NOTES

Signal Transduction Genes Required for Heterocyst Maturation in *Anabaena* sp. Strain PCC 7120

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How heterocyst differentiation is regulated, once particular cells start to differentiate, remains largely unknown. Using near-saturation transposon mutagenesis and testing of transposon-tagged loci, we identified three presumptive regulatory genes not previously recognized as being required specifically for normal heterocyst maturation. One of these genes has a hitherto unreported mutant phenotype. Two previously identified regulatory genes were further characterized.

Because cyanobacterial heterocysts are micro-oxic cells within oxygen-producing filaments (13, 42), they are hospitable to oxygen-sensitive processes, including nitrogen fixation and hydrogen production (12–14, 38). Which vegetative cells differentiate into heterocysts depends upon HetR (7), whose dimer binds to DNA but with unknown specificity (20), and upon a peptide, PatS (44), that inhibits differentiation. However, the regulation of heterocyst maturation remains largely undefined. Bacteria use signaling systems, often based on protein phosphorylation, to coordinate adaptive processes (1, 36). Several proteins that participate in the regulation of heterocyst differentiation and function are known: (i) canonical DNA-binding proteins, especially NtcA (17, 18) and the related protein DevH (16, 35); (ii) unorthodox DNA-binding proteins (20, 24); and (iii) components of phosphorylated two-component regulatory systems and a Ser/Thr kinase (8, 9, 27, 30, 32, 45, 46, 48). Other processes, e.g., transport (22), may also critically regulate differentiation. Despite these insights, ignorance of molecular participants in regulation hinders understanding of the process of heterocyst differentiation as a whole. Fox genes, by definition, are required specifically for fixation of dinitrogen in the presence of oxygen (10). We have identified additional regulatory Fox genes upon which heterocyst maturation depends.

Regulatory-family genes with Fox[−] mutant phenotypes. We mutated *Anabaena* sp. with the transposon (Tn) Tn5-1063 and identified mutants that are capable of aerobic growth on the nitrate-containing medium AAN but not on (N-free) AA medium (19). Transposon insertions present in 1,076 Fox[−] mu-

tants were localized in 491 open reading frames (ORFs) that included 83% of the previously known Fox genes but also genes known not to be Fox genes. We sought to determine, in part by complementation, whether transposon-mutated ORFs that were annotated (21) as members of a regulatory family and that had not previously been identified as playing a regulatory role in heterocyst formation were responsible for the corresponding mutant phenotypes. We did so by previously described methods (11, 19), testing all such genes for the ability of plasmids (Tables 1 and 2) to restore corresponding mutants to a Fox⁺ phenotype.

As discussed below, ORFs all0187, alr1086, and all2760, presumptively encoding, respectively, a transcriptional regulator, a response regulator, and a Ser/Thr kinase, were shown for the first time to be Fox genes (see below and Table 1). We also confirm and extend findings presented by Ning and Xu (32) regarding alr0117, whose presumptive product is a histidine kinase, and by Fan et al. (11) concerning alr5348, one of whose predicted domains shows limited similarity to a Spo0J transcriptional regulator.

Testing of presumptive Fox genes. Plasmids pRL2855a, pRL2858a, pRL3055, and pRL3194 (Table 2) replicate in *Anabaena* sp. and contain, intact, only the wild-type gene assessed for complementation. FQ671 and other alr0117 mutants were complemented by plasmid pRL2855a. pRL3141 contains two genes 3' from all0187 and on the same strand of DNA. The all0187 mutant FQ1062 grew aerobically on N₂ when bearing pRL3055 with or without pRL3141 but not when bearing pRL3141 alone (data not shown). PCR analysis (11) of complementation of fully segregated FQ1062 by pRL3055 provided no evidence of recombination of pRL3055 with the genome (data not shown), implying that pRL3055 complemented in *trans*. As noted for the ORF 3' from alr0117 (32), the ORFs 3' from alr1086 and all2760 are encoded on the opposite strand of DNA (21), so no polar effect of transposon mutation ac-

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TABLE 1. Fox[−] regulatory ORFs mutated by Tn5-1063, complementation, and predicted functions of their products

