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A Major Facilitator Superfamily Protein, HepP, Is Involved in Formation of the Heterocyst Envelope Polysaccharide in the Cyanobacterium *Anabaena* sp. Strain PCC 7120

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Some filamentous cyanobacteria such as *Anabaena* sp. strain PCC 7120 produce cells, termed heterocysts, specialized in nitrogen fixation. Heterocysts bear a thick envelope containing an inner layer of glycolipids and an outer layer of polysaccharide that restrict the diffusion of air (including O_2) into the heterocyst. *Anabaena* sp. mutants impaired in production of either of those layers show a Fox$^-$ phenotype (requiring *f*ixed nitrogen for growth under *ox*ic conditions). We have characterized a set of transposon-induced Fox$^-$ mutants in which transposon Tn*5*-1063 was inserted into the *Anabaena* sp. chromosome open reading frame *all1711* which encodes a predicted membrane protein that belongs to the major facilitator superfamily (MFS). These mutants showed higher nitrogenase activities under anoxic than under oxic conditions and altered sucrose uptake. Electron microscopy and alcian blue staining showed a lack of the heterocyst envelope polysaccharide (Hep) layer. Northern blot and primer extension analyses showed that, in a manner dependent on the nitrogen-control transcription factor NtcA, *all1711* was strongly induced after nitrogen step-down. Confocal microscopy of an *Anabaena* sp. strain producing an All1711-green fluorescent protein (All1711-GFP) fusion protein showed induction in all cells of the filament but at higher levels in differentiating heterocysts. All1711-GFP was located in the periphery of the cells, consistent with All1711 being a cytoplasmic membrane protein. Expression of *all1711* from the P_{glnA} promoter in a multicopy plasmid led to production of a presumptive exopolysaccharide by vegetative cells. These results suggest that All1711, which we denote HepP, is involved in transport of glycoside(s), with a specific physiological role in production of Hep.

Cyanobacteria such as those of the genera *Anabaena* and *Nostoc* grow as filaments of cells that, when growing actively in the absence of a source of combined nitrogen, comprise two types of cells: vegetative cells that perform oxygenic photosynthesis and heterocysts—which differentiate from vegetative cells at semiregular intervals along filaments—that perform N_2 fixation. During heterocyst differentiation, structural and metabolic changes take place that result in a micro-oxic, intracellular environment appropriate for the expression and function of nitrogenase (19, 62). Among other adaptations, thick cell envelope layers are formed that greatly impede the entry of gases (58) and dedicated terminal respiratory oxidases are expressed (55) that reduce free oxygen. The process by which heterocysts differentiate from vegetative cells involves the execution of a specific program of gene expression (25, 33, 64, 65). Two positive-acting regulators are required for the initiation of heterocyst differentiation: NtcA, the global nitrogen-control transcription factor of cyanobacteria (24), and HetR, a differentiation-specific protein (5, 7). NtcA binds to DNA sites with the consensus sequence GTA-N_8-TAC (24). The net result of differentiation is that in the N_2-fixing filament, the vegetative cells provide the heterocysts with photosynthate and the heterocysts provide the vegetative cells with fixed nitrogen (reviewed by Wolk et al. [62]).

Cyanobacteria are diderm bacteria (53), i.e., they have a Gram-negative type of cell wall that comprises an outer membrane (OM) and, internal to it, a peptidoglycan layer, both of which are outside the cytoplasmic membrane (CM; 19, 33). Lipopolysaccharide (LPS), the major component of the OM of Gram-negative bacteria, comprises a lipid A region, a core region, and an O antigen. In filamentous cyanobacteria, whereas the CM and peptidoglycan layer surround each cell, the OM is continuous along the filament, defining a continuous periplasm (20, 41, 61). During differentiation, a heterocyst envelope polysaccharide (Hep) layer is deposited outside the wall of the differentiating cell. Subsequently, a layer of heterocyst envelope glycolipids (Hgl; 3, 62) is deposited in a position internal to the Hep layer. In the genome of the model cyanobacterium *Anabaena* sp. strain PCC 7120 (sometimes called a *Nostoc* sp.), genes involved in the formation of both of these layers are clustered (2, 14, 17, 28), although functionally related *hep* genes are also present elsewhere in the chromosome (39, 59).

