

NOTES

Predicted Glycosyl Transferase Genes Located outside the HEP Island Are Required for Formation of Heterocyst Envelope Polysaccharide in *Anabaena* sp. Strain PCC 7120^{∇†}

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During maturation, heterocysts form an envelope layer of polysaccharide, called heterocyst envelope polysaccharide (HEP), whose synthesis depends on a cluster of genes, the HEP island, and on an additional, distant gene, *hepB*, or a gene immediately downstream from *hepB*. We show that HEP formation depends upon the predicted glycosyl transferase genes *all4160* at a third locus and *alr3699*, which is adjacent to *hepB* and is cotranscribed with it. Mutations in the histidine kinase genes *hepN* and *hepK* appear to silence the promoter of *hepB* and incompletely down-regulate *all4160*.

The filamentous nitrogen-fixing cyanobacterium *Anabaena* sp. strain PCC 7120 produces terminally differentiated cells, called heterocysts, when it is deprived of fixed nitrogen. These specialized cells are internally microoxic as a result of (i) cessation of oxygen production; (ii) intensification of respiration; and (iii) deposition of a thick envelope, comprising an outer polysaccharide layer and an inner glycolipid layer, that impedes the entry of oxygen (35). The glycolipid layer is the principal barrier to the entry of oxygen, whereas the polysaccharide layer is evidently required for the integrity of the glycolipid layer. The homogeneous layer of heterocyst envelope polysaccharide (HEP) has been chemically analyzed in two other *Anabaena* sp. strains and in a *Cylindrospermum* sp. strain. In those strains, HEP is composed of oligosaccharide repeating units whose mannose-(glucose)₃ backbone is decorated with side chains that are different in the different strains. In the *Anabaena* sp. strains, a highly similar polysaccharide was found in the envelopes of akinetes, a kind of spore. It was crudely estimated that, exclusive of regulatory genes, on the order of 20 genes are required for synthesis of the concatenated subunits (33).

Through DNA microarray analysis and near-saturation

transposon mutagenesis, numerous *Anabaena* sp. strain PCC 7120 open reading frames in the HEP island (genes *alr2825* through *alr2841*) of the chromosome were found to be required specifically for formation of the envelope polysaccharide layer (7, 17). Mutants with mutations in the presumptively glycosyl transferase-encoding genes *hepB* (*alr3698*) (23, 36), *alr3699*, and *all4160* (this study) that are distant from the HEP island in the genome also lack the polysaccharide layer. Regulation of HEP deposition depends upon signal transduction systems that comprise, at least, the histidine kinase *HepN* (11, 26), the response regulator *HenR* (11), the serine/threonine kinase *HepS* (11), and the interacting two-component regulatory elements *HepK* and *DevR_A* (39, 40). In addition, proteins *Abp2* and *Abp3* bind to the DNA sequence upstream of *hepC*, and inactivation of *abp2* and *abp3* greatly reduces the expression of *hepA* and *hepC* (20). *abp2* and *abp3* mutants, however, lack the glycolipid layer of the heterocyst envelope rather than the polysaccharide layer.

In the 1,076 sites of insertion mapped in a transposon mutagenesis project screening for *Fox⁻* mutants (i.e., mutants unable to fix dinitrogen in the presence of oxygen) (9, 10, 11, 17), two insertions of transposon Tn5-1063 were found in *hepB*, four insertions were found in the downstream neighboring gene *alr3699*, and nine insertions were found in *all4160*. Previously, we showed that there was up-regulation of *hepB* in heterocysts using *gfp* (encoding green fluorescent protein [GFP]) as a transcriptional reporter gene (32). By screening random promoter:*gfp* fusions for fusions that are regulated during heterocyst differentiation, we also found that the promoter of *all4160*, like that of the HEP island gene *hepA* (34), is up-regulated specifically in proheterocysts and heterocysts. In this paper, we show that *all4160* and *alr3699* are required for synthesis of the HEP layer and that whereas *hepB* and *alr3699*, like genes of the HEP island (17, 22, 26, 40), are strongly

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TABLE 1. Cyanobacterial strains, plasmids, and primers used

