alr0117, a two-component histidine kinase gene, is involved in heterocyst development in Anabaena sp. PCC 7120

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Anabaena sp. PCC 7120 was mutagenized by transposon Tn5-1087b, generating a mutant whose heterocysts lack the envelope polysaccharide layer. The transposon was located between nucleotides 342 and 343 of alr0117, a 918 bp gene encoding a histidine kinase for a two-component regulatory system. Complementation of the mutant with a DNA fragment containing alr0117 and targeted inactivation of the gene confirmed that alr0117 is involved in heterocyst development. RT-PCR showed that alr0117 was constitutively expressed in the presence or absence of a combined-nitrogen source. hepA and patB, the two genes turned on during wild-type heterocyst development, were no longer activated in an alr0117-null mutant. The two-component signal transduction system involving alr0117 may control the formation of the envelope polysaccharide layer and certain late events essential to the function of heterocysts.

INTRODUCTION

Anabaena sp. PCC 7120 is a filamentous nitrogen-fixing cyanobacterium that produces heterocysts at semi-regular intervals when grown in nitrogen-free medium. Heterocysts are specialized cells surrounded by an outer polysaccharide layer and an inner glycolipid layer that prevents the entry of oxygen. Respiratory activity is enhanced in heterocysts to maintain a low-oxygen microenvironment and protect the activity of nitrogenase (Walsby, 1985; Murry & Wolk, 1989). It is known that the genes hepA, hepB, hepC, hepK and devR are involved in the formation of the polysaccharide layer (Holland & Wolk, 1990; Zhu et al., 1998; Wolk, 2000; Zhou & Wolk, 2003) and that the genes hetM (hglB), hglCD, devBCA and hglK are involved in the formation of the glycolipid layer (Black & Wolk, 1994; Bauer et al., 1997; Fiedler et al., 1998; Black et al., 1995) in Anabaena sp. PCC 7120. hetN, a gene positioned next to hetM and presumptively encoding a ketoacyl reductase, is required for maintenance of heterocyst pattern (Black & Wolk, 1994; Callahan & Buikema, 2001).

In the timetable of heterocyst development, genes required for initiation and morphogenesis are basically divided into two groups: (1) transcription of hetR, a gene essential to initiation of heterocyst development, and of hetC, a gene required for very early heterocyst development, is up-regulated within 3-5 h after deprivation of fixed nitrogen (Black & Wolk, 1993; Xu & Wolk, 2001); (2) from 5 to 10 h, hepA, hetM and devA, the genes involved in formation of the heterocyst envelope, are induced (Holland & Wolk, 1990; Cai & Wolk, 1997; Maldener et al., 1994). hetN is induced between 6 and 12 h after transfer into nitrogen-free medium (Bauer et al., 1997). hetR, hetC, hepA, devA and hetN are expressed specifically or primarily in heterocysts or proheterocysts.

patB is a gene required for nitrogen fixation but not morphogenesis of heterocysts. Its deduced product resembles a transcriptional regulator with a Fe4S4 ferredoxin region near its N-terminus and a helix–turn–helix motif near its C-terminus. A patB deletion mutant is unable to fix nitrogen, while a patB mutant defective in its N-terminal ferredoxin domain or without the C-terminal helix–turn–helix domain grows very slowly and produces multiple contiguous heterocysts in stationary-phase culture in nitrogen-free medium. Genes regulated by patB and those regulating patB are unknown. patB is specifically expressed in heterocysts starting from 12 to 18 h after nitrogen step-down (Liang et al., 1992; Jones et al., 2003).

Two-component signal transduction systems are important machineries for bacteria to regulate cell differentiation and other physiological processes in response to environmental or intracellular changes (Albright et al., 1989). The simplest two-component regulatory systems consist of a sensor histidine kinase, often located in the cytoplasmic membrane, and a cytoplasmic response regulator. Upon sensing a certain signal, an input domain of the sensor histidine kinase modulates the activity of its transmitter domain, which then auto-phosphorylates an internal histidine residue and transfers the phosphoryl group to a response regulator. Consequently, transcription of particular genes, or various other functions, are regulated. However, many
prokaryotic signalling systems have multiple components, interconnections with other regulatory circuits or feedback loops (Stock et al., 2000).