Hep from *Anabaena cylindrica* comprises a β-1,3-linked mannosyl-glucosyl-glucosyl-glucose tetrasaccharide backbone to which sidebranches of mannose, glucose and glucosyl glucose, galactose, and xylose are attached (8). Hep of *Anabaena variabilis* ATCC 29413 (very closely related to *Anabaena* sp. strain PCC 7120) and Hep of *Cylindrospermum licheniforme* are apparently very similar to Hep of *A. cylindrica* (9), but with minor changes in

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the side branches (reviewed by Wolk et al. [62]). Bacterial biosynthesis of an extracellular polysaccharide generally entails assembly of sugars from sugar nucleotides into an oligosaccharide on a lipid carrier in the cytoplasmic face of the CM. The oligosaccharide, in some cases bound to the lipid carrier, is transferred across the CM, polymerized to form a polysaccharide, and moved through the periplasm and the OM to the cell surface (60). Isolated heterocysts of *Anabaena variabilis* incubated with [^{14}C]mannose produce an envelope polysaccharide containing [^{14}C]mannose, [^{14}C]arabinose, and [^{14}C]glucose and contain corresponding [^{14}C]sugar nucleotides and [^{14}C]sugar phosphoglycolipids that are presumed intermediates in polysaccharide biosynthesis (10). Which pathway the activated sugar follows to produce Hep could possibly be deduced from the identity of genes whose mutation results in a Fox$^-$ phenotype (requiring *f*ixed nitrogen for growth under *ox*ic conditions). Extensive mutagenic analysis has, however, been performed in *Anabaena* sp. strain PCC 7120 (see, e.g., references 17, 28, 34, 39, 59, and 65) but not in *A. variabilis*. We here characterize a gene, open reading frame (ORF) *all1711*, that encodes a major facilitator superfamily (MFS) protein that is needed for diazotrophic growth and production of Hep and which, when overexpressed, elicits production of extracellular (presumed) polysaccharide in vegetative cells.

MATERIALS AND METHODS

Strains and growth conditions. For transposon mutagenesis, electron microscopy, and initial complementation experiments, *Anabaena* sp. (also known as *Nostoc* sp.) strain PCC 7120 was grown, with shaking, in flask cultures of AA/8 liquid medium, with or without nitrate (26), or in medium AA, with or without nitrate, solidified with 1.2% purified (Difco) Bacto agar (26) at 30°C and illuminated with white light (ca. 30 μmol s^{-1} m^{-2}). For other experiments, *Anabaena* sp. strain PCC 7120 was grown in BG11 medium modified to contain ferric citrate instead of ferric ammonium citrate (50); BG11$_0$ medium (BG11 further modified by omission of $NaNO_3$); or BG11$_0$ medium plus ammonium [BG11$_0$ containing 3 or 5 mM NH_4Cl and, respectively, 6 or 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer, pH 7.5] at 30°C in the light (ca. 25 μE m^{-2} s^{-1}), in shaken (100-rpm) liquid cultures or in medium solidified with 1% Difco agar. Alternatively, cultures (referred to as bubbled cultures and denoted BG11C or BG11$_0$C) were supplemented with 10 mM $NaHCO_3$ and bubbled with a mixture of air and 1% (vol/vol) CO_2 in the light (ca. 75 μE m^{-2} s^{-1}). Ammonium-supplemented BG11$_0$C contained, in addition, 8 mM NH_4Cl and 16 mM TES-NaOH buffer (pH 7.5).

When appropriate, and unless otherwise indicated, antibiotics were added to the cyanobacterial cultures at the following concentrations (or as described elsewhere [17]): in liquid cultures, streptomycin sulfate (Sm) (2 to 5 μg ml^{-1}), spectinomycin dihydrochloride pentahydrate (Sp) (2 to 5 μg ml^{-1}), erythromycin (Em) (5 μg ml^{-1}), and neomycin sulfate (Nm) (5 μg ml^{-1}); and in solid media, Sm (5 μg ml^{-1}), Sp (5 μg ml^{-1}), Em (5 μg ml^{-1}), and Nm (30 to 40 μg ml^{-1}).