Strain, plasmid, or primer	Derivation and/or relevant characteristics ^a	Reference(s) or source
<i>Anabaena</i> sp. strains		
1017	Em ^r , alr0117 (<i>hepN</i> ::Tn5-1087b)	26
ABP2	Nm ^r	20
DRHB2429	Em ^r , all4160::C.CE2	This study
FQ689	Bm ^r Nm ^r Sm ^r , alr3699::Tn5-1063 at chromosomal bp 4467495	This study
FQ749	Bm ^r Nm ^r Sm ^r , alr3699::Tn5-1063 at chromosomal bp 4467724	This study
FQ792	Bm ^r Nm ^r Sm ^r , all4160::Tn5-1063 at chromosomal bp 5007183	This study
FQ873	Bm ^r Nm ^r Sm ^r , all4160::Tn5-1063 at chromosomal bp 5006680	This study
FQ1351	Bm ^r Nm ^r Sm ^r , alr3699::Tn5-1063 at chromosomal bp 4468098	This study
FQ1421	Bm ^r Nm ^r Sm ^r , all4160::Tn5-1063 at chromosomal bp 5007278	This study
PCC 7120	Wild type	R. Haselkorn, University of Chicago
Y7	Bm ^r Nm ^r Sm ^r , <i>hepK</i> ::Tn5-1065	9
Plasmids		
anc0304	Cm ^r Em ^r , sequencing bridging clone, bp 5002439 through bp 5019236 (for comparison, all4160 extends from bp 5007439 to bp 5006384)	19
anc2015	Cm ^r Em ^r , sequencing bridging clone, bp 4462687 through bp 4477773 (for comparison, alr3699 extends from bp 4467059 to bp 4468207)	19
anp01253	Ap ^r , sequencing clone, bp 4998732 through bp 5007158	19
anp02990	Ap ^r , sequencing clone, bp 4466571 through bp 4474909	19
pHB912	Km ^r Sm ^r Sp ^r , GFP-reporting vector	32
pHB1726	Km ^r Sm ^r Sp ^r , pHB912 derivative bearing chromosomal bp 5009091 to bp 5007155 ^b	This study
pHB2416	Ap ^r , 2.4-kb fragment containing all4160, amplified with PCR using primers all4160-1 and all4160-2, cloned into pMD18-T	This study
pHB2427	Ap ^r Cm ^r Em ^r ; cassette C.CE2 (Cm ^r Em ^r) excised with BamHI from plasmid pRL598 was blunted with T4 DNA polymerase and ligated to pHB2416 that had been linearized at the ClaI site within all4160 and blunted	3; this study
pHB2429	PstI-SmaI fragment, from pHB2427, that bears all4160::C.CE2 was blunted with T4 DNA polymerase and ligated with XbaI-cut, blunted pRL277	2; this study
pMD18-T	Ap ^r , cloning vector	http://www.takara.com.cn/bioxiew/bioview02_14.pdf
pRL2881b	Km ^r , 2,866-bp HinPII fragment of anp02990 ligated to AccI-cut pK18	27; this study
pRL2890	Sm ^r Sp ^r , insert of pRL2881b, containing alr3700 and alr3701 as its only intact open reading frames, excised with BamHI and PstI, ligated between the unique BamHI and NsiI sites of pRL2831a	17; this study
pRL3169a	Km ^r , 1,267-bp, alr3699-bearing fragment of RsaI- plus HaeIII-cut anp02990 ligated to SmaI-cut pK18	27; this study
pRL3176	Cm ^r Em ^r , 1,338-bp fragment of anp01253, excised with BsrGI plus DraI and containing all4159 as its only intact open reading frame, ligated between the BsiWI and StuI sites of the polylinker of pRL2833a	10; this study
pRL3177	Cm ^r Em ^r , insert of pRL3169a, bearing alr3699 as its only intact open reading frame, excised with SacI plus SphI and inserted between the SacI and SphI sites of pRL2833b	17; this study
pRL3182	Sm ^r Sp ^r , 1,375-bp fragment, excised from anc0304 with Asp718I and XbaI and bearing all4160 as its only intact open reading frame, inserted between the BsiWI site and the Dam-insensitive XbaI site of pRL2831a	17; this study
Primers		
7120pool-1	5'-AGCGTGCATAATAAGCCCTACA-3'	
7120pool-2	5'-CGTCTTGTAGITCCCGTCATC-3'	
all4160-1	5'-CGATGAGAGTGGTTAGAGGGA-3'	
all4160-2	5'-CAGCTTGG AGCATTTCCATCGT-3'	
hepB-1	5'-CATACAGTACTAGGAGATAAGAGTGA-3'	
hepB-2	5'-GTCGGCTGATAGAACAACCACA-3'	
hepB-3	5'-ACTGCTGGCGAGCTTGTG-3'	
hepB-4	5'-TGTCGCCAGTATGCTGTGAC-3'	
alr3699-1	5'-CTGGTTTCCCCTCTGGTCT-3'	
alr3699-2	5'-CTGTTTGACTGGCTTGGGA-3'	
IDT141	5'-ACTAGCCAACCCACAGAGGTA-3'	
IDT142	5'-CATCAAGCTCCAGTTTTTCTGA-3'	
IDT641	5'-TTGAAGGTTTTGGGTTAGCAAT-3'	
IDT642	5'-GGAGTCTGTCCAGGAAGGTTGTC-3'	

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

^b See Fig. 1.

down-regulated in *hepK* and *hepN* mutants, all4160 is only modestly down-regulated by mutations in *hepK* and *hepN*.

