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Clustered Genes Required for the Synthesis of Heterocyst Envelope Polysaccharide in *Anabaena* sp. Strain PCC 7120

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As demonstrated with *alr2835* (*hepA*) and *alr2834* (*hepC*) mutants, heterocysts of *Anabaena* sp. strain PCC 7120, a filamentous cyanobacterium, must have an envelope polysaccharide layer (the Hep$^+$ phenotype) to fix dinitrogen in an oxygen-containing milieu (the Fox$^+$ phenotype). Transpositions presumptively responsible for a Fox$^-$ phenotype were localized in open reading frames (ORFs) near *hepA* and *hepC*. A mutation in each of nine of these ORFs was complemented by a clone bearing only that single, intact ORF. Heterocysts of the nine mutants were found to lack an envelope polysaccharide layer. Complementation of mutations in *alr2832* and *alr2840* may have resulted from recombination. However, *alr2825*, *alr2827*, *alr2831*, *alr2833*, *alr2837*, *alr2839*, and *alr2841*, like *hepA* and *hepC*, are required for a Hep$^+$ Fox$^+$ phenotype.

When *Anabaena* spp. and certain other filamentous cyanobacteria are deprived of combined nitrogen in the presence of O_2, 5 to 10% of their vegetative cells differentiate into cells called heterocysts. Differentiation takes place at semiregular intervals along the filaments, forming a spacing pattern (34, 38). The process of differentiation involves changes in cellular biochemistry that collectively produce a micro-oxic intracellular milieu in which O_2-labile nitrogenase in heterocysts is protected from inactivation by O_2 (15, 16; J. Elhai and C. P. Wolk, Abstr. 7th Int. Symp. Photosynthetic Prokaryotes, abstr. 114B, 1991). Protection involves inactivation of the O_2-producing photosystem II of vegetative cells (1), deposition of extracellular layers of polysaccharide and glycolipid that greatly decrease entry of O_2 (25, 33), increased respiration by which O_2 that does enter is reduced to H_2O (25), and intercellular interactions that provide heterocysts with the requisite reductant (38).

We shall denote as Fox genes genes that are required specifically for nitrogen fixation in the presence of oxygen. Genes required for the formation of heterocyst envelope polysaccharide are Fox genes and include the following: $devR_A$ (*alr0442*) and *hepK* (*all4496*), whose products interact as parts of a two-component regulatory system (42); *alr0117* and *alr1086* (26; our unpublished results), whose products also resemble elements of such a system; *hepB* (*alr3698*) and *hepC* (*alr2834*), whose predicted products show greatest similarity to a glycosyl transferase and a UDP-galactose-lipid carrier transferase, respectively (23, 34, 35, 39, 44); and *hepA* (*alr2835*), which encodes a member of the family of ATP-binding proteins of ATP-binding cassette transporters and is activated in response to nitrogen deprivation (12, 18, 37). These genes are dispersed at Mb positions 0.12, 0.52, 1.27, 3.45 (*hepC* and *hepA*), 4.47, and 5.38 in the 6.41-Mb chromosome of *Anabaena* sp. strain PCC 7120. Heterocyst differentiation in *Anabaena* sp. strain PCC 7120 was analyzed with microarrays whose elements contained part or all of one to eight open reading frames (ORFs)(12). In addition to *hepA*, ORFs close to it in the *Anabaena* sp. genome (20), including (i) *alr2823* and/or *alr2824*, (ii) *alr2826*, *alr2828*, *alr2832*, *alr2833*, *alr2836*, and *alr2839*, and (iii) one or more of *alr2841* through *all2843*, were found to be up-regulated early in heterocyst differentiation (12, 18). Because up-regulation does not imply essentiality for differentiation or function, we asked whether ORFs other than *hepA* and *hepC* in this region are Fox genes, and we identified seven that are.

MATERIALS AND METHODS

Growth conditions for cyanobacteria. *Anabaena* sp. strain PCC 7120 was maintained in AA/8 medium (19) at 30°C in the light (ca. 3,500 ergs cm^{-2} s^{-1}) on a rotary shaker. Liquid cultures to be transposon mutagenized and liquid cultures of derivatives of *Anabaena* sp. (Table 1) were grown under the same conditions but in AA/8+N medium (19) in the presence of appropriate antibiotics, unless specified otherwise. Tests of complementation (see below) and some preparation for microscopy made use of medium AA or AA+N solidified with 1.2% home-purified (Difco) Bacto agar (19), plus antibiotics as appropriate.

Transposon mutagenesis, identification of mutants, and localization of transposons. *Anabaena* sp. cells were mutagenized by transposon Tn*5*-1063 (36), mutant colonies were selected by growth in the presence of 400 μg of neomycin sulfate (Nm) per ml, and those that were presumptively Fox$^-$ were identified by their persistent change of color from blue-green to yellow and lack of protracted growth in response to nitrogen deprivation. Such colonies were grown with nitrate supplemented with Nm for selection, and their DNA was extracted (7). DNA contiguous with the transposon was amplified by inverse PCR (27; Q. Fan et al., unpublished data), the PCR products were sequenced, and the sequences were localized within the genome of *Anabaena* sp. (20; http://www.kazusa.or.jp/cyano/Anabaena/).

Induction of heterocysts, staining with Alcian blue, and extraction of glycolipids. To induce heterocyst formation, strains that had been grown in AA/8+N medium were washed three times with nitrogen-free AA/8 medium, and a 5-μl portion of suspension was spotted on agar-solidified AA medium. One week later, the agar bearing a spot was excised, stained with 2 μl of a 1% aqueous solution of Alcian blue for 40 s, washed with distilled water to remove nonad-

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TABLE 1. Bacterial strains and plasmids used