In the genome of *Anabaena* sp. PCC7120, there are 203 two-component signal transduction genes, 73 encoding sensory kinases, 77 encoding response regulators and 53 encoding hybrid sensory kinases and response regulators (Kaneko et al., 2001). *pata*, a gene involved in patterning of heterocysts, is predicted to be a response regulator without a DNA-binding domain (Liang et al., 1992). *hepK*, a gene involved in the formation of the polysaccharide layer, encodes a sensor histidine kinase, which controls the induction of *hepA* by interacting with DevRA (Zhu et al., 1998; Zhou & Wolk, 2003). In the work described here, by transposon mutagenesis of *Anabaena* sp. PCC 7120, we obtained a heterocyst development mutant, 1017, whose heterocyst envelope could not be stained by alcian blue, indicating that the polysaccharide layer was lacking or that heterocyst development was blocked at an early stage. Inverse PCR and DNA sequencing localized the transposon to the N-terminal portion of a two-component histidine kinase gene.

**METHODS**

**Cyanobacterial strains, culture conditions and conjugation.** *Anabaena* sp. PCC 7120 was obtained from the Freshwater Algae Culture Collection of the Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan (Song et al., 1999), and cultured in BG11 medium (Stanier et al., 1971) without shaking in the light (∼30 μE m⁻² s⁻¹). For the culturing of mutants or complemented strains, erythromycin (5 μg ml⁻¹) or spectinomycin (10 μg ml⁻¹) was added as required to the BG11 medium. Before transfer to BG110 (BG11 without nitrate), cyanobacterial cells were washed twice with the nitrogen-free medium. Conjuga
tions between *Escherichia coli* and *Anabaena* were conducted as described by Elhai & Wolk (1988).

**Transposon mutagenesis and selection of mutants.** *Anabaena* sp. PCC 7120 was mutagenized with Tn3-1087b as described by Ernst et al. (1992). The resulting transconjugants were inoculated on erythromycin-containing BG11 plates for further growth, then tested on BG110 plates for their capacity for diazotrophic growth under aerobic conditions. The mutants that turned yellow were tested in BG110 and then grown on BG11 plates for further growth, then tested to see if they were able to grow on BG11 plates containing erythromycin (5 μg ml⁻¹) or spectinomycin (10 μg ml⁻¹). Mutants that were unable to grow in the presence of these antibiotics were then tested on BG11 plates containing 30 μg ml⁻¹ of spectinomycin or spectinomycin and 5 μg ml⁻¹ of erythromycin. Mutants that did not grow in the presence of these antibiotics were then considered to be transposon mutants.

**Construction of a gene library for *Anabaena* sp. PCC 7120.** Total DNA of *Anabaena* sp. PCC 7120 was partially digested with *Sau3A*I at 4°C until most DNA fragments were between 0.5 and 9 kb, as revealed by gel electrophoresis. DNA fragments of 2–9 kb were then retrieved from the agarose gel and ligated with BamHI-cut and dephosphorylated pRL25C (Wolk et al., 1988). The ligated products were electroporated into E. coli DH10B, resulting in more than 2500 kanamycin-resistant colonies, of which about 60% contained inserts averaging 3–6 kb in size.

**Identification of transposon-interrupted genes and DNA fragments.** One microgram of genomic DNA was completely digested with *Alu*I and self-ligated in 200 μl reaction mixture at 16°C for 20 h. PCR amplifications were conducted using 2 μl of the ligation product as the template and 1087b-2 and 1087b-3 (Table 1) as the primers. The PCR products were sequenced with primer 1087b-1 (Table 1). Plasmid DNA extracted from complemented mutants was electroporated into E. coli DH10B and sequenced with primers pRL25V-1 and pRL25Cseq from both ends. Locations of the sequences were determined by searching the Kazusa genome database (www.kazusa.or.jp/cyano/cyano.html). The *alr0117* region was sequenced from PCR products generated using primers alr0117-4 and alr0117-5.