Methods. *Anabaena* sp. strain PCC 7120 and its derivatives (Table 1) were grown in BG-11 medium or in AA/8 plus nitrate as previously described (16, 26). All mutants, complemented mutants, and strains containing pHB912-derived plasmids were grown in the presence of appropriate antibiotics. To

induce heterocyst formation, rapidly growing cultures were washed twice with nitrogen-free medium, resuspended, and incubated on a shaker in the presence of light (~30 microeinstein m⁻² s⁻¹). Molecular cloning was performed by standard protocols. Plasmids (Table 1) were introduced into *Anabaena* strains by conjugation (8). Bright-field microscopy, fluorescence microscopy, and Alcian blue staining for observation of

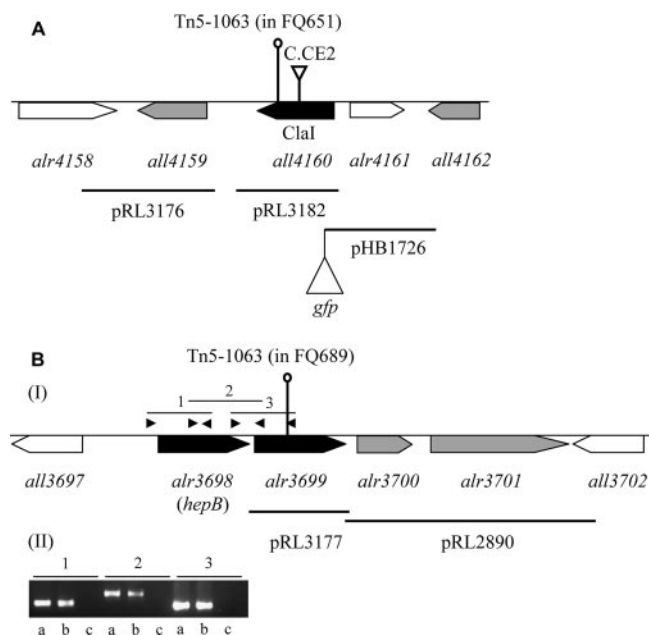


FIG. 1. Two genomic regions involved in the formation of the HEP layer. (A) Map of the all4160 region, showing the locations of Tn5-1063 and C.CE2, complementing DNA fragments, and the cloned upstream region that is present in pHB1726. (B) Locations of Tn5-1063 and complementing DNA fragments in the *hepB*-*alr3699* region (I) and cotranscription of *hepB* and *alr3699* as visualized with RT-PCR (II). The arrowheads in panel B-I indicate primers for PCR whose products are shown in panel B-II. The combinations of primers used are as follows: lanes 1, *hepB*-1/*hepB*-3; lanes 2, *hepB*-2/*alr3699*-1; and lanes 3, *hepB*-4/*alr3699*-2. Lanes a, b, and c show electrophoretograms of products of PCR obtained using genomic DNA, cDNA, and RNA as the templates, respectively.

the envelope polysaccharide layer were performed as described previously (14, 17, 32). Images showing the expression of a gene in different strains were taken in parallel during one session of microscopic observations. To minimize GFP bleaching, an image viewed with a microscope equipped with a JVC3 charge-coupled device color video camera was fixed immediately, assisted by the software 10moons-2000/PRO (version 4.5). Multiple alignments of DNA sequences were performed using CLUSTAL X (30) and the SEAVIEW alignment editor (13). Aligned sequences were analyzed using the neighbor-joining method (28) assisted by MEGA 3.1 (21). The sequence divergence between species pairs was calculated using the p-distance model. Branch support was assessed using 1,000 bootstrap replicates. Four glycosyl transferases, ExoY of *Sinorhizobium meliloti* (25), Atu3560 of *Agrobacterium tumefaciens* strain C58 (GenBank accession no. NP 534056), and WcaJ and WcaL of *Escherichia coli* (29), were included in the phylogenetic analysis of the predicted glycosyl transferases in cyanobacteria.