Escherichia coli strains were grown in LB medium, supplemented when appropriate with antibiotics at standard concentrations (1). *E. coli* strain DH5α or DH5αMCR was used for plasmid constructions. *E. coli* strain DH5α or ED8654 bearing a conjugative plasmid and strain HB101 or DH5αMCR bearing a methylase-encoding helper plasmid and one or two cargo plasmids were used for conjugation with the *Anabaena* sp. as previously described by (e.g.) Wolk et al. (63).

Transposon mutagenesis and electron microscopy. Cells of *Anabaena* sp. strain PCC 7120 were mutagenized using transposon Tn*5*-1063, and interrupted genes identified, as described elsewhere (17, 28). Electron microscopy was performed as described by Black et al. (4).

Complementing plasmids and complementation. Complementation was performed using compatible vectors (63). Plasmid anp00110 is a sequencing clone (chromosomal bp 2047194 to 2056558) that contains *all1711* and its neighboring genes cloned into the BamHI site of pUC18 (29). pRL3161 (Cm^r Em^r; Cm represents chloramphenicol; the superscript "r" denotes resistance) bears *all1711* within the 2,607-bp SnaBI-BstYI fragment of anp00110 cloned between the StuI and BamHI sites of pRL2833a (17). pRL2880 (Sm^r Sp^r) bears *all1710*, the open reading frame downstream of *all1711*, within the 3,544-bp NheI-PvuII fragment of anp00110 in pRL2831a (17) digested with XbaI and StuI. pRL3845 was constructed as follows: (i) an NarI-FspI, neomycin phosphotransferase (*npt*)-bearing fragment of pRL3810 (34) was replaced by a chloramphenicol acetyltransferase (*cat*), erythromycin resistance (*erm*), AccI-EcoRV fragment from pRL1075 (5), yielding pRL3812; and (ii) an SphI-DraI, P$_{glnA}$-*all1711*-containing fragment of pRL3161 was ligated between the SphI and StuI sites of pRL3812.

GFP fusion and analysis. To fuse the C terminus of All1711 to green fluorescent protein (GFP) via a flexible linker, a 620-bp fragment containing 602 bp from the 3′ terminus of *all1711* (stop codon removed) was first amplified by PCR using DNA from strain PCC 7120 as the template and oligodeoxynucleotide primers all1711-5 (5′-ACT**AGTCCCACCTCCAC**CATAAGGCAATATACGAAATCTTCTGC-3′) and all1711-6 (5′-CGCC TAAACATTCG-3′). Primer all1711-5 bears a potential SpeI restriction site (underlined) and a series of nucleotides whose inverted sequence provides a (Gly)$_4$Thr linker (in bold). pCSLR88 is a derivative of Sm^r Sp^r plasmid pRL277 (5), between whose SacI and XhoI restriction sites are located the following (in physical succession): (i) AAGCTTGAT; (ii) the aforementioned PCR product starting with all1711-6; (iii) the SpeI derived from all1711-5, fused to an NheI site; (iii) *gfp-mut2* (12) less its first two codons; and (iv) immediately after the termination codon of *gfp-mut2*, a polylinker sequence (5′-AATCAGGCCTCCCGGGGAATTCAC TAGTCATATATGGGATCCGTCGA-3′), the last five nucleotides of which (underlined) are part of a SalI site that was used for insertion into the XhoI site of pRL277.

Transfer of pCSLR88 to *Anabaena* sp. strain PCC 7120 was effected by a triparental mating with *E. coli* HB101 carrying pCSRL88 and helper and methylation plasmid pRL623 and *E. coli* ED8654 bearing plasmid pRL443 (16), with selection for resistance to Sm and Sp. The genetic structure of a selected clone, strain CSRL22, was tested by PCR with DNA from that clone and primers all1711-3 (5′-TTATTAGCTGCCATCATCTTCA-3′) and gfp-4 (36) to check whether integration of the plasmid had occurred in the correct genomic location and primers all1711-3 and all1711-4 (5′-GACATTGCCCACTCTACTCATA-3′) to check whether the clone was homozygous for the mutant chromosomes.