ORF (name of gene)	No. of mutants with a Tn in that ORF	Mutant(s) studied	Chromosomal site (21) of Tn insertion (bp)	Complementing fosmid clone	Complementing replicating plasmid	Predicted function	Heterocyst envelope phenotype ^a
alr0117 (<i>hepN</i>)	6	FQ671	120135	anc0709	pRL2855a	Two-component sensory histidine kinase	Hgl ⁺ Hep [−]
all0187 (<i>conR</i>)	1	FQ1062	201581	anc1418	pRL3055	Transcriptional regulator	Hgl ⁺ Hep ⁺ ^b
alr1086 (<i>henR</i>)	11	FQ621	1272510	anc3515	pRL3194	Two-component response regulator	Hgl [−] Hep [−] (has fibrous layer)
all2760 (<i>hepS</i>)	6	FQ1487, FQ1641	3356965, 3357475	anc1982	pRL2858a	Serine/threonine kinase	Hgl ⁺ Hep [−]
alr5348	3	FQ1281 (11)	— ^c	—	—	Bears Spo0J-like domain	Hgl ⁺ (11) Hep [−]

^a Within the context of this study, Hgl⁺ and Hgl[−] phenotypes are, respectively, those in which heterocysts bear or do not bear a laminated layer of envelope glycolipids. More generally, an Hgl mutant can be one in which heterocyst envelope glycolipids are not synthesized (11) or are synthesized but not deposited in an organized array, as in an *hglK* mutant (2). Hep⁺, heterocysts bear a homogeneous envelope layer of compacted polysaccharide; Hep[−], they bear no such layer.

^b In FQ1062, polar junctions of heterocysts to vegetative cells are usually abnormally little constricted.

^c —, see reference 11.

counts for the phenotypes of their mutants. FQ621 and another alr1086 mutant were complemented by pRL3194. In addition, an alr1086 mutant was reconstructed by insertional mutagenesis with pRL2814, and the insertions were determined to be where expected by (i) PCR with primers 5'-AGC AGCCGGGACAAAATTA-3' and, separately, 5'-TTTTACA AAAACAGGGTTATCAAA-3' and 5'-TTGTAAAACGAC GGCCAGT-3'; and (ii) recovery of the integrated plasmid from genomic DNA by excision with HindIII, religation, and transfer to *Escherichia coli*, followed by restriction, separately, with PstI and XmnI. FQ1487, FQ1641, and other all2760 mutants were complemented by plasmid pRL2858a. We conclude that ORFs all0187, alr1086, and all2760, like alr0117 (32) and alr5348 (11), are Fox genes.

Many genes that are involved in heterocyst differentiation are transcriptionally activated, often after an extended delay, in response to nitrogen step-down (15). Plasmid pRL3183 bears an all2760:*luxAB* fusion. Strain SR3183 is derived from single, homologous recombination of pRL3183 with the chromosome of *Anabaena* sp. strain SR3183, and the transpositions in FQ671 (alr0117), FQ1062 (all0187), and FQ621 (alr1086) position *luxAB* to measure transcription of the corresponding genes. As quantified by use of a luminometer (41), in none of those mutants was a significant increase in transcription in excess of twofold observed in response to deprivation for fixed nitrogen (data not shown). Neither was such an increase observed by use of microarrays for the same ORFs except, at 8 h after nitrogen step-down, for all2760 (9). Therefore, these may be developmentally active genes that, like *hetF* (43), are transcribed constitutively. It remains possible that their transcription may increase in heterocysts while diminishing in vegetative cells and that their activity may be regulated posttranscriptionally.

Structural and lipid-related phenotypes of Fox[−] mutants.

(i) **all0187.** The methods of Black et al. (2) and Nichols and Wood (31) were used for fixation for electron microscopy and thin-layer chromatography of lipid extracts, respectively. Strains were incubated for 7 days (for electron microscopy) or 10 days (for thin layer chromatography) on agar-solidified AA medium. Because the all0187 mutant FQ1062 forms heterocysts with a polysaccharide layer (i.e., is Hep⁺) and a glycolipid layer (i.e., is Hgl⁺) (Fig. 1C and D; Fig. 2), its mutation does not appear to affect the biosynthesis, per se, of the components of the heterocyst envelope. Rather, the constriction of the cell

and cell wall that normally takes place at the poles of the heterocyst is very often aborted. As a result, the constituents of the heterocyst envelope are deposited aberrantly, leading to the formation of heterocyst envelopes whose ends are widely open (Fig. 1C, arrowheads). Consequently, more oxygen can enter heterocysts and inactivate nitrogenase, presumptively accounting for their inability to fix N₂ under a normal atmosphere. Seldom are the heterocysts of FQ1062 as nearly normal at their poles as seen in the right-hand side of Fig. 1C. In an all0187 mutant, the junctions between vegetative cells are also often less clearly constricted than those in the wild-type strain (data not shown). Thus, all0187, like *hglK*, is a Fox gene whose influence can be seen in the shapes of vegetative cells in series (2). All0187 shows alignment along 87% of the length of a LytR transcriptional regulator domain (COG1316 [28]) that, in other bacteria, is involved in autolysis (6). *Bacillus subtilis* LytR is described as a transcriptional attenuator of its own gene and of the *lytABC* operon, where LytC is *N*-acetylmuramoyl-L-alanine amidase. Although HcwA, also an *N*-acetylmuramoyl-L-alanine amidase, is a Fox gene product (47), it remains unknown whether All0187 regulates itself and (or) *hcwA*. FQ1550 (26) and other mutants in our collection show defects in heterocyst envelope formation similar to those seen in FQ1062; the corresponding wild-type genes may possibly be regulated by All0187. Because the phenotype of an all0187 mutant appears to be constriction specific rather than heterocyst specific and All0187 appears to be regulatory, we denote the gene *conR*.