Identification of DNA fragments that can up-regulate expression of GFP in heterocysts of *Anabaena* sp. strain PCC 7120. To identify genes up-regulated in developing heterocysts, as visualized at the level of single cells, we constructed an *E. coli* library of *Anabaena* sp. strain PCC 7120 genomic DNA in plasmid pHB912 (Table 1). About 80% of the 50,000 colonies of the library carried, at the BglIII site of the vector, ca. 2- to

9-kb fragments derived by partial digestion of genomic DNA with Sau3AI. The library was introduced into *Anabaena* sp. strain PCC 7120 by conjugation. Twenty thousand resulting exconjugants were grown in petri dishes on agar with spectinomycin, heterocyst differentiation was induced in medium lacking fixed nitrogen, and the exconjugants were examined individually by fluorescence microscopy. Exconjugants in which GFP was observed to be up-regulated in proheterocysts or heterocysts were grown and treated as follows. Plasmids were extracted from the exconjugants and reintroduced into *Anabaena* sp. strain PCC 7120, and the resulting derivatives were examined. The cloned DNA fragments were identified by sequencing their ends with primers 7120pool-1 and 7120pool-2 (Table 1) and localizing the ends in the genome using the Kazusa database (www.kazusa.or.jp/cyano/cyano.html). Forty-nine DNA fragments clearly up-regulated *gfp* and 39 fragments clearly down-regulated *gfp* in developing heterocysts. We report here on a plasmid, pHB1726, whose chromosomal insert overlaps the 5' end of all4160 and up-regulates *gfp* in developing heterocysts (Fig. 1 and 2). By following the expression of GFP, we found, consistent with the results of Ehira and Ohmori (6), that up-regulation of all4160 began between 6 and 12 h after nitrogen stepdown (Fig. 2). Because all4160 and the *hepB* gene cluster had both been shown by use of microarrays to be up-regulated during heterocyst development, were shown by use of *gfp* to be up-regulated in developing heterocysts, and were identified in a screen for Fox⁻ mutants, we analyzed the physiological function and regulation of these genes.

all4160 and alr3699 are predicted to encode glycosyl transferases. Amino acids 163 to 340 of the 351-amino-acid protein All4160 show substantial similarity (58% positive; E value, 5×10^{-33}) to amino acids of WcaJ, a putative glycosyl transferase. WcaJ may transfer glucose-1-phosphate to a lipid carrier, probably undecaprenylpyrophosphate, during biosynthesis of the colanic acid exopolysaccharide of *E. coli* (29). All4160 is most closely related to homologues in other heterocyst-forming species; is more distantly related to a homologue in a non-heterocyst-forming, filamentous, nitrogen-fixing species, *Trichodesmium erythraeum*; and is related even more distantly to other predicted glycosyl transferases (e.g., All2285) of *Anabaena* sp. strain PCC 7120 and to glycosyl transferases from unicellular cyanobacteria (see Fig. S1 in the supplemental material). A 64-amino-acid sequence (amino acids 42 to 105) close to the N terminus of All4160 shows similarity to SpoIIAA (62% positive; E value, 10^{-6}), the antagonist of an anti-sigma factor, SpoIIAB, in *Bacillus subtilis* (1). The presence of an N-terminal, anti-sigma factor antagonist-like region in All4160 suggests that in addition to its glycosyl transferase activity, All4160 may act as a regulatory factor. As predicted with TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) and SOSUI (http://bp.nuap.nagoya-u.ac.jp/sosui/sosui_submit.html), All4160 is probably a transmembrane protein. This is consistent with its function as a glycosyl transferase and does not necessarily contradict the prediction that it may be a regulatory factor (38).

All4160, HepB (Alr3698), and Alr3699 are predicted to be glycosyl transferases. All4160 and All3699 are required for formation of the envelope polysaccharide layer (see below), and HepB may be required for formation of this layer. HepB and Alr3699 are more closely related to each other than either