Strain or plasmid	Derivation and/or relevant characteristics[a]
Strains of *Anabaena* sp.	
PCC 7120	Wild type, from R. Haselkorn
DR1069	Sm^r Sp^r; *alr2835* (*hepA*) mutation resulting from double recombination with pRL1069 (5)
FQ57	Bm^r Nm^r Sm^r; *alr2827*::Tn*5*-1063
FQ344	Bm^r Nm^r Sm^r; *alr2841*::Tn*5*-1063
FQ428	Bm^r Nm^r Sm^r; *alr2837*::Tn*5*-1063
FQ470	Bm^r Nm^r Sm^r; *alr2833*::Tn*5*-1063
FQ610	Bm^r Nm^r Sm^r; *alr2838*::Tn*5*-1063
FQ773	Bm^r Nm^r Sm^r; *alr2832*::Tn*5*-1063
FQ794	Bm^r Nm^r Sm^r; *alr2839*::Tn*5*-1063
FQ885	Bm^r Nm^r Sm^r; *alr2825*::Tn*5*-1063
FQ1630	Bm^r Nm^r Sm^r; *alr2840*::Tn*5*-1063
FQ1631	Bm^r Nm^r Sm^r; *alr2831*::Tn*5*-1063
Strains of *E. coli*	
DH10B	17
DH5αMCR	Invitrogen Corp., received courtesy of J. C. Meeks
HB101	6
Plasmids	
anc0293	Cm^r Em^r; *Anabaena* sp. strain PCC 7120 chromosomal DNA from bp 3436903 to 3449816 in the BamHI site of pRL838
anc0626	Cm^r Em^r; *Anabaena* sp. strain PCC 7120 chromosomal DNA from bp 3451665 to 3468470 in the BamHI site of pRL838
anc0901	Cm^r Em^r; *Anabaena* sp. strain PCC 7120 chromosomal DNA from bp 3446424 to 3461510 in the BamHI site of pRL838
anp02206	Ap^r; *Anabaena* sp. strain PCC 7120 chromosomal DNA from bp 3440325 to 3447227 in the BamHI site of pUC18
anp04627	Ap^r; *Anabaena* sp. strain PCC 7120 chromosomal DNA from bp 3444424 to 3452207 in the BamHI site of pUC18
anp08632	Ap^r; *Anabaena* sp. strain PCC 7120 chromosomal DNA from bp 3457063 to 3463731 in the BamHI site of pUC18
pGEM-T Easy	Ap^r; cloning vector (Promega Corp.)
pK18	Km^r; cloning vector (28)
pRL443	Ap^r Tc^r; conjugative plasmid (13)
pRL838	Cm^r Em^r; BAC vector (20) (GenBank accession no. AF403425)
pRL1124	Km^r; methylase-encoding plasmid (13)
pRL1383a	Sm^r Sp^r; RSF1010-based cloning vector (GenBank accession no. AF403428)
pRL2665b	Ap^r Cm^r Em^r; source of C.CE3-*oriT*(RK2) cassette to make derivatives of pUC18 and pGEM-T Easy conjugable to, and selectable in, *Anabaena* sp. strain PCC 7120 (GenBank accession no. AY661563)
pRL2683a	Km^r Sm^r Sp^r; pRL1124 made mobilizable by the insertion, at its unique *Pst*I site, of an *oriT* (RK2) fragment (32) in whose SalI site is inserted Sm^r Sp^r cassette C.S4 (3, 37)
pRL2686	Km^r; Sm^r Sp^r marker removed from pRL2683a by SalI digestion and religation
pRL2792	Ap^r; internal fragment of *alr2833* amplified by PCR using genomic DNA from *Anabaena* sp. strain PCC 7120 as template and primers IDT145 and IDT146, cloned in pGEM-T Easy
pRL2815	Ap^r Cm^r Em^r; PstI fragment containing C.CE3-*oriT* cassette from pRL2665b transferred to the PstI site of pRL2792
pRL2831a	Sm^r Sp^r; glutamine synthetase promoter P_{glnA} on a 0.7-kb EcoRI fragment from pRL559 (14) transferred to the EcoRI site of pRL1383a, oriented toward the BamHI site
pRL2831b	Sm^r Sp^r; same as pRL2831a, but P_{glnA} oppositely oriented
pRL2862	Sm^r Sp^r; *alr2831*-containing StuI-HindIII fragment of anc0293, subcloned between the Ecl136II and HindIII sites of pRL2831b
pRL2863	Sm^r Sp^r; *alr2832*-containing EcoRV-XhoI fragment of anc0293 transferred between the StuI and XhoI sites of pRL2831a
pRL2864	Sm^r Sp^r; *alr2827*-containing XmnI-XbaI fragment of anc0293, subcloned between the StuI and XbaI sites of pRL2831a
pRL2865a	Sm^r Sp^r; *alr2833*-containing HindIII fragment of anp04627 transferred to the HindIII site of pRL2831b
pRL2866	Sm^r Sp^r; *alr2841*-containing HindIII fragment of anc0626, transferred to the HindIII site of pRL2831b
pRL2871	Km^r; *alr2825*-containing KpnI-XmnI fragment of anp02206 transferred between the KpnI and HincII sites of pK18
pRL2872	Km^r; *alr2839*-containing EcoRV fragment of anp08632 inserted in the HincII site of pK18
pRL2873	Sm^r Sp^r; *alr2840*-containing HincII-XbaI fragment of anp08632, transferred between the StuI and XbaI sites of pRL2831a
pRL2875	Sm^r Sp^r; *alr2825*-containing KpnI-SphI fragment of pRL2871, transferred between the same sites of pRL2831b
pRL2876	Sm^r Sp^r; *alr2839*-containing KpnI-SphI fragment of pRL2872, transferred between the same sites of pRL2831b
pRL2877	Sm^r Sp^r; *alr2837*-containing XbaI-BglII fragment of anp08632 transferred between the XbaI and BamHI sites of pRL2831a
pUC18	Cloning vector (41)

[a] Sm, streptomycin; Bm, bleomycin; Cm, chloramphenicol; Ap, ampicillin; Tc, tetracycline; Km, kanamycin.

sorbed stain, and examined by microscopy. For extraction of glycolipids, the agar bearing a spot of cells was immersed for 5 min in a solution of chloroform and methanol (2:1, vol/vol) and the adhering organic solution was permitted to evaporate. Photographs were taken with a Nikon CoolPix 4300 digital camera mounted on a Wild microscope.

Construction of plasmids. RSF1010-based vectors pRL2831a and pRL2831b (plasmids are described in Table 1) were constructed to express single ORFs from the *glnA* promoter. "a" and "b" refer to two orientations of that promoter, allowing cloning into one end or the other of an extensive polylinker. Plasmid RSF1010 was chosen because it can be mobilized to, and can replicate in, cells of