**Southern blot hybridization.** This was performed using a Dig High Prime DNA Labelling and Detection Starter Kit (Roche). Total DNA was restricted with HindIII, separated by electrophoresis on 0.7% agarose gel, and blotted onto a nylon filter by capillary transfer. The PCR fragment of *alr0117* amplified using primers alr0117-1 and alr0117-2 (Table 1) was used as the probe.

**Extraction of RNA.** Total RNA was extracted according to a method described for *Frankia* (Xu et al., 2002) with modifications. About 200 ml of *Anabaena* sp. PCC 7120 grown in BG11, or 24 h after transfer into BG110, was collected by centrifugation, washed once with 10 ml TE (10 mM Tris/HCl and 1 mM EDTA, pH 8–9), and resuspended in 2 ml LETS (100 mM LiCl, 10 mM EDTA, 10 mM Tris/HCl, 1% SDS, pH 8–9), which was then mixed with 2 ml glass beads (kept in double-distilled H₂O) and 2 ml phenol/ chloroform (1:1, v/v). The cells were broken by four rounds of vortexing (1 min) intermitted by cooling on ice (1 min) and the mixture was centrifuged at 6000 r.p.m. for 15 min. The supernatant was transferred into Eppendorf tubes. After addition of 0–2 mM LiCl and 2–5 vols ethanol to each tube, the nucleic acids were precipitated with 30 μl diethyl pyrocarbonate (DEPC)-treated double-distilled H₂O. The nucleic acid solution was treated with 6 units RNase-free DNase I (Takara) at 37°C for 40 min and repeated for six times.

**Table 1. Primers**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
</tr>
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<tbody>
<tr>
<td>glnA-1</td>
<td>GGGAGCAGCCTACCTTTG</td>
</tr>
<tr>
<td>glnA-2</td>
<td>GTGTGGGATCGAGAACC</td>
</tr>
<tr>
<td>hepA-1</td>
<td>TGGAGCTGTCGTCACCTT</td>
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<tr>
<td>hepA-2</td>
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<tr>
<td>hetM-1</td>
<td>TGGATTCGGCCAGGGCAG</td>
</tr>
<tr>
<td>hetM-2</td>
<td>ACTGGGTCAGAACTACCTC</td>
</tr>
<tr>
<td>patB-1</td>
<td>AGTGCGTTGAGTGTCGAAC</td>
</tr>
<tr>
<td>patB-2</td>
<td>AGTTGACATCTCCGGTTC</td>
</tr>
<tr>
<td>hglE-1</td>
<td>TGACTGCGTTAGCGGAGT</td>
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<td>hglE-2</td>
<td>AGCCTCGAGTATGCGGAC</td>
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<td>hetN-1</td>
<td>TGTCTGCTGGTTGCGGA</td>
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<td>hetN-2</td>
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<tr>
<td>prRL25V-1</td>
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<tr>
<td>prRL25Cseq</td>
<td>GCATTGGTAACGTGTCAGACC</td>
</tr>
<tr>
<td>alr0117-1</td>
<td>AGAGCGCGGATTATTTTGG</td>
</tr>
<tr>
<td>alr0117-2</td>
<td>ACAATACCTGTCTTAGCCATCT</td>
</tr>
<tr>
<td>alr0117-3</td>
<td>AGATGCTGCGATGCTGGAGC</td>
</tr>
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<td>alr0117-4</td>
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</tr>
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<td>alr0117-5</td>
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<td>1087b-1</td>
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<td>ACCTCGATACATTCGCCGTAG</td>
</tr>
<tr>
<td>1087b-3</td>
<td>TCAGATCTCCGGCGGATATCTCATC</td>
</tr>
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to eight rounds until no PCR product could be detected with the primers for RT-PCR. After each round, total RNA was precipitated and dissolved in RNase-free double-distilled H2O.

**PCR and RT-PCR.** PCRs were conducted in 50 µl volumes containing 10 mM Tris/HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 50 µM dNTPs, 100 pmol of each of the primers, 2 units Taq DNA polymerase (MBI) and appropriate template DNA. The reactions were initiated by 94°C for 5 min, followed by 30 cycles of: 94°C 1 min, 60°C 1 min and 72°C 1 min, and a final extension at 72°C for 5 min. For RT-PCR, first-strand cDNA was synthesized in a 25 µl reaction containing 50 mM Tris/HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM DTT, 50 µM dNTPs, 25 units RNasin, 200 units M-MLV reverse transcriptase (Promega), 2 µg total RNA and 0.5 µg random primers at 37°C for 60 min. The relative concentration of cDNA was evaluated after serial dilutions by PCR using primers glnA-1 and glnA-2 and adjusted to the same level according to the brightness of PCR bands. Two microlitres of the adjusted cDNA was used for PCR to detect the induced expression of genes. The primers are listed in Table 1.