GFP fluorescence was analyzed by confocal microscopy. Samples from cultures of the *Anabaena* sp. set atop solidified medium were visualized using a Leica HCX PLAN-APO 63× 1.4-numerical aperture (NA) oil immersion objective attached to a Leica TCS SP2 confocal laser-scanning microscope. GFP was excited using 488-nm irradiation from an argon ion laser, and fluorescent emission was monitored from 500 to 538 nm. GFP fluorescence intensity was analyzed using ImageJ 1.43m software. To determine the relative fluorescence intensities in different cell zones, integrated density was recorded in squares of 1.35 μm^2. A total of 34 to 50 measurements were made for each of the lateral and septal walls of vegetative cells at each of two time points, 12 and 24 h after nitrogen step-down. At those same respective times, 14 and 16 measurements were made of the lateral walls of (pro)heterocysts and 12 and 16 measurements at the poles of (pro)heterocysts.

Northern blot and primer extension analyses. RNA was isolated from bubbled cultures of the *Anabaena* sp. as described previously (44). For Northern blot analysis, an amount of 10 μg of RNA was loaded per lane and electrophoresed in denaturing 1% agarose formaldehyde gels. DNA probes were generated by PCR using genomic DNA from the *Anabaena* sp. and oligodeoxynucleotide primers all1711-1 (5′-ATCGAT CGCCTAAACATTCG-3′; potential ClaI restriction site underlined) and

all1711-2 (5′-GATATCATAAGGCAATATACGAAATCTTCT GC-3′; potential EcoRV restriction site underlined). Hybridizations were performed as previously described (49). As a control for RNA loading and transfer efficiency, the filters were hybridized with a probe of the RNase P RNA gene (*rnpB*) from *Anabaena* sp. strain PCC 7120 (57). Probes were labeled with a DNA labeling kit (Ready to Go; GE Healthcare) and [α-^{32}P]dCTP.

Primer extension analysis was performed as previously described (46). RNA was prepared as described above, and primers all1711-9 (5′-CGGC TAATCCCCAAACTTGTCGC-3′) and all1711-10 (5′-CCATCCAACTG AAGATGATGGC-3′) were used. For convenience, unrelated sequence ladders were used as size markers (see below). Radioactive areas in Northern blot membranes and primer extension gels were visualized with a Cyclone storage phosphor system (Packard).

Nitrogenase activity. Cells grown in BG11$_0$C plus ammonium (bubbled cultures), with antibiotics for the mutants, were incubated 24 h under the same growth conditions but without combined nitrogen (BG11$_0$C medium) and without antibiotics for induction of nitrogenase. Nitrogenase activity was determined by the acetylene reduction assay as described previously (45) under oxic or anoxic conditions. For the anoxic assays, the cell suspensions were supplemented with 10 μM DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and bubbled with argon for 5 min. Chlorophyll *a* (Chl) content of the cell suspensions was determined by the method of Mackinney (38).

Sucrose uptake. Filaments grown in BG11$_0$C-plus-ammonium bubbled cultures (supplemented with antibiotics for the mutants) and, when indicated, incubated in BG11$_0$C bubbled cultures for 16 h were passed 8 times through a 25-gauge needle (length, 5/8 in.), and the filaments in the resulting suspension were harvested by centrifugation, washed, and suspended in 25 mM TES-NaOH buffer (pH 7.0) (47). Suspensions containing an amount of cells corresponding to about 4 μg Chl ml^{-1} were incubated at 30°C in the light (ca. 180 μE m^{-2} s^{-1}), and the assays were started by the addition of 50 μM sucrose (final concentration), which was prepared by mixing unlabeled sucrose with [U-^{14}C]sucrose (American Radiolabeled Chemicals, Inc.) (500 to 700 mCi mmol^{-1}). Samples (1 ml) were taken every 10 min for up to 40 min (for which time uptake was linear [47]), filtered through Millipore 0.45-μm-pore-size HA filters, and washed on the filters with the same buffer. Radioactivity in the cells was determined in a scintillation counter, and the radioactivity retained by boiled cells was used as a blank.