TABLE 2. Primers used

Primer	Sequence	Use
IDT93	5′-TAA CCA GAA TCA TGA CCG TTT G-3′	For PCR amplification of *hepA* (IDT93 and -94)
IDT94	5′-CAC CAC AAC CTT ATC TGC TTT G-3′	
IDT145	5′-ATT ATA CTT CTG GCG ACC CTG A-3′	To clone an internal fragment of *alr2833* (IDT145 and -146)
IDT146	5′-ACT GTT TCA TCC GTG GAG AAC T-3′	
IDT249	5′-AAT ATA CAG GTG ATT CGA CAA AGG-3′	P3 for *alr2825*
IDT250	5′-TAA ATC TCC GCC TTG TTG TTG AAT-3′	P4 for *alr2825*
IDT251	5′-TTG GAA ACT TGC AGG CAA ACA CGC-3′	P3 for *alr2827*
IDT252	5′-TAA CCA TCC AAT GGC TAG CAC CAA-3′	P4 for *alr2827*
IDT253	5′-GAA TTT TAT ACC CCA GGA TAT GAA-3′	P3 for *alr2831*
IDT254	5′-TCT TTG CGT TGG TTG TCA CTA ACA-3′	P4 for *alr2831*
IDT255	5′-AAA TAC TAT TAT TTA CTA GCG CGC-3′	P3 for *alr2832*
IDT256	5′-AAT AAA TGA ATG TTG AGT AAT CAG-3′	P4 for *alr2832*
IDT259	5′-ATA ACT TCG TAT AGC ATA CAT T-3′	P1 for *alr2827*, *alr2832*, *alr2837*, and *alr2840*; P2 for *alr2825*, *alr2833*, *alr2839*, and *alr2841*
IDT260	5′-GAT CTT ATT TCA TTA TGG TGA-3′	P2 for *alr2827*, *alr2832*, *alr2837*, and *alr2840*; P1 for *alr2825*, *alr2833*, *alr2839*, and *alr2841*
IDT271	5′-GGC GTT GGC GGT TGC AGA CC-3′	For PCR amplification of *rnpB* (IDT271 and -272)
IDT272	5′-AGT TGG TGG TAA GCC GGG TTC-3′	
IDT295	5′-GAA AGA GAA AGA TTA CAA GCC-3′	P3 for *alr2833*
IDT296	5′-TTG AGA ATT ATT TGC TGA ATA-3′	P4 for *alr2833*
IDT297	5′-ATC GAC GCA CAA AAT ATT ATC-3′	P3 for *alr2837*
IDT298	5′-AGC AAT TTT CCA AAT CCT CAG-3′	P4 for *alr2837*
IDT299	5′-ATG GTA AAA GTT CCT TGA TGG-3′	P3 for *alr2839*
IDT300	5′-TTT CAA TTT GCG CTA ACC AAT-3′	P4 for *alr2839*
IDT301	5′-TTA CTG CTA GTT CCA CAG GTG-3′	P3 for *alr2840*
IDT302	5′-AAA TAG ATA AAA AGC AAA GGT-3′	P4 for *alr2840*
IDT303	5′-GCA GCG ATT TGA TGA AGA CCA-3′	P3 for *alr2841*
IDT304	5′-TGA TTG GAC TCG GCT TGT TAG-3′	P4 for *alr2841*
IDT347	5′-GGC AAC CTC ATG TCC TCA TC-3′	P1 for *alr2831*
IDT348	5′-GCC GCA TAC GAT TTA GGT GA-3′	P2 for *alr2831*
IDT384	5′-CGC ACA CAT CTT TTT ATT CAG C-3′	Sequencing primer to test plasmid recovered from *Anabaena* sp.::pRL2815
IDT390	5′-AAT GAT TGG CGC TGG TTT TA-3′	To amplify an internal fragment of *alr2831* by PCR (IDT390 and -391)
IDT391	5′-TCA CGT TTG AGA CGA CAT CC-3′	
IDT392	5′-GGC AGA ACG GGT ATT TGA A-3′	To amplify an internal fragment of *alr2832* by PCR (IDT392 and -393)
IDT393	5′-AAA GAA AAC TGG CTC AGA AAA A-3′	
IDT394	5′-CCT GAT GAC TGC AAG AAC CA-3′	To amplify an internal fragment of *alr2833* by PCR (IDT394 and -395)
IDT395	5′-GGC TGT AGC TTG AGC GAT TT-3′	
IDT396	5′-TGA CAA GCG TAA TAG TTC CAA A-3′	To amplify an internal fragment of *alr2834* by PCR (IDT396 and -397)
IDT397	5′-TTT TGT AGG CTT GCG TTC CT-3′	

Anabaena sp. (31), and a *glnA* promoter was chosen because it is expressed both in heterocysts and vegetative cells (14). To facilitate transfer of inserts, the parental plasmids bore the same polylinker as did BAC vector pRL838 (see the next paragraph).

Complementation experiments. Complementation experiments were carried out initially with mapped, pRL838-based BAC clones from the *Anabaena* sp. strain PCC 7120 sequencing project (20) to determine whether the mutation that was responsible for the mutant phenotype was localized near the transposon insertion. Conjugative plasmid pRL443 and a mobilizable methylating plasmid, either pRL2683a or pRL2686, were introduced into *Escherichia coli* strain DH10B bearing a BAC. The resulting strain was mated diparentally with a corresponding mutant with selection for resistance to Nm, conferred by the transposon, and erythromycin (Em), conferred by the BAC. Recombination of the BAC with the genome, permitting replication of the selective marker, resulted routinely in the growth of hundreds of colonies. Three or four colonies from such a mating were grown individually in medium AA/8+N supplemented with 50 μg of Nm per ml and 2 μg of Em per ml and then spotted on petri dishes containing AA agar, AA+N agar, and AA+N agar plus 400 μg of Nm and 10 μg of Em per ml. Liquid cultures of wild-type *Anabaena* sp. strain PCC 7120 and of the original mutant were also spotted. The mutation was considered complemented (i) if spots from two or more of the cultures and the wild type greened and grew, while the mutant yellowed, remained yellow, and failed to grow, on AA agar; (ii) if all cultures grew on AA+N agar; and (iii) if the presumptive exconjugants grew and only the original mutant and the wild-type strain died on AA+N agar containing the two antibiotics.

In further complementation experiments, RSF1010-based plasmids in *E. coli* strain DH5αMCR were transferred by triparental matings with *E. coli* strain DH10B bearing pRL443 and pRL2686. Exconjugants were selected on agar-solidified AA+N medium with 200 μg of Nm per ml, to select for the presence of the transposon, plus 10 μg of spectinomycin dihydrochloride (Sp) per ml to select for a derivative of RSF1010. Subsequent testing of exconjugants was performed as described for BAC-bearing exconjugants, but with Sp replacing Em for selection.

Construction of an *alr2833* mutant by insertional mutagenesis. An internal fragment of *alr2833* was generated by PCR using primers IDT145 and IDT146 (Integrated DNA Technologies, Coralville, Iowa) (Table 2) with DNA from wild-type *Anabaena* sp. as template. The PCR product was cloned into vector pGEM-T Easy (Promega). A cassette (from pRL2665b) permitting mobilization into, and selection in, *Anabaena* sp. was introduced into the PstI site of the resulting plasmid, yielding pRL2815 in *E. coli* HB101. Mating with *E. coli* DH10B containing pRL443 and pRL2686 resulted in coresidence of the three plasmids in *E. coli* HB101. pRL2815 was introduced into *Anabaena* sp. by a diparental mating, and the phenotypes of Em-resistant exconjugants were determined. The position of the insertion was verified by recovery (36) in *E. coli*

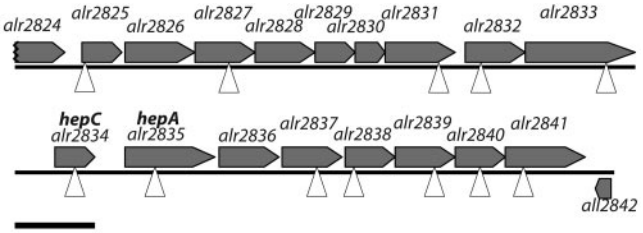


FIG. 1. Map (20) of genes in an "expression island" (12) of the genome of *Anabaena* sp. strain PCC 7120. The positions of transposons in mutants that were successfully used for complementation are indicated by triangles. The sole insertion found in *alr2834* was reported earlier (44). The marker extends 1.5 kb.

DH5αMCR of pRL2815 and contiguous DNA, following digestion of genomic DNA with HindIII, and analysis of transformants by restriction, separately, with PstI and XmnI, and by sequencing through the unique HindIII site of the plasmid with primer IDT384.