**RESULTS**

**A mutant with alr0117 interrupted by Tn5-1087b**

Anabaena sp. PCC 7120 was mutagenized with Tn5-1087b, resulting in many mutants defective in heterocyst development (D. Ning & X. Xu, unpublished). In one of these mutants, 1017, the heterocysts could not be stained by alcin blue, did not produce prominent polar nodules and were generally smaller than those of the wild-type (Fig. 1). The fragment from nucleotides 27 to 568 of alr0117 was amplified using primers alr0117-1 and alr0117-2 and cloned into pMD18-T, a T-vector. The cloned fragment was excised by homologous single-crossover recombination. The fragment containing complete alr0117 and alr0120 (fragment I, Fig. 3). DNA fragments from this region were also able to complement several other mutants of similar phenotype but with Tn5-1087b inserted in other genes. For instance, mutant 2384 harbours the transposon within all5309, which encodes a two-component hybrid sensor and regulator, and was complemented by a fragment containing complete alr0115, alr0116 and alr0117 (fragment II, Fig. 3). Sequencing of the alr0117 region showed that there was a G to A substitution at nucleotide 552 in alr0117 of mutant 2384, which changed a codon for Trp (UGG) into a stop codon (UAG). The fragment complementing mutant 2384 also complemented mutant 1017, and vice versa.

**Inactivation of alr0117 by homologous single-crossover recombination**

To confirm the role of alr0117 in heterocyst development, we inactivated this gene by single-crossover recombination. The fragment from nucleotides 27 to 568 of alr0117 was amplified using primers alr0117-1 and alr0117-2 and cloned into pMD18-T, a T-vector. The cloned fragment was excised with PstI and BamHI and cloned into PstI/BglII double-digested pRL277 (Black & Wolk, 1993), resulting in plasmid pHB296, which is non-replicative in Anabaena. The plasmid was introduced into Anabaena sp. PCC 7120 and integrated into the genome by homologous single-crossover recombination (Fig. 4a), resulting in SR296. Because of the
filamentous structure of Anabaena and the fact that individual cells contain multiple copies of the genome, full segregation of the single recombinant SR296 was attained by sonication and plating. Four of the resulting colonies showed an identical phenotype to that of mutant 1017. The single-crossover recombination and complete segregation were confirmed by Southern blot hybridization (Fig. 4b).

**Expression of alr0117 and regulation of heterocyst development genes by alr0117**

To test whether alr0117 is induced by nitrogen step-down, we detected its transcriptional activity under nitrogen-replete and nitrogen-depleted conditions by RT-PCR, using primers alr0117-2 and alr0117-3. hepA, a gene induced by lack of nitrogen (Wolk *et al*., 1993), and glnA, a gene encoding glutamine synthetase and expressed in BG11 and BG110 at approximately the same level (Wei *et al*., 1994), were used as the controls. No PCR product was detected using RNA as the template. As shown in Fig. 5(a), the transcription level of alr0117 was not upregulated when cultures were shifted from nitrogen-containing to nitrogen-free medium.

As for its potential role in heterocyst development, we hypothesized that the histidine kinase gene alr0117 may...
control the expression of certain genes responsible for formation of the heterocyst envelope and differentiation of protoplasm. Using RT-PCR, we tested the transcription of five genes, namely hepA, hetM, hglE, hetN and patB, in wild-type and the alr0117::Tn5-1087b mutant. These five genes were selected because they were clearly and reproducibly shown to be inducible upon nitrogen step-down by this method under our conditions. hglE was first identified in Nostoc punctiforme ATCC 29133 (Campbell et al., 1997). In the genome of Anabaena 7120, there are two ORFs, alr5351 (BLAST score 2244; identity 74±2%; positive 83±5%) and all1646 (BLAST score 1282; identity 52±0%; positive 67±4%), similar to it (Kaneko et al., 2001). Because alr5351 shows a higher similarity to hglE and a transposon insertion in it caused an inability of diazotrophic growth of Anabaena sp. PCC 7120 (D. Ning & X. Xu, unpublished), we tentatively named alr5351 as hglE. In the alr0117::Tn5-1087b mutant, the induction of hepA was completely blocked, patB appeared to be constitutively transcribed at a low level, while the expression pattern of hetM, hglE and hetN were essentially unaffected (Fig. 5b). Two independent experiments showed consistent results.