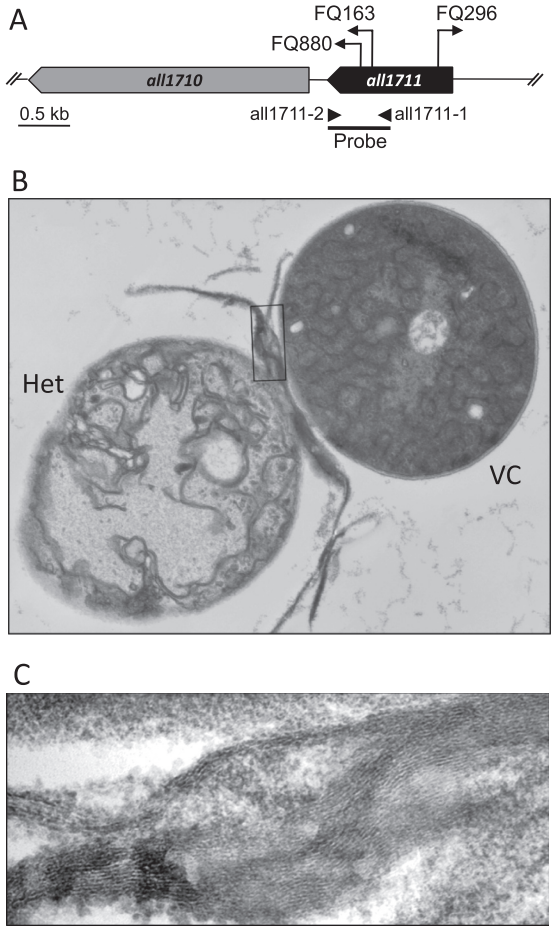

FIG 1 Mutants of *all1711*. (A) Schematic of the *all1711* locus in strain PCC 7120, with indication of the insertion sites and orientation of transposon Tn*5*-1063 in mutant strains FQ163, FQ296, and FQ880. The locations of the primers used to generate the probe for Northern blot analysis (Fig. 3 and 8) are indicated. (B and C) Electron micrograph of a defective heterocyst (Het) and its adjacent vegetative cell (VC) in mutant strain FQ880 (B) and further magnification of the area shown by a rectangle in panel B (C).

RESULTS

Isolation and phenotype of *all1711* mutants. From transposon mutagenesis experiments screening for Fox$^-$ mutants, five clones were isolated that bore transposon Tn*5*-1063 in ORF *all1711* of the *Anabaena* sp. genome. Three of those clones, strains FQ163, FQ296, and FQ880, were used in this work. The locations of their sites of insertion are shown in Fig. 1A. ORF *all1711* encodes a 399-amino-acid residue protein of the Major Facilitator Superfamily (MFS) with some relation to proteins in the Glycoside-Pentoside-Hexuronide (GPH):cation symporter family (http://www.tcdb.org/; 51), which includes some sucrose transporters. In the same DNA strand, starting 206 bp downstream from *all1711*, ORF *all1710* encodes a 904-amino-acid residue protein (Fig. 1A). All1710, which bears a putative N-terminal transmembrane segment and is strongly predicted to be extracytoplasmic (http://www.psort.org/), somewhat resembles some outer membrane proteins, including porins. The fact that five independent transposon insertions into *all1711* produced a Fox$^-$ phenotype suggests that such an insertion, and not independent mutations in other places in the chromosome, is responsible for the Fox$^-$ phenotype. Although a polar effect of the insertion is also considered, below, our results indicate that inactivation of *all1711*, *per se*, accounts for the mutant phenotype.

Mutant strains FQ163, FQ296, and FQ880 showed very low nitrogenase activity (determined by the acetylene reduction assay) in the presence of O_2 but showed appreciable activity—a Fix$^+$ phenotype—when the assay was performed under anoxic conditions (Table 1). The Fox$^-$ Fix$^+$ phenotype of these mutants suggests that they are affected in the heterocyst-provided protection of nitrogenase against O_2. Electron microscopy of a heterocyst from strain FQ880 clearly showed lack of a Hep layer (Fig. 1B). In contrast, the laminations of the Hgl layer are evident in strain FQ880 (Fig. 1C). In wild-type *Anabaena* sp. strain PCC 7120, alcian blue colors the Hep layer of the heterocyst envelope (22). Alcian blue did not stain the heterocysts produced by strains FQ163, FQ296, and FQ880 (Fig. 2), consistent with a lack of Hep as shown above for FQ880 by electron microscopy. These results indicate that these mutants specifically lack the Hep layer. The *all1711* gene appears, therefore, to be required for production of Hep, accounting for the low nitrogenase activity observed in the mutants in the presence of O_2.