(ii) **all2760.** The all2760 mutants tested are Hep[−] and Hgl⁺, as assessed by electron microscopy (Fig. 1E and F) and thin-layer chromatography of lipid extracts (Fig. 2) of mutant and wild-type strains, and as corroborated by staining with Alcian Blue (unpublished technique of M. Gantar, J. Elhai, J. Jia, and M. Ow, cited in references 4, 19, and 26—see also references 16 and 32; data not shown). Other mutants were examined similarly. The sole putative regulatory motif in predicted All2760 is characteristic of serine/threonine kinases. Therefore, we denote all2760 *hepS*. Thirteen ORFs present in *Anabaena* sp. that have both Ser/Thr and His kinase domains (33, 39) provide presumptive precedents for direct coupling of His and Ser/Thr kinases in signal transduction pathways. What protein(s) All2760 may phosphorylate is unknown.

(iii) **alr0117.** Ning and Xu (32) described the predicted product of alr0117 and showed that an alr0117 mutant pro-

TABLE 2. Cyanobacterial strains and plasmids used

Strain or plasmid	Derivation and/or relevant characteristics ^a
Strains of <i>Anabaena</i> sp.	
FQ621.....	Bm ^r Nm ^r Sm ^r ; alr1086::Tn5-1063
FQ671.....	Bm ^r Nm ^r Sm ^r ; alr0117::Tn5-1063
FQ1062.....	Bm ^r Nm ^r Sm ^r ; all0187::Tn5-1063
FQ1281 (11).....	Bm ^r Nm ^r Sm ^r ; alr5348::Tn5-1063
FQ1487.....	Bm ^r Nm ^r Sm ^r ; all2760::Tn5-1063
FQ1490 (11).....	Bm ^r Nm ^r Sm ^r ; Tn5-1063 upstream from <i>hglA</i> ; Hgl ⁺
FQ1641.....	Bm ^r Nm ^r Sm ^r ; all2760::Tn5-1063
PCC 7120.....	Wild type, from R. Haselkorn
Plasmids	
anc0709.....	Cm ^r Em ^r ; <i>Anabaena</i> sp. chromosomal DNA from bp 111372 to bp 129814 in BamHI site of pRL838 (21)
anc1418.....	Cm ^r Em ^r ; <i>Anabaena</i> sp. chromosomal DNA from bp 197711 to bp 215670 in BamHI site of pRL838 (21)
anc1982.....	Cm ^r Em ^r ; <i>Anabaena</i> sp. chromosomal DNA from bp 3353565 to bp 3377720 in BamHI site of pRL838 (21)
anc3515.....	Cm ^r Em ^r ; <i>Anabaena</i> sp. chromosomal DNA from bp 1262840 to bp 1283836 in BamHI site of pRL838 (21)
pRL2814.....	Ap ^r Cm ^r Em ^r ; an internal fragment of alr1086, PCR amplified from <i>Anabaena</i> sp. with primers 5'- GCGAGTAATGGTGAAGAAGGAA-3' and 5'- CGGTAAGGCAATTTAA GACCTG-3', was inserted in pGEM-T Easy (Promega, Madison, WI), and a PstI-Cm ^r Em ^r - <i>oriT</i> (RK2)-PstI fragment from pRL2665b (19) was placed in the unique PstI site of the resulting plasmid
pRL2833b.....	Cm ^r Em ^r ; same as pRL2833a (11), but <i>PglA</i> oppositely oriented
pRL2855a.....	Cm ^r Em ^r ; alr0117-containing BsrFI fragment from anc0709 fused with XmaI-BamHI linkers from pIC20H (29) to the BamHI site of pRL2833a (11)
pRL2858a.....	Cm ^r Em ^r ; all2760-containing MfeI fragment from anc1982 fused with EcoRI-SacI linkers from pIC20H (29) to the SacI site of pRL2833b
pRL3055.....	Sm ^r Sp ^r (34); all0187-containing PvuI (blunted)-AclI fragment of anc1418 transferred between the StuI and ClaI sites of pRL2831a (19)
pRL3120.....	Ap ^r ; alr1086-containing PCR product, generated with primers 5'-TCTCCCGAATTCATGT TTCAAATTTTGATAATTGATGAT-3' and 5'-GCTTCTCGAGTTAGTCAAACCTTAAC TAGTAGGA-3' and wild-type <i>Anabaena</i> sp. DNA as template, cloned between the EcoRI and XhoI sites of pGilda (BD Biosciences Clontech)
pRL3141.....	Cm ^r Em ^r ; all0185- and all0186-containing XbaI-ScaI fragment of anc1418 linked with the short XbaI-PstI and SmaI-EcoRI fragments of pIC20H (29) to the NsiI and StuI sites of pRL2833a (11)
pRL3178.....	Cm ^r Em ^r ; <i>sacB</i> vector based on pRL271 (3), deleted from SpeI to XbaI
pRL3183.....	Bm ^r Cm ^r Em ^r Km ^r Sm ^r ; the all2760-bearing SstI fragment from pRL2858a was first inserted into the SstI site of pRL3178, and into the unique XbaI site of that fragment was then added a SacI- <i>luxAB</i> -XbaI- <i>oriV</i> -Km ^r Bm ^r Sm ^r -BglII fragment from pRL1063a by means of BlnI-containing linkers; finally, a supernumerary <i>oriV</i> sequence was deleted by cutting with SgrAI and XbaI, blunting, and religating
pRL3190.....	Ap ^r ; alr1086-containing EcoRI-XhoI fragment of pRL3120 transferred between the same sites of pIC20H (29)
pRL3194.....	Cm ^r Em ^r ; alr1086-containing XhoI-PstI fragment of pRL3190 transferred between the XhoI and NsiI sites of pRL2833a (11)

