered and nodules fail to fix nitrogen (54, 55), showing the importance of the stringent response in symbiosis. In *Sorangium cellulosum* So ce56, the Srel mutant is unable to form multicellular fruiting bodies under conditions of nutrient starvation (56). ppGpp also affected secondary metabolite biosynthesis (56–58).

In this study, we characterized a mutant in which the rel_{ana} gene was disrupted in *Anabaena*. The phenotype of the mutant can be complemented by a wild-type copy provided on a plasmid. Although a portion of the 5′-terminal end is still present and thus may encode a partial protein capable of ppGpp synthesis, we have two arguments indicating that even if this is true, the level of ppGpp present in this mutant must be very low. First, the 5′ portion of the rel_{ana} gene present in the chromosome gives only a low level of expression that is even lower, after induction of heterocyst differentiation, than the basal level detected at time zero; second, after treatment with SHX, the ppGpp accumulates rapidly in the wild type but not in the mutant. Thus, we conclude that the interruption of Rel_{ana} results in a very low level of ppGpp synthesis in the mutant. It was reported a long time ago that ppGpp levels increase at the early stage of heterocyst differentiation (59, 60). Our data further indicate that this ppGpp synthesis activity is required for heterocyst development. Indeed, no heterocysts are formed in the mutant that cannot grow under nitrogen-fixing conditions. The enhanced expression of *hetR* does not occur in the mutant compared to the wild type. While this may account for the failure in the initiation of heterocyst differentiation in many mutants, it is not the case for the *all1549*Ωsp/sm mutant since the defect cannot be rescued by overexpression of either *hetR* or its direct targets *hetZ* and *hetP*. Our data indicate that the inactivation of rel_{ana} produced a profound physiological defect, affecting the survival ability of *Anabaena* at the early stage of the transfer to nitrogen-fixing conditions. This effect makes the filament unable to respond properly to combined-nitrogen deprivation and thus to initiate heterocyst differentiation.

Heterocyst differentiation is a relatively long process, taking about 20 h before heterocysts can provide fixed nitrogen for filament growth. Therefore, during this long period of time, the cells must readjust their metabolism, in the absence of external nitrogen input, to maintain the viability of the filaments. We propose that ppGpp plays a role in the regulation of such metabolic rebalancing. Such a role mirrors what happens in *E. coli* under conditions of amino acid starvation or other nutrient starvation, during which major nutrient-consuming activities are shut down so that the cellular resources are redirected toward those activities essential for cell survival (5, 61). A similar situation may occur in *Anabaena*, where ppGpp accumulation at the early stage of heterocyst differentiation allows the cells to reprogram their metabolic activities so that the differentiation can proceed; otherwise, uncontrolled, nutrient-demanding activities may make the cells unable to keep pace with the demand for nutrients. The failure to properly activate expression of *hetR*, as well as that of several other genes (*hetP*, *hetZ*, *hetC*, and *ntcA*) involved in heterocyst development (data not shown), can be regarded as a consequence of such a misregulation. Our studies reveal a new mechanism involved in heterocyst development that ensures filament survival during the early stage of the differentiation process. Interestingly, we found that the amount of cyanophycin, an important nitrogen reserve for cyanobacteria (62), fell rapidly following nitrogen starvation in the *all1549*Ωsp/sm mutant, in contrast to the wild type, in which it remains stable or even increases, possibly as a safeguard response to nitrogen starvation and cell survival strategy (data not shown). This observation is consistent with the metabolic rebalancing necessary for the proper functioning of the developmental process.

ACKNOWLEDGMENTS

This work was supported by the National Natural Science Foundation of China (grant no. 31170048 and 30970088) and the Fundamental Research Funds for the Central Universities (program no. 2011QC070 and 2011PY090).

We are very grateful to Emmanuelle Bouveret for the gift of *E. coli* strains.

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