PCR analysis. An ORF cloned in a replicating plasmid may complement either in *trans* or, if the clone recombined into the *Anabaena* sp. genome, in *cis*. PCR analysis was used to look for possible single or double recombination. Primers P1 and P2 were chosen that flanked the cloning region of the vector in question; for each gene to be tested, primers P3 and P4 were chosen that were specific to the genome outside of the region of the clone. PCR with *Taq* polymerase (Invitrogen) was performed by denaturation at 94°C for 3 min; 30 cycles of 94°C for 1 min, 50°C for 1 min, and 72°C for 2 or 4 min; and then 72°C for 10 min. DNA templates were isolated (7) from the wild-type strain and the mutants and from complemented mutants that were subcultured to liquid AA/8 from spots growing on AA agar.

Reverse transcription-PCR analysis. Cells of a 50-ml culture of wild-type *Anabaena* sp. strain PCC 7120 were sedimented after deprivation of fixed nitrogen for 8 h and resuspended in 400 μl of T0.1E buffer (10 mM Tris, 0.1 mM EDTA; pH 8.0), and RNA was extracted at 65°C in the same way as was DNA (6), except that acidic phenol (pH 4.8) was used. RNA was precipitated with a one-fourth volume of 8 M LiCl and 1 volume of isopropanol at −80°C for 15 min. The pellet was suspended in 100 μl of 0.1 M sodium acetate and 5 mM $MgSO_4$ and treated twice at 37°C for 15 min with RNase-free DNase (Roche). RNA was then loaded on an RNeasy column (QIAGEN) and treated again twice with RNase-free DNase (QIAGEN) for 15 min each according to the manufacturer's instructions, and purified RNA was then eluted from the column and tested for the absence of contaminating DNA by PCR amplification with primers for *hepA* (IDT93 and IDT94) and *mpB* (IDT271 and IDT272). cDNA was generated with random primers using 0.5 μg of RNA and Superscript II (Invitrogen) according to the manufacturer's instructions. PCR with *Taq* polymerase (Invitrogen) was performed by denaturation at 94°C for 3 min; 29 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 4 min; and then 72°C for 10 min.

RESULTS AND DISCUSSION

Localization of transposons within the genome of *Anabaena* sp. *Anabaena* sp. strain PCC 7120 was mutagenized with transposon Tn*5*-1063 and screened for Fox$^-$ mutants. The positions of representatives of 63 of the transposition loci thereupon identified and found to be clustered in its chromosome from *alr2825* to *alr2841* are shown in Fig. 1. The corresponding mutants listed in Tables 1 and 3 were selected for further study. As illustrated by the lack of an insertion in *hepC*, our transposon mutagenesis achieved only near-saturation (Fan et al., unpublished).

Phenotypes of presumptive Fox$^-$ mutants. In response to nitrogen deprivation on an agar-solidified medium, each transposon mutant herein described formed heterocysts (Fig. 2A). Solid medium was used because the shear in shaken liquid cultures can disperse a heterocyst envelope glycolipid layer that lacks a protective envelope polysaccharide layer. To determine whether the mutants, like *hepA* and *hepC* mutants, lacked heterocyst envelope polysaccharide, we tried to stain them with Alcian blue. This stain colors the envelope of heterocysts of wild-type *Anabaena* sp. strain PCC 7120 blue (M. Gantar, J. Elhai, J. Jia, and M. Ow, Abstr. 5th Cyanobacterial Mol. Biol. Workshop, p. 25, 1995) (Fig. 2B) but did not stain the heterocyst envelope of *hepA* mutant DR1069, which lacks

TABLE 3. ORFs in the *hepA* region, annotated functions, and complementation

ORF	No. of transposon mutants	Representative mutant	Complementing BAC	Complementing replicating plasmid	Annotated function[a]
alr2822	0				Similar to hypothetical protein YegL of *E. coli*
alr2823	0				Similar to hypothetical protein YegK of *E. coli*
alr2824	0				Similar to hypothetical protein YegI of *E. coli*
alr2825	6	FQ885	anc0293	pRL2875	Glucose-1-P cytidyltransferase[b]
alr2826	0				Dehydrogenase
alr2827	1	FQ57	anc0293	pRL2864	Epimerase/dehydratase[b]
alr2828	0				Glycosyltransferase[b]
alr2829	0				Hypothetical protein
alr2830	0				dTDP-4-dehydrorhamnose 3,5-epimerase and related enzymes[b]
alr2831	12	FQ1631	anc0293	pRL2862	NAD(P)-dependent oxidoreductase[b]
alr2832	3	FQ773	anc0293	pRL2863	Glycosyltransferase
alr2833	7	FQ470	anc0901	pRL2865a	Hypothetical protein (see text)[b]
alr2834	0				*hepC*[b]
alr2835	3				*hepA*[b]
alr2836	0				Glycosyltransferase
alr2837	8	FQ428	anc0901	pRL2877	Glucosyltransferase (see text)
alr2838	1	FQ610[c]	anc0901	—[c]	Glycosyltransferase
alr2839	6	FQ794	anc0901	pRL2876	Glycosyltransferase
alr2840	6	FQ1630	anc0626	pRL2873	Glycosyltransferase
alr2841	10	FQ344	anc0626	pRL2866	Unknown[b]

[a] http://www.kazusa.or.jp/cyano/Anabaena/.
[b] The text discusses possible relationships of these genes to the biosynthesis of an LPS.
[c] Analysis of FQ610, whose mutation was incompletely segregated and not complemented by a single-gene construct (data not shown), is continuing.

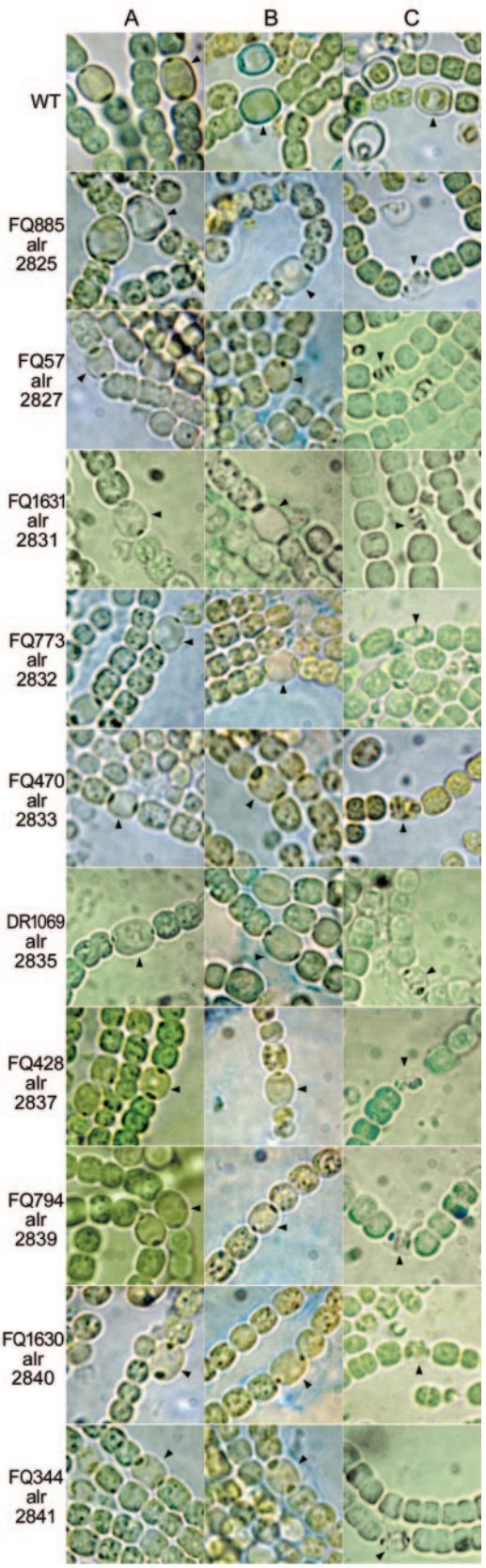


FIG. 2. Wild-type and mutant strains of *Anabaena* sp. strain PCC 7120 unstained (A), stained with Alcian blue (B), and lipid extracted with chloroform and methanol (2:1, vol/vol) (C). Only the wild-type strain retained heterocyst envelope polysaccharide, as assessed by staining with Alcian blue and retention of an envelope layer evident after lipid extraction. Aberrant overall coloration resulting from various intensities of illumination was partially corrected with Photoshop 7.0. Arrowheads point to heterocysts.