^a Ap, ampicillin; Bm, bleomycin; Cm, chloramphenicol; Em, erythromycin; Nm, neomycin; Sm, streptomycin; Sp, spectinomycin.

duces transcripts of two of the seven biosynthetic genes for heterocyst envelope glycolipids that have been documented (11) and lacks staining of heterocyst envelope polysaccharide. Our observations by electron microscopy (Fig. 1A and B) confirm their implication that those heterocysts synthesize heterocyst envelope glycolipids and their interpretation that heterocysts of an alr0117 mutant lack a heterocyst envelope polysaccharide layer. Because alr0117 presumptively encodes a two-component histidine kinase but because a histidine kinase that regulates synthesis of heterocyst envelope polysaccharide has already been named *hepK* (46, 48), we and X. Xu (personal communication) agree to name alr0117 *hepN*. Facile loss of the glycolipid layers from Hep[−] heterocysts can account for the description of those heterocysts as proheterocysts (see the leg-

end to Fig. 1b in reference 32), and breakage of the glycolipid layers into fragments so small that they are not recovered following centrifugal sedimentation can account for the paucity of envelope glycolipid seen in thin-layer chromatograms of lipid extracts of FQ671 (Fig. 2). Concordantly, we were able to visualize the glycolipid layers of the alr0117 mutant FQ671 by transmission electron microscopy only when filaments were scraped from petri dishes.

(iv) **alr5348.** In the Fox[−] mutant FQ1281, the transposon is inserted downstream from the Spo0J-like domain of ORF alr5348. Although present within a cluster of genes required for the synthesis and normal deposition of heterocyst envelope glycolipids (11), fully segregated FQ1281 is Hgl⁺ (11) and Hep[−] (Fig. 1G, H). That mutant is complemented by a plasmid

FIG. 1. Electron microscopy of heterocyst sections from regulatory *Fox⁻* mutants. (A and B) *alr0117* mutant FQ671. (C and D) *alr0187* mutant FQ1062, most of whose heterocysts have terminal pores that are unusually wide (C, arrowheads: the heterocyst on the right is more nearly normal in structure). (E and F) *all2760* mutant FQ1641. (G and H) *alr5348* mutant FQ1281. (I and J) *alr1086* mutant FQ621. Panels B, D, F, and H, magnified from the boxed regions in panels A, C, E, and G, respectively, show glycolipid laminations. im, inner membrane; pg, peptidoglycan; om, outer membrane; GL, envelope glycolipid; P, envelope polysaccharide; FL, fibrous material. Magnifications ($10^3\times$): A, $\times 11$; B, $\times 142$; C, $\times 9.3$; D, $\times 148$; E, $\times 19$; F, $\times 147$; G, $\times 15$; H, $\times 118$; I, $\times 137$; J, $\times 69$.