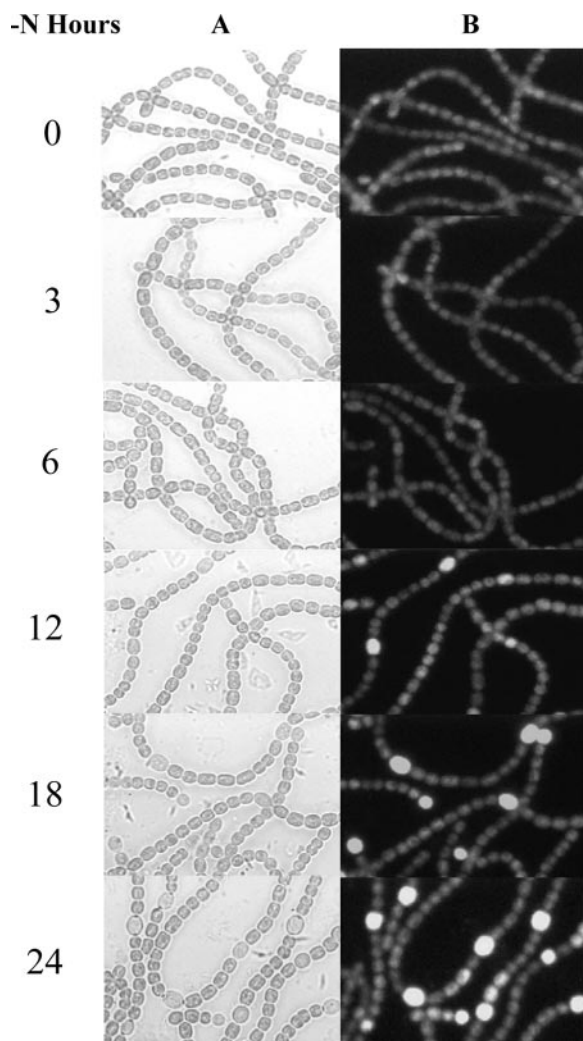


FIG. 2. Time course of the up-regulation of all4160 in developing heterocysts after nitrogen stepdown, as reported by the GFP-based fluorescence of *Anabaena* sp. strain PCC 7120 bearing plasmid pHB1726. (A) Bright-field images; (B) images of fluorescence of GFP.

one is to All4160 (see Fig. S1 in the supplemental material). HepB shows some similarity to WcaL (45% of 247 amino acids positive in a total of 389 amino acids; E value, 2×10^{-9}), another glycosyl transferase that is active in the biosynthesis of colanic acid (29). Proteins similar to Alr3699 are found in heterocyst-forming species and in a non-heterocyst-forming species, *Lyngbya* sp. strain PCC 8106 (see Fig. S1 in the supplemental material). After the suggested WcaJ-catalyzed sugar transfer to the lipid carrier, WcaL and other enzymes may catalyze the sequential transfer of additional sugars (29). HepB, Alr3699, and WcaL are members of glycosyl transferase family 4 (5), while Alr4160 and WcaJ are not included in the currently posted 89 families of glycosyl transferases (http://www.cazy.org/fam/acc_GT.html).

Effects of *hepK* and *hepN* on the up-regulation of all4160, alr3698 (*hepB*), and alr3699 during heterocyst differentiation. In the microarray analysis of Ehira and Ohmori (6), alr3699 was clearly shown to be up-regulated upon N stepdown, and a

reevaluation of the data of these workers (37) showed strong statistical support also for up-regulation of *hepB* (alr3698) but not for up-regulation of alr3700. Our previous analyses (22, 26, 40) showed that with few exceptions, HEP island genes depend very significantly on both *hepK* and *hepN* for up-regulation during heterocyst differentiation, and the same was observed for *hepB* at 14 h after nitrogen stepdown. Our report on the effect of regulatory genes on the expression of open reading frames of *Anabaena* sp. strain PCC 7120 (22) inadvertently omitted alr3699; data for *hepK* and *hepN* mutants showed reduced expression of alr3698, alr3699, and alr3700 with high statistical support. We have identified no stem-loop structure that might terminate transcription in the 62-bp sequence between *hepB* and alr3699.

We tested whether *hepB* and alr3699 are cotranscribed in filaments after nitrogen stepdown using reverse transcription-PCR (RT-PCR) (Fig. 1B), as reported previously (10). RNA was extracted from wild-type *Anabaena* sp. strain PCC 7120 after 24 h of N deprivation and freed of DNA until no PCR products could be detected with the RNA sample. We then used primers that started upstream from the coding region of *hepB* and extended to near the middle of alr3699, with the second RT-PCR overlapping the first and third RT-PCRs (Fig. 1B-I). The PCR products obtained with cDNA as templates matched the products predicted for, and obtained with DNA as templates, whereas the use of RNA as templates resulted in no products (Fig. 1B-II). Our results support the idea that in response to nitrogen deprivation, the *hepB* and alr3699 genes are not just both up-regulated with a dependence on *hepK* and *hepN* but are jointly transcribed.