a polysaccharide layer (34) (Fig. 2B), or of any other transposon mutant shown in Fig. 2. In response to extraction with organic solvents, the protoplasts of heterocysts shrank. In the wild-type strain, a gap was seen within the residual polysaccharide layer, whereas in those mutants no thick envelope remained (Fig. 2C). The lack of staining with Alcian blue and loss of a pronounced envelope layer upon lipid extraction suggest that transposon insertion in any one of those mutated ORFs is sufficient to block deposition of heterocyst envelope polysaccharide. Alcian blue did stain heterocyst envelopes of incompletely segregated mutant FQ610 (Tables 1 and 3 and data not shown).

High-resolution electron microscopy had earlier shown that heterocysts of *hepA* (*alr2835*) and *hepC* (*alr2834*) mutants deposit only glycolipids (34, 44). A thicker glycolipid layer appears to be formed in Hep$^-$ mutants than in the wild-type strain, a finding reminiscent of the observation that increased pO_2 increases formation of heterocyst envelope glycolipid (21); deposition of heterocyst envelope glycolipid may be adjusted in response to permeant O_2 (38).

Complementation of presumptive Fox$^-$ mutants with BACs and with replicating plasmids. The phenotype of a transposon-generated mutant may result from a second mutation elsewhere in the genome combined with antibiotic resistances conferred by the transposon. To provide an initial test of which developmental changes were due to insertions of the transposon, we attempted to complement the mutations by recombination with clones containing the wild-type ORFs that had been intercepted in the mutants. The BAC-based plasmids used as bridging clones in the *Anabaena* sp. sequencing project bore inserts that averaged ca. 18 kb in size. Because of the very low copy number of these F-based plasmids, they are believed to be less liable to deletions than are plasmids of higher copy number. Mutations in the newly mutated genes indicated in Fig. 1 were complemented by recombination with predicted BAC clones.

Although a mutation was complemented by a BAC, the Fox$^-$ mutant phenotype might have been due to a mutation in a position near the transposon-mutated ORF and, if the ORF were in an operon but not at the 3′ terminus of the operon, might have resulted from a polar effect of the transposon on the expression of a downstream gene or genes in that operon. Tests of complementation by a single gene in a replicating plasmid were predicated on the following idea. If a gene complemented in *trans*, the effect could be attributed to no other gene, because no other entire gene would have been present in the complementing DNA. In particular, the mutant phenotype could not be attributed to a polar effect of the mutation on the transcription of a downstream gene because transcription of that gene would not have been affected in *trans*.

Complementation by a single gene was tested by subcloning the entirety of that gene together with at most a fragment of its neighboring genes into vectors pRL2831a or pRL2831b. Mu-

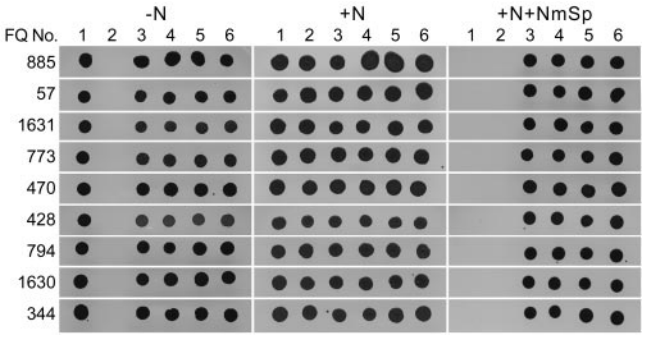


FIG. 3. Tests of complementation by clones bearing, intact, only the gene mutated in the corresponding mutant: FQ885 (*alr2825*) plus pRL2875, FQ57 (*alr2827*) plus pRL2864, FQ1631 (*alr2831*) plus pRL2862, FQ773 (*alr2832*) plus pRL2863, FQ470 (*alr2833*) plus pRL2865a, FQ428 (*alr2837*) plus pRL2877, FQ794 (*alr2839*) plus pRL2876, FQ1630 (*alr2840*) plus pRL2873, and FQ344 (*alr2841*) plus pRL2866. Spots were grown from cells of wild-type *Anabaena* sp. strain PCC 7120 (lanes 1), a particular FQ mutant (lanes 2), and four independent, exconjugant clones of the particular FQ mutant bearing the corresponding pRL plasmid (lanes 3 to 6). The left, center, and right panels present, respectively, the results observed (for any one mutant, simultaneously) 2 to 3 weeks after spotting cells on agar-solidified media AA, AA+N, and AA+N plus 200 μg of Nm ml^{-1} and 10 μg of Sp ml^{-1}. Cells of the wild-type strain grew in the presence or absence of nitrate but failed to grow in the presence of antibiotic; cells of each mutant grew in the presence of nitrate unless counterselected by antibiotics, but failed to grow in the absence of nitrate; addition of the corresponding cloned genes permitted the mutants to grow with only N_2 as nitrogen source or in the presence of antibiotics.