**DISCUSSION**

The barrier to the penetration of oxygen into heterocysts depends upon both the polysaccharide layer and the glycolipid layer of the heterocyst envelope. A mutant lacking the polysaccharide layer or the glycolipid layer is unable to fix dinitrogen under aerobic conditions (Murry & Wolk, 1989). The polysaccharide layer of heterocysts or proheterocysts can be easily observed under the light microscope after staining with alcian blue. The transposon-induced mutant 1017 of Anabaena sp. PCC 7120 was unable to grow on nitrogen-free medium and could not be stained by alcian blue, suggesting that it was defective in the formation of this polysaccharide layer. Since transposon mutagenesis may be accompanied by secondary random mutation, it is important to confirm the cause of a phenotype apparently due to transposon interruption of a gene by (1) complementation of the mutant with a DNA fragment containing the gene or (2) targeted inactivation of that gene. By conjugative transfer of a gene library into mutant 1017, we found that DNA fragments containing alr0117 complemented the mutant. We also inactivated alr0117 by

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**Fig. 4.** Inactivation of alr0117 by single-crossover recombination. (a) Schematic diagram showing the single recombination. (b) Detection of single recombination by Southern blot hybridization. Lane 1, wild-type; lane 2, single recombinant SR296.

**Fig. 5.** Detection of transcription by RT-PCR. (a) Transcription of alr0117 in BG11 (+N) or BG11o (−N). (b) Transcription of heterocyst development genes in the wild-type strain (WT) and mutant 1017.
homologous single-crossover recombination, which resulted in a copy lacking 26 bases of the 5′-end of the coding region and a copy lacking 350 bases of the 3′-end. This mutant showed an identical phenotype to mutant 1017. Additionally, we found several other mutants complemented by DNA fragments containing alr0117, which are phenotypically similar to 1017 but carrying the transposon in different genes. Sequencing of the alr0117 region of these mutants showed a nonsense mutation in alr0117. Because there is no gene downstream of alr0117 in the same direction, these lines of evidence firmly established that alr0117 is involved in heterocyst development.

alr0117 encodes a two-component histidine kinase with a transmembrane segment at its 5′-terminus. Using RT-PCR, we tested the whole-filament transcription of this gene before and after nitrogen deprivation and found no upregulation of the gene. However, the result of such a whole-filament test does not exclude the possibility that the gene was upregulated in nonheterocysts and downregulated in other vegetative cells. It should also be noted that the RT-PCR results provide qualitative rather than quantitative evaluation of gene expression. Using the same method, we found that the induction of hepA and patB was blocked in an alr0117 null mutant. The regulatory effect on hepA, the first gene known to be essential for the formation of the polysaccharide layer, explains the lack of that layer from the heterocyst envelope in mutant 1017. hetM and hglE, the two genes involved in formation of the glycolipid layer, and hetN, which is required for maintenance of heterocyst pattern, remained inducible in alr0117::Tn5-1087b. Because the upregulation of hetM and hetN is known to occur between the onset of induced expression of hepA and patB, alr0117 may control multiple genes that are apparently unrelated to each other during heterocyst development.

It is clear that both alr0117 and hepK control the expression of hepA and the formation of the polysaccharide layer. However, it was not reported whether hepK also affects the expression of patB or other genes. One possibility is that hepK only controls the expression of hepA; therefore it acts downstream of alr0117. The second possibility is that hepK controls other gene(s) in addition to hepA; therefore hepK and alr0117 may act in the same or different regulatory systems with overlapping effects.

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**REFERENCES**