To examine at a single-cell level whether the transcription of all4160 and of the *hepB*-alr3699 cluster is affected by *hepK* and *hepN*, we introduced pHB912-based plasmids bearing presumptive promoter regions of all4160 and *hepB* (32) into strains Y7 (*hepK*::Tn5-1065) (9) and *hepN*::Tn5-1087b (26). As controls to compare the specificity of the regulation of all4160 and *hepB* with the regulation of other genes, we also introduced fusions of *gfp*, as a reporter, to the presumptive promoter regions of *hglD*, *hglE_A*, *devB*, *coxBII*, and *nifB* (32) into the same *hepK* and *hepN* strains. *hglD* and *hglE_A* are required for synthesis of heterocyst envelope glycolipids (10), *devB* is thought to be involved in transport of those lipids (12), *coxBII* encodes a component of a cytochrome oxidase that helps to maintain microoxic conditions in heterocysts (18, 31), and *nifB* encodes a protein on which a precursor of the FeMo-co prosthetic group of dinitrogenase is assembled (15). For further comparison, the same plasmids were introduced also into an *abp2* mutant that forms the polysaccharide layer, but not the glycolipid layer, of the heterocyst envelope (20).

As shown in Fig. 3 and as previously reported (32), *hepB*, *hglD*, *hglE_A*, *devB*, *coxBII*, and *nifB* were up-regulated in heterocysts of wild-type *Anabaena* sp. strain PCC 7120. Consistent with the results of microarray analyses, *hepB* was shown here to be up-regulated in heterocysts of wild-type *Anabaena* sp. strain PCC 7120 but not in heterocysts of *hepK* and *hepN* mutants (Fig. 3). However, we observed no significant decrease in expression of *hglD*, *hglE_A*, *devB*, *coxBII*, or *nifB* in *hepK* and *hepN* mutants, which in general is consistent with the results of microarray analyses (22). Although the expression of all4160 was more reduced in the *hepK* mutant than in the *hepN* mu-