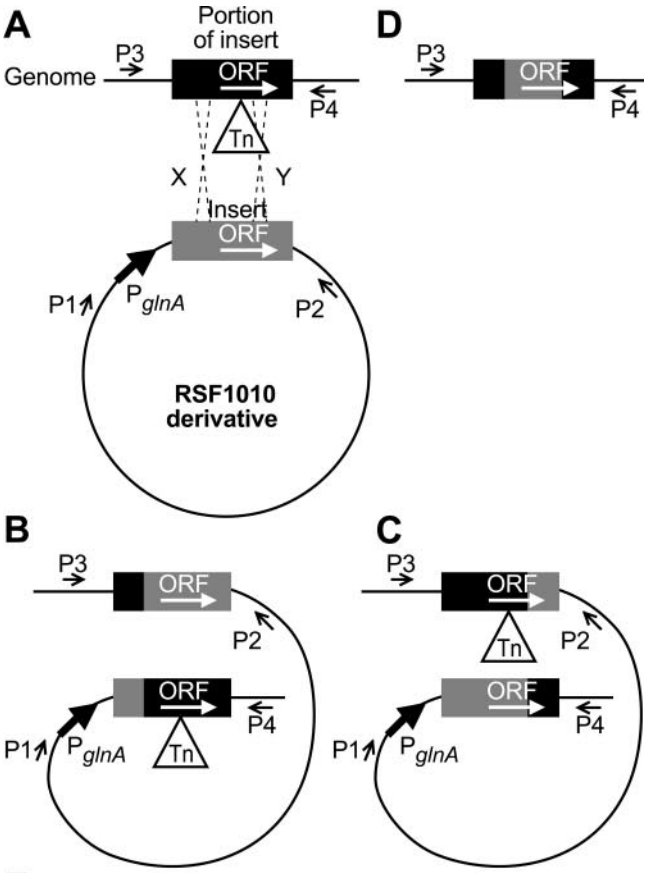
tations in nine of the BAC-complemented, presumptive, newly identified Fox genes (Fig. 1) were complemented by clones bearing a corresponding wild-type gene (Table 3; Fig. 3). These results were consistent with the interpretation that each mutant phenotype resulted from insertion of the transposon in the corresponding gene. However, complementation might have resulted from recombination between the complementing plasmid and the mutated chromosome. A presumptive *recA* gene (*all3272*) has been identified in *Anabaena* sp. strain PCC 7120 (4; http://www.kazusa.or.jp/cyano/Anabaena/), but no cyanobacterial *recA* mutant has been isolated despite extensive attempts (24). Therefore, our work was carried out with a recombination-proficient strain. *alr2840* overlaps *alr2839* and is separated from *alr2841* by only 24 bp, suggesting that those genes may be cotranscribed. In turn, complementation of mutations in *alr2839* and *alr2840* by their respective genes suggested that recombination might have occurred.

PCR analysis (Fig. 4 and 5) was used to determine whether complementation may have resulted from recombination. Complemented mutations in (i) *alr2825*, (ii) *alr2827*, and (iii) *alr2839* showed no evidence of recombination (Fig. 5A, B, and G, respectively), implying that those ORFs are Fox genes. (iv) Complemented *alr2837* mutant FQ428 showed evidence (Fig. 5F) of a single recombination event in which only that ORF could be expressed from its natural promoter. The clone in complementing plasmid pRL2877 extended 183 bp into *alr2836* (and 38 bp 3′ from *alr2837*, 134 bp 5′ from *alr2838*). There is a small chance that the mutation conferring the phenotype of FQ428 may lie within *alr2836*. However, it seems exceedingly unlikely that the phenotype of all eight independent mutations in *alr2837* would be similarly attributable. Therefore, we conclude that *alr2837* is a Fox gene.

(v) Despite showing no single recombination, *alr2833* showed evidence of double recombination (Fig. 5E). An *alr2833* mutation was constructed by insertional mutagenesis with pRL2815, and the locus of insertion was confirmed by restriction and sequencing as described in Materials and Methods. Like *alr2833* mutant FQ470, the resulting mutant was Fox^- and Hep^- (data not shown). Thus, the phenotype of FQ470 may be due to a transposition affecting *alr2833*, a 3′ gene, or both. Its 3′ neighbor, *alr2834*, is 776 bp distant. The intergenic region has 13 or more stop codons in each reading frame, probably blocking further translation. PCR with primer pairs IDT394-IDT395 and IDT396-IDT397 produced products of the predicted size with, as template, DNA from wild-type cells and (separately) cDNA from cells deprived of fixed nitrogen for 8 h. Therefore, *alr2833* and *alr2834* were transcribed. Primers IDT394 and IDT397 produced, with the same sample of DNA as template, a predicted band of 3.63 kb and, with the same sample of cDNA as template, no band (data not shown). Therefore, (a) no transcript bridged the two ORFs, so (b) *alr2834* has its own promoter, implying (c) that the phenotypes of the insertional mutant with pRL2815 and, by extension, the seven *alr2833* transposon mutants are due not to a polar effect but to the effect on *alr2833*, and so (d) *alr2833* is a Fox gene. (vi) Because in complemented FQ773, Fox gene *alr2833* may have been expressed from a promoter in pRL2863 (see the faint band in Fig. 5D, lane 5), the transposon in *alr2832* may have had a polar effect on *alr2833*. Therefore, *alr2832* may not be a Fox gene.

(vii) Plasmid pRL2862 showed single and double recombination with *alr2831* mutant FQ1631 (Fig. 5C). A transcript from *alr2831* to *alr2832* would start with a highly extended stem-loop structure (AAUUGAUUGUUUGGUAGGGUGCGUCAGUAUGAAGAUUUCUGAGUAUAGUUAGGUUCUAUCGCACUGACGCACCCUACUGGAUAGUCUAUU) (http://www.bioinfo.rpi.edu/applications/mfold/old/rna/form1.cgi [45]) and would have at least nine stop codons in each reading frame, presumably blocking further translation. As described for *alr2833* and *alr2834*, PCR and reverse transcription-PCR with primer pairs IDT390 through IDT393 indicated that *alr2831* and *alr2832* were transcribed, but the two ORFs were not bridged by a transcript (data not shown). The mutations in *alr2831* therefore lacked a polar effect. Although the complementing sequence in pRL2862 extends 139 bp into *alr2830*, where a spontaneous mutation could have been corrected by recombination, it is implausible that the phenotype of all 12 transposon mutations in *alr2831* (Table 3) were attributable to such an upstream mutation. Therefore, we conclude that *alr2831* is a Fox gene.

(viii) Plasmid pRL2866 showed evidence of single and double recombination with *alr2841* mutant FQ344 (Fig. 5I), implying that complementation may have been due to recombinant correction of a spontaneous mutation in *alr2840* or *all2842*, into which the insert of pRL2866 extends 268 and 8 bp, respectively (for comparison, *alr2841* and its 5′ and 3′ intergenic regions total 1,608 bp). However, it is hardly credible that such a mutation was parent to the phenotype of all 10 independent mutations observed in *alr2841* (Table 3). Moreover, the effect of the *alr2841* mutations that we identified cannot be



E

Lane	Source of template	Primer pair	Comment
1	Wild type	P3, P4	Wild type (positive control)
2	Mutant	P3, P4	No product expected, or segregation incomplete
3	Complemented mutant	P3, P4	Double recombination (if mutation fully segregated)
4	Complemented mutant	P2, P3	Single recombination upstream from Tn (at X)
5	Complemented mutant	P1, P4	Single recombination downstream from Tn (at Y)
6	Complemented mutant	P1, P2	Free plasmid

F

			primer		*bp predicted, lanes*			
Orf alr	**mut't FQ**	**pl'd pRL**	**P3 IDT**	**P4 IDT**	**1 (2,3)**	**4**	**5**	**6**
2825	885	2875	249	250	2251	2318	3101	3168
2827	57	2864	251	252	2198	2293	3012	3102
2831	1631	2862	253	254	2727	2749	3581	3584
2832	773	2863	255	256	1317	1390	2078	2153
2833	470	2865a	295	296	2841	2873	3734	3799
2837	428	2877	297	298	1408	1390	2245	2229
2839	794	2876	299	300	1883	1913	2738	2795
2840	1630	2873	301	302	1708	1791	2533	2618
2841	344	2866	303	304	1971	1982	2591	2871

attributed to a polar effect, because *all2842*, 3′ from *alr2841*, is oppositely oriented. Therefore, we conclude that *alr2841* is a Fox gene.