whose only intact *Anabaena* sp. gene is *alr5348* (11). Mutations in *hepS*, *hepN*, *hepK*, and *devR_A*, like that of the *alr5348* mutation in FQ1281, all result in *Hep⁻ Hgl⁺* ultrastructural mutant phenotypes. Whether the products of any or all of *hepS*, *hepN*, and *alr5348* form a phosphotransfer cascade with the interacting, two-component regulatory elements *HepK* and *DevR_A* (46) remains to be determined. *alr5348* is the only one of these genes whose product bears what resembles a known DNA-binding domain, *spo0J*. We are pursuing determination of the role, if any, of the *Spo0J*-like domain of *alr5348* in the *Hep⁻* attribute of FQ1281.

(v) ***alr1086***. Although heterocysts of the *alr1086* mutant FQ621 may synthesize traces of envelope glycolipids (Fig. 2), they appear to lack both the heterocyst envelope glycolipid layer present in the wild-type strain (40) and a normal heterocyst envelope polysaccharide layer (Fig. 1). However, those heterocysts produce fibrous extracellular material of variable thickness that appears to be layered parallel to the cell membrane, hinting at lamination (Fig. 1I and J). The fibrous ma-

terial that lies outside of the plasmalemma of an *alr1086* mutant may correspond to the fibrous material identified by Lang and Fay (25) as formed earliest and most peripherally during heterocyst formation in *Anabaena cylindrica*. Such a mutant phenotype has not previously been reported. Whether or not the material is chemically related to the material that forms the bulk of the polysaccharide layer of the heterocyst envelope remains unknown, but Alcian Blue did not stain it noticeably (data not shown). Because an *alr1086* mutation pleiotropically affects regulation of the two principal layers of the heterocyst envelope, we denote that ORF *henR*.

Whereas sigma factors play important roles in other bacterial differentiation processes (see, e.g., reference 37), no major role of sigma factors has heretofore been recognized in heterocyst differentiation (5, 23). Like the *Anabaena* sp. developmental proteins *DevR* (8, 46) and *PatA* (27), *HenR* resembles response regulators that lack known DNA-binding motifs. *HenR* belongs to a group of response regulators in *Anabaena* sp. that have "a weak but significant similarity to the catalytic

FIG. 2. Analysis of heterocyst envelope glycolipids in regulatory *Fox*⁻ mutants, mutant FQ1490 (11; see Table 2), and wild-type *Anabaena* sp. Heterocyst envelope glycolipids (arrows) are distinguishable from other *Anabaena* sp. lipids by their violet coloration when incompletely oxidized. The upper glycolipid is less abundant than the lower and may for that reason sometimes not be visualized together with the latter. Concordant with the results in Fig. 1, mutants bearing transposon insertions in ORFs all0187 and all2760 produce abundant heterocyst envelope glycolipids, little of such glycolipid is seen in an alr0117 mutant (see the text), and no more than a trace is seen in an alr1086 mutant, whereas mutant FQ1490 is known to form none (11).

domain of Ser/Thr phosphatases of type PP2C" (39). HenR most closely resembles the *Bacillus* sp. response regulator RsbU, which exhibits phosphatase activity and interacts with RsbV. In turn, RsbV resembles the product of *Anabaena* sp. ORF all087, which overlaps the 3' terminus of *henR*. RsbV and All087 show similarity to an anti-sigma factor antagonist. Just beyond all087 lies all1088, whose predicted product is a two-component histidine kinase. If the effect of HenR should prove to be mediated by All087, sigma factors may have a more major role than heretofore recognized in heterocyst differentiation. In this regard, it is notable that ORFs alr4800 and all1087, which presumptively encode anti-sigma factor antagonists, show strongly significant increases in transcription at 3 and 8 h, respectively, of nitrogen deprivation (9).

The apparently pleiotropic effect of a *henR* mutation suggests that HenR is part of a master system that regulates synthesis of, at least, the heterocyst envelope. That system may bifurcate after HenR, one branch controlling deposition of the glycolipid layer and the other regulating deposition of the polysaccharide layer. It remains to be determined

how regulation by *henR* is coordinated with that by the known ORFs (i) *abp2*, *abp3* (24), and *devH* (35), which encode DNA-binding proteins and whose mutation blocks production of heterocyst envelope glycolipids; and (ii) *devR_A-hepK* (46), *hepN* (32), *hepS*, and alr5348, which evidently also regulate synthesis of the polysaccharide layer of the heterocyst envelope.

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