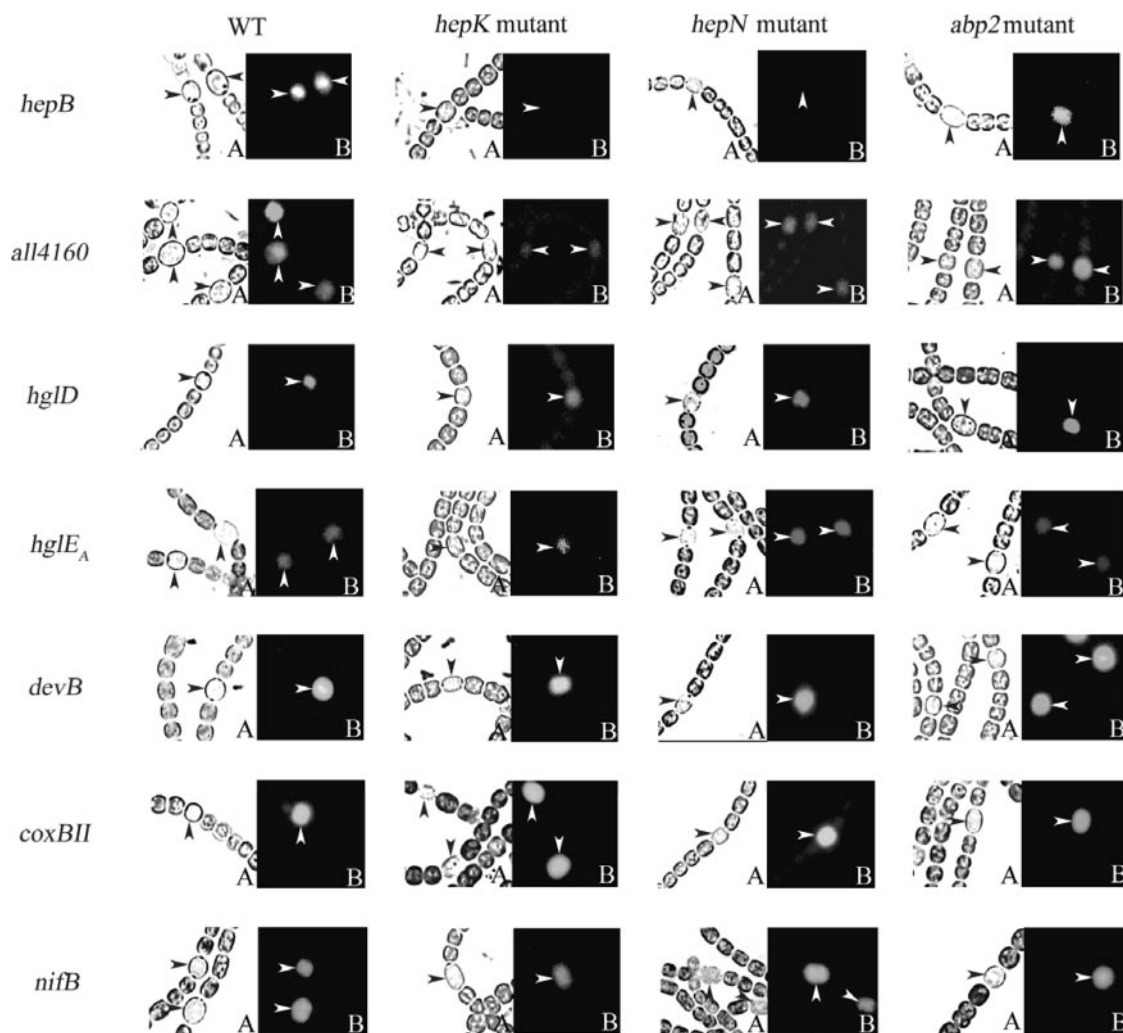


FIG. 3. Expression of fusions of *gfp* to presumptive promoter regions of *all4160* and of other genes in *Anabaena* sp. strain PCC 7120 and its Y7 (*hepK*::Tn5-1065), *hepN*::Tn5-1087b, and *abp2* derivatives at 24 h after nitrogen stepdown. The arrowheads indicate heterocysts. (A) Bright-field images; (B) images of fluorescence of GFP.

tant, in neither of these mutants was *all4160* expression reduced nearly as greatly as the expression of HEP island genes was (Fig. 3). In contrast, both *all4160* and *hepB* were expressed similarly in the wild type and in the *abp2* mutant; despite the lack of a glycolipid layer in the *abp2* mutant (Fig. 3), so were *hglD*, *hglE_A*, and *devB*.

***all4160* and *alr3699* are involved in the formation of the HEP layer in *Anabaena*.** Transposon mutants and targeted insertional *all4160* or *alr3699* mutants were defective in the formation of the HEP layer. Plasmid pHB2429 (Table 1) was used to inactivate *all4160*, as described previously (4), by insertion of cassette C.CE2 into the *Cla*I site of this gene through homologous double recombination (Fig. 1). Complete segregation of the resulting mutant, DRHB2429, was confirmed by PCR with primers *all4160*-1 and *all4160*-2 (Table 1; data not shown). Similarly, complete segregation of the transposon-generated *all4160* mutants FQ873 and FQ1421 was confirmed by PCR with primers IDT141 and IDT142, and complete segregation of the transposon-generated *alr3699* mutant FQ689 was confirmed by PCR with primers IDT641 and IDT642 (Ta-

ble 1; data not shown). Mutants DRHB2429 and FQ689 lacked the polysaccharide layer of the heterocyst envelope, as determined by staining with Alcian blue (Fig. 4), and were unable to grow aerobically in the absence of fixed nitrogen.

Because the independent transposon mutants and the targeted insertion mutants had the same phenotype, the lack of a HEP layer in the *all4160* and *alr3699* mutants was very unlikely to be attributable to the presence of additional mutations elsewhere in the genome. If *all4160* and *all4159* are cotranscribed, a mutation in *all4160* might exert a polar effect on *all4159*. However, because these genes are separated by 674 bp of noncoding sequence, it is very improbable that they are cotranscribed. Moreover, the genes 3' from *alr3699* presumably encode a defective *Pst*I methylase and endonuclease (24), for which no role in synthesis of HEP is evident. Nonetheless, we sought to test experimentally whether *all4160* and *alr3699* play essential roles in heterocyst development or whether mutation of these genes has only a polar effect.

all4160 mutants FQ873 and FQ1421 were first successfully complemented with BAC clone *anc0304*, and *alr3699* mutants