(ix) Plasmid pRL2876 bearing *alr2839* showed no evidence of recombination with mutant FQ794, implying that in complemented FQ794, *alr2840* was not expressed from a promoter upstream from *alr2839*. Therefore, if *alr2840* is a Fox gene, a sequence within the 3′ end of *alr2839* can promote it. Although six transposon insertions were observed in *alr2840* (Table 3), the effects of each of these may be attributable to a polar effect of the mutation on transcription of *alr2841* and, because a PCR product attributable to double recombination of pRL2873 with mutant FQ1630 was observed (Fig. 5H), we cannot now conclude that *alr2840* is itself a Fox gene.

Evidence of recombination does not imply a need of recombination for complementation. For unknown reasons, perhaps that protein synthesis is more sensitive in heterocysts than in vegetative cells to certain antibiotics, *Anabaena* sp. shows greater sensitivity to those antibiotics when growing on N_2 than on fixed nitrogen. Not wanting to chance counterselecting recombinants, we grew complemented strains without antibiotics. Therefore, it may be more noteworthy that some strains lacked, than that others showed, recombination.

We note the utility of sequencing clones as a source of material for constructions and other purposes. Because their ends have been sequenced, their entire sequences are presumed known once a genomic sequence is finalized, and they

FIG. 4. Use of PCR to analyze possible recombination products in complemented mutants. (A) A plasmid (RSF1010 derivative) bears a fragment (in gray) that contains a single, intact (wild-type) copy of an ORF (shown as a white arrow) that has not recombined with the transposon (Tn)-interrupted copy of the same ORF in the genome. The ORF in the plasmid may be expressed from the *glnA* promoter or, perhaps, from an intervening, native promoter. PCR primers P3 and P4 are genomic sequences, up- and downstream from the cloned region, respectively, and primers P1 and P2 are vector sequences present, respectively, up- and downstream from the cloning region of the plasmid (P1 is also upstream from the *glnA* promoter). (B) Single, homologous recombination upstream from the transposon (at X in diagram A) would give rise to structure B, in which the uninterrupted ORF is positioned downstream from its natural upstream sequence. PCR would be expected to yield a product of predictable size with primers P2 and P3. (C) Single, homologous recombination downstream from the transposon (at Y in diagram A) would give rise to structure C, in which the uninterrupted ORF and any 3′ cotranscribed sequences would be placed under the influence of the *glnA* promoter and/or an intervening native promoter. PCR would be expected to yield a product of predictable size with primers P1 and P4. (D) Double, homologous recombination at both sides of the transposon would give rise to structure D, identical with that of the wild-type strain. With DNA from the wild-type strain or a double recombinant as template, PCR would be expected to yield a product of predictable size with primers P3 and P4. Such a band should be obtained with DNA from the mutant strain as template only if segregation of the mutation were incomplete, because the extension period was too short for the polymerase to traverse the transposon. If DNA from the mutant yielded no band, a band of the same size as from the wild-type strain, using DNA from the complemented mutant as template, would indicate that a double recombination event had cured the transposon from one or more copies of the genome. Lane numbers in inset tables E and F refer to lane numbers in Fig. 5, below. Inset table F shows PCR band sizes predicted (lanes 1 and 6) or predicted conditionally upon incomplete segregation (lane 2) or single (lanes 4 and 5) or double (lane 3) recombination.