TABLE 1 Nitrogenase activity of wild-type *Anabaena* sp. strain PCC 7120, *all1711* insertion mutants, and complemented strains[a]

Strain	Genotype	Nitrogenase activity (μmol ethylene [mg Chl]$^{-1}$ h^{-1} ± SD) (no. of independent cultures) under indicated condtions	
		Oxic	Anoxic
PCC 7120	Wild type	31.85 ± 2.45 (6)	36.67 ± 2.10 (6)
FQ163	*all1711*::Tn5-1063	0.12 ± 0.05 (2)	6.79 ± 1.28 (2)
FQ296	*all1711*::Tn5-1063	0.29 ± 0.13 (3)	10.75 ± 1.66 (3)
FQ880	*all1711*::Tn5-1063	0.17 ± 0.07 (3)	13.61 ± 2.21 (3)
FQ163-C1	FQ163 (pRL3161)	5.03 ± 0.37 (2)	4.47 ± 0.00 (2)
FQ163-C2	FQ163 (pRL3161, pRL2880)	2.09 ± 0.49 (2)	3.91 ± 1.57 (2)
FQ163-C3	FQ163 (pRL3845)	3.11 ± 2.17 (2)	4.09 ± 2.87 (2)
FQ296-C	FQ296 (pRL3845)	1.62 ± 0.20 (3)	2.46 ± 0.21 (3)
FQ880-C	FQ880 (pRL3845)	1.92 ± 0.67 (3)	4.65 ± 0.90 (3)

[a] Nitrogenase activity was determined by the acetylene reduction assay under oxic or anoxic conditions as described in Materials and Methods. Data represent means and standard deviations of the results obtained, with the numbers of independent cultures indicated in parentheses.

Expression of *all1711*. Expression of *all1711* was investigated by Northern blot analysis using RNA isolated from bubbled cultures of *Anabaena* sp. strain PCC 7120 grown with ammonium and incubated for different periods of time in the absence of combined nitrogen (Fig. 3A). A transcript of 1.38 kb was induced during the period of 3 to 12 h after nitrogen step-down. This transcript was present at very low levels in ammonium-grown cells and was detected at low to very low levels in the period of 24 to 35 h after nitrogen step-down. Because *all1711* is 1,200 bp long (29), the 1.38-kb RNA species appears to be a monocistronic transcript that is expressed during heterocyst differentiation. The Northern blot analysis was also performed with RNA isolated from an *ntcA* mutant, strain CSE2 (21), and from a *hetR* mutant, strain 216 (6, 7). Whereas induction of *all1711* was barely observed in strain CSE2, induction in strain 216 took place at high levels and, in contrast to the wild type, expression did not decrease after 12 h of nitrogen step-down (Fig. 3A). Because *hetR* mutations normally block heterocyst formation (6, 7), these results suggest that induction of *all1711* does not take place specifically in cells that are differentiating into heterocysts.