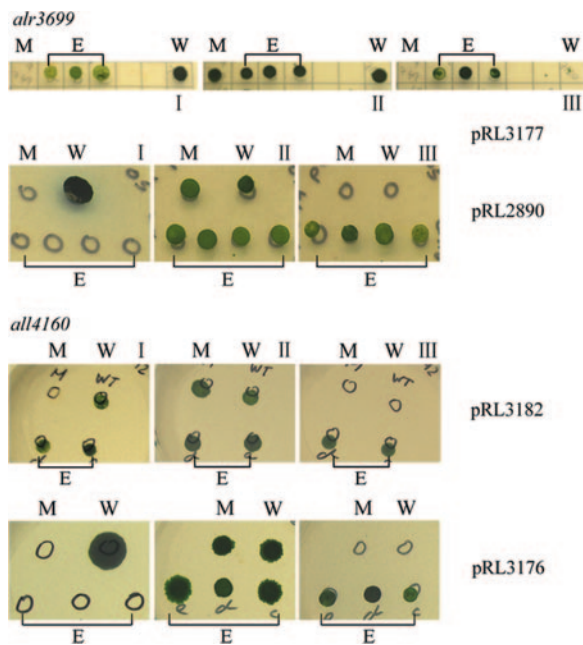


FIG. 5. Complementation of *alr3699* mutant FQ749 and *all4160* mutant FQ792. E, plasmid-bearing exconjugants; M, mutant; W, wild-type *Anabaena* sp. strain PCC 7120. Petri dishes contained agar-solidified medium AA (I), AAN (II), and AAN plus neomycin (to which the transposon in the mutant confers resistance) and either erythromycin (pRL3176, pRL3177) or spectinomycin (pRL2890, pRL3182), to which the potentially complementing plasmid confers resistance (III). Petri dishes were photographed at the end of the following periods of incubation: 4 weeks (pRL3177 series), 6 weeks (pRL2890 series), 2 weeks (pRL3182 series), and 2 months (pRL3176 series). All strains spotted grow in the presence of nitrate and the absence of antibiotics, only the exconjugant strains grow in the presence of neomycin plus a second antibiotic, and only the complemented mutant strains and wild-type *Anabaena* sp. strain PCC 7120 grow in the absence of fixed nitrogen. There was complementation of the mutants (i.e., growth of exconjugants in the absence of fixed nitrogen) by clones bearing a wild-type copy of the transposon-mutated gene (pRL3177, pRL3182), and there was no complementation by clones bearing a copy of the downstream gene(s) whose transcription may be affected as a polar effect of the transposon insertion. Especially when there is no spot of growth, the circles on some petri dishes show the approximate positions of spots.

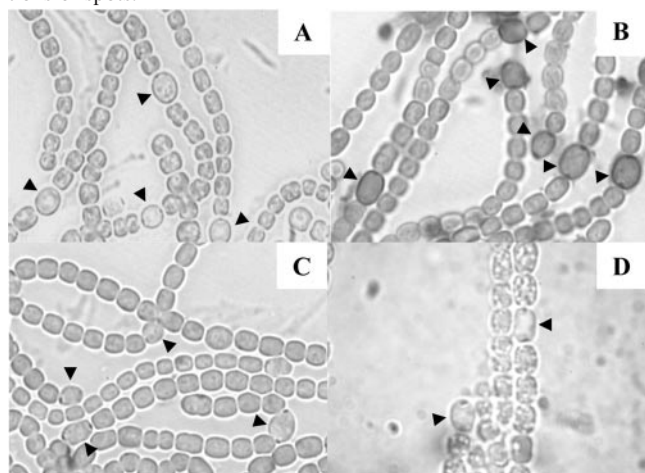


FIG. 4. Micrographs of unstained *Anabaena* sp. strain PCC 7120 (A) and, after attempted staining with Alcian blue, of the wild type (B), mutant *all4160::C.CE2* (C), and *alr3699* mutant FQ689 (D). The arrowheads indicate heterocysts.

FQ689 and FQ1351 were first complemented with BAC clone *anc2015* (data not shown). In addition, complementation of *all4160* mutant FQ792 was sought with plasmids pRL3182 and pRL3176, whose only intact *Anabaena* sp. strain PCC 7120 genes are *all4159* and *all4159*, respectively, and complementation of *alr3699* mutant FQ749 was sought with plasmids pRL3177 and pRL2890, which contain *alr3699* and both *alr3700* and *alr3701*, respectively, as their only intact *Anabaena* sp. strain PCC 7120 genes (Fig. 1 and Table 1). The cloned fragments were all positioned to have their genes driven in the sense orientation by the *glnA* promoter in their vectors. pRL3182 complemented the mutation in FQ792, but pRL3176 did not, and pRL3177 complemented the mutation in FQ749, but pRL2890 did not (Fig. 5). These results support the idea that *all4160* and *alr3699* are required for complementation of their respective mutations, i.e., that the phenotypes of mutations in these genes are not due only to polar effects on their downstream genes.

This study, together with previous reports (23, 32, 36), shows that at least three predicted glycosyl transferase genes—*all4160*, *hepB*, and *alr3699*—located outside of the HEP island are up-regulated in developing heterocysts and that at least *all4160* and *alr3699* are required for the formation of HEP. The up-regulation of HEP island genes and of the *hepB*-*alr3699* cluster appears to be highly dependent on *hepK* and *hepN* during heterocyst differentiation, and the up-regulation of *all4160* is only partially dependent on these genes. Our unpublished results also indicate that other predicted glycosyl transferase genes (for example, *all0919*) are located outside of the HEP island and are up-regulated in developing heterocysts but are not essential for diazotrophic growth under aerobic conditions.

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