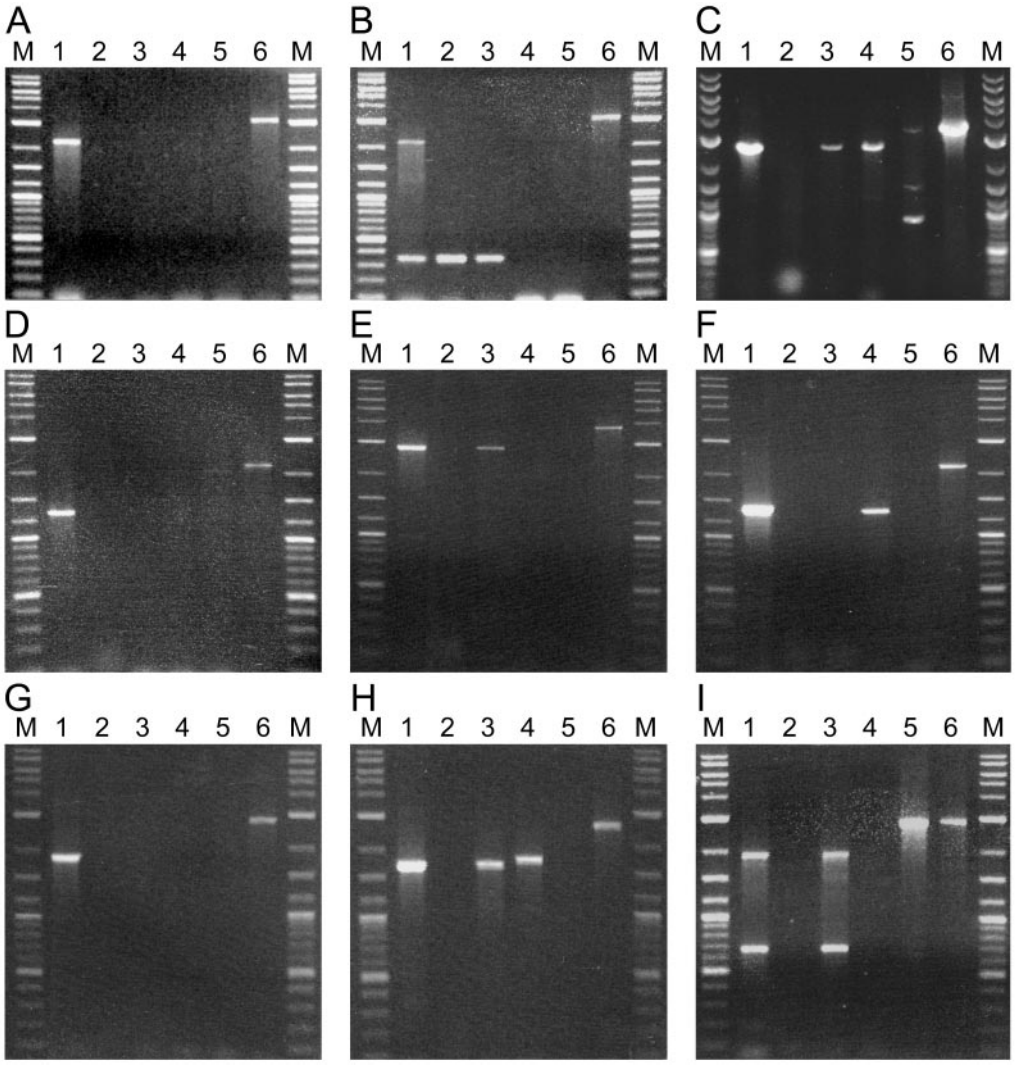


FIG. 5. PCR analyses of N_2-grown cultures derived from complemented mutants. Panels correspond to analysis of total DNA, as template, from wild-type *Anabaena* sp. strain PCC 7120 (lanes 1), or (in lanes 2) the following mutants: FQ885 (*alr2825*) (A); FQ57 (*alr2827*) (B); FQ1631 (*alr2831*) (C); FQ773 (*alr2832*) (D); FQ470 (*alr2833*) (E); FQ428 (*alr2837*) (F); FQ794 (*alr2839*) (G); FQ1630 (*alr2840*) (H); FQ344 (*alr2841*) (I). Lanes 3 to 6: complemented strains FQ885 plus pRL2875, FQ57 plus pRL2864, FQ1631 plus pRL2862, FQ773 plus pRL2863, FQ470 plus pRL2865a, FQ428 plus pRL2877, FQ794 plus pRL2876, FQ1630 plus pRL2873, and FQ344 plus pRL2866. Lanes M are New England BioLabs 2-log DNA size-standard markers (10, 8, 6, 5, 4, and 3 kb [bright]; 2, 1.5, 1.2, and 1.0 kb [bright]; 0.9, 0.8, 0.7, 0.6, and 0.5 kb [bright]; and 0.4, 0.3, 0.2, and 0.1 kb). Primers were ORF-specific P3 and P4 (lanes 1 to 3), P2 plus P3 (lanes 4), P1 plus P4 (lanes 5), and P1 and P2 (lanes 6), specific for the ends of the cloning region of pRL1383a and its derivatives pRL2831a and pRL2831b. In the panels (see inset table F of Fig. 4), the band in lane 1 shows the size expected for wild-type DNA, indicating the efficacy of P3 and P4 to prime the expected PCR with genomic DNA; lanes 2 lack any band other than from primer dimerization, indicating that segregation of the mutations was complete; and lanes 3 show in several instances (panels C, E, H, and I) the presence of a band in the complemented mutant, indicative of double recombination having taken place in these recombination-proficient cells. Single recombination shown by bands in lanes 4 and 5 is discussed in the text. The band in each lane 6 shows the size expected for the plasmid added to the corresponding strain, indicating the efficacy of P1 and P2 to prime the expected PCR with the plasmid present in total DNA from the complemented strain.

are far less subject to point mutations (and, therefore, for a need for confirmatory sequencing) than would be PCR-amplified fragments. If generated from appropriate vectors, e.g., pRL838, they could provide a minimally redundant library of known completeness for complementing mutations of unknown genomic position.

Relationships between syntheses of LPS and heterocyst envelope polysaccharide. Mutations in *all4829* and *all4830*, respective orthologs of lipopolysaccharide (LPS) biosynthetic genes *rfbP* and *rfbZ*, affect synthesis of vegetative cell LPS. The mutants are Fox^-; the *rfbP* mutant synthesizes at least some heterocyst envelope polysaccharide (40). An *all4828* (*rfbD*) mutant is Fox^+ (40). Like *rfbP* and *rfbZ*, autolysin-encoding *hcwA* (43) and a gene that encodes a putative penicillin-binding protein (22) are Fox genes. LPS is a constituent of the walls of vegetative cells, HcwA degrades those walls, and penicillin blocks synthesis of those walls. Therefore, the refashioning of cell wall components of the

differentiating vegetative cell may be a prerequisite for correct transport and deposition of components of the heterocyst envelope.

Lipid A is the portion of LPS that roots it in the outer cell membrane (29), and HepA itself (Alr2835), used in a BLAST (2) search against *Pseudomonas aeruginosa* strain PAO1 and *E. coli* strain CFT073, shows greatest similarity to the lipid A export ATP-binding–permease protein MsbA (in PAO1, 282 bits [Expect = 9e-77] versus 214 bits [Expect = 4e-56] for the next closest match; and in CFT073, 270 bits [Expect = 2e-73] versus 227 bits [Expect = 2e-60] for the next closest match). Conversely, MsbA from *E. coli* K-12 (GenBank P60752; in *E. coli*, an *msbA* knockout mutation is lethal [29]) shows greatest similarity, among the more than 80 proteins annotated as ATP-binding transporters in *Anabaena* sp. strain PCC 7120, to Alr2835 (313 bits [Expect = 3e-86]) and Alr5199 (316 bits [Expect = 4e-87]).

Other observations also hint that the *hepA* region may contain genes related to synthesis of LPS in addition to genes required for synthesis of heterocyst envelope polysaccharide. (i) Alr2833 bears a motif (http://www.ebi.ac.uk/interpro/DisplayIproEntry?ac = IPR003856) of a chain length determinant protein (or wzz protein) involved in LPS biosynthesis. (ii) The structure of the heterocyst envelope polysaccharide has been defined in detail for *Anabaena cylindrica* (9, 10), and in lesser detail for *Anabaena variabilis* strain ATCC 29413, in which its synthesis has been studied (8, 11), and *Cylindrospermum licheniforme* (11). Because *A. variabilis* and *Anabaena* sp. strain PCC 7120 are genetically highly similar (http://genome.jgi-psf.org/draft_microbes/anava/anava.home.html; http://www.kazusa.or.jp/cyano/Anabaena/), their heterocyst envelope polysaccharides and corresponding biosynthetic pathways are probably very similar. *A. variabilis* has a cluster of genes that corresponds with the cluster *alr2823-alr2841* in *Anabaena* sp. strain PCC 7120 (we thank Jeff Elhai for assistance with this analysis). Neither rhamnose nor any other deoxy sugar was found in the heterocyst envelope polysaccharide of *A. variabilis* (11). The predicted product of *alr2830* (its ortholog in *A. variabilis* shows 98% amino acid identity) is similar (as is also Alr4489) to dTDP-4-dehydrorhamnose 3,5-epimerase, a diagnostic gene (*rfbC* in *E. coli*) in the synthesis of activated rhamnose en route to LPS synthesis in gram-negative bacteria. The biosynthesis of dTDP-rhamnose also requires a glucose-1-phosphate nucleotide transferase, an epimerase, and a reductase, to which the products of ORFs *alr2825*, *alr2827*, and *alr2831* show similarity. (iii) Alr2841 (annotated as an unknown protein) shows sequence similarity to O-antigen polymerases that are involved in LPS and exopolysaccharide synthesis in different bacteria (InterPro family PD416824). (iv) Alr2828 shows similarity to glycosyl transferases that are involved in biosynthesis of the O-antigen portion of LPS. Finally, (v) Alr2834 (HepC) shows overall similarity (Expect = 7e-42) to COG2148, WcaJ, sugar transferases involved in LPS synthesis (30). Whether mutations in the *hepA* region affect LPS and whether ORFs in that region (excluding *hepC*) in which we found no transposon insertion are Fox genes remain untested.

If genes for synthesis of an LPS and of heterocyst envelope polysaccharide are both present in the *hepA* region, future studies should clarify whether they are related or merely interspersed. A possibility not hitherto considered is that heterocyst envelope polysaccharide may itself be an LPS that differs from, but may share some biosynthetic genes with, vegetative cell LPS. The possible localization of rhamnose (see above) in the single-copy core region of such an LPS (29), coupled at one end to lipid A and at the other to the highly repeated subunits of heterocyst envelope polysaccharide (10), could account for that sugar not having been observed in the envelope polysaccharide of *A. variabilis* heterocysts (11).

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