Primer extension analysis was performed to identify the promoter of and to investigate further the regulation of expression of *all1711*. RNA isolated from bubbled cultures of *Anabaena* sp. strain PCC 7120 that had been grown with ammonium and incubated for 3 or 9 h in the absence of combined nitrogen was subjected to retrotranscription with oligodeoxynucleotide primers all1711-9 (Fig. 3B) and all1711-10 (not shown). Four RNA 5′ ends, corresponding to nucleotide positions 28 (RNA I), 51 (*), 106 (RNA II), and 115 (RNA III) upstream of the start codon of the gene, were detected with primer all1711-9 (Fig. 3B). Only the data for RNA I, RNA II, and RNA III were confirmed using primer all1711-10, indicating that the RNA 5′ end marked by an asterisk was a nonspecific product of extension with primer all1711-9. The positions corresponding to RNA I and (displaced by 1 nucleotide) RNA II are transcription start sites of *Anabaena* sp. strain PCC 7120 according to Mitschke et al. (43). The three RNA species show a regulatory pattern consistent with that observed in the Northern blot analysis for the *all1711* transcript (Fig. 3A), i.e., strong induction in response to withdrawal of combined N and impaired expression in the *ntcA* mutant but not in the *hetR* mutant. However, induction of RNA I in the period from 3 to 9 h was impaired in strain 216. Both RNA I and RNA III are preceded by appropriately located putative −10 promoter hexamers (Fig. 3C), and a putative NtcA-binding site (GAA-N_8-TAC) is found centered at −41.5 nucleotides with respect to RNA III. The promoter producing RNA III is therefore a class II NtcA-activated promoter (25). A possible, suboptimal NtcA-binding site (GCA-N_8-TGC) is also found centered at −41.5 nucleotides with respect to RNA I (Fig. 3C). No obvious promoter element(s) are, however, located in front of RNA II, which, although detected at levels higher than RNA III, shows a regulatory pattern similar to it, suggesting that they might be related (i.e., RNA II might be a degradation product or a site of halted retrotranscription of RNA III).

PSIPRED (http://bioinf.cs.ucl.ac.uk/psipred/) predicts that All1711 bears 12 transmembrane segments, with its N and C termini in the cytoplasmic side of the cytoplasmic membrane. To identify the loci of expression of *all1711* in the cyanobacterial filament, as well as the possible subcellular localization of its encoded protein, *all1711* less its termination codon was fused by a linker to *gfp-mut2* (Fig. 4A). Strain CSRL22 that bears this construct is homozygous for chromosomes bearing the inserted plas-

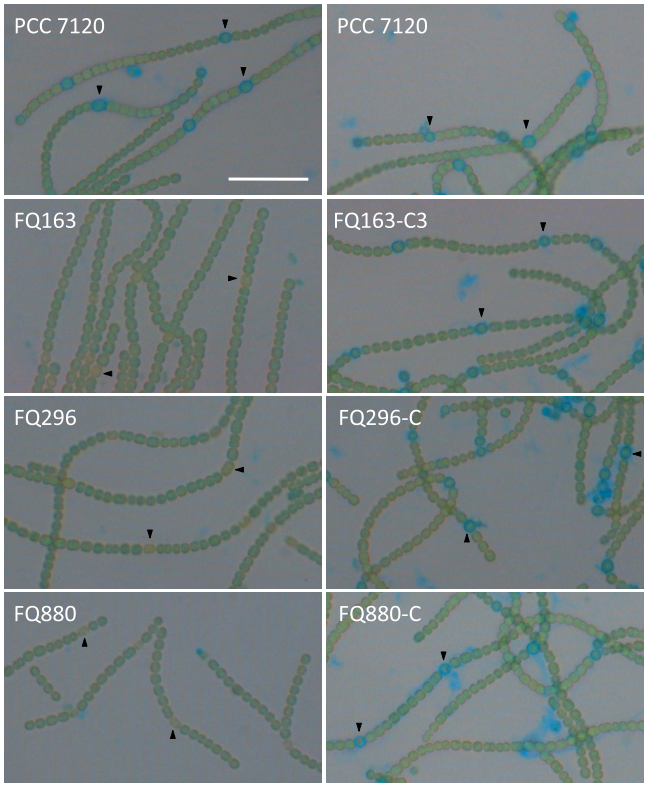

FIG 2 Wild-type *Anabaena* sp. strain PCC 7120 (two samples of which are shown), *all1711* mutants, and complemented mutant strains. Filaments from bubbled BG11C cultures of the indicated strains (see genotypes in Table 1) were incubated in $BG11_0C$ medium for 24 h. Samples from these cultures were then supplemented with a solution of 1% alcian blue 8GX (Sigma) and visualized by light microscopy. The heterocyst envelope polysaccharide (Hep) layer of the wild type and of the complemented mutants is stained blue. Some examples of heterocysts or, in the case of the mutants, presumptive heterocysts are indicated by arrowheads. Bar, 25 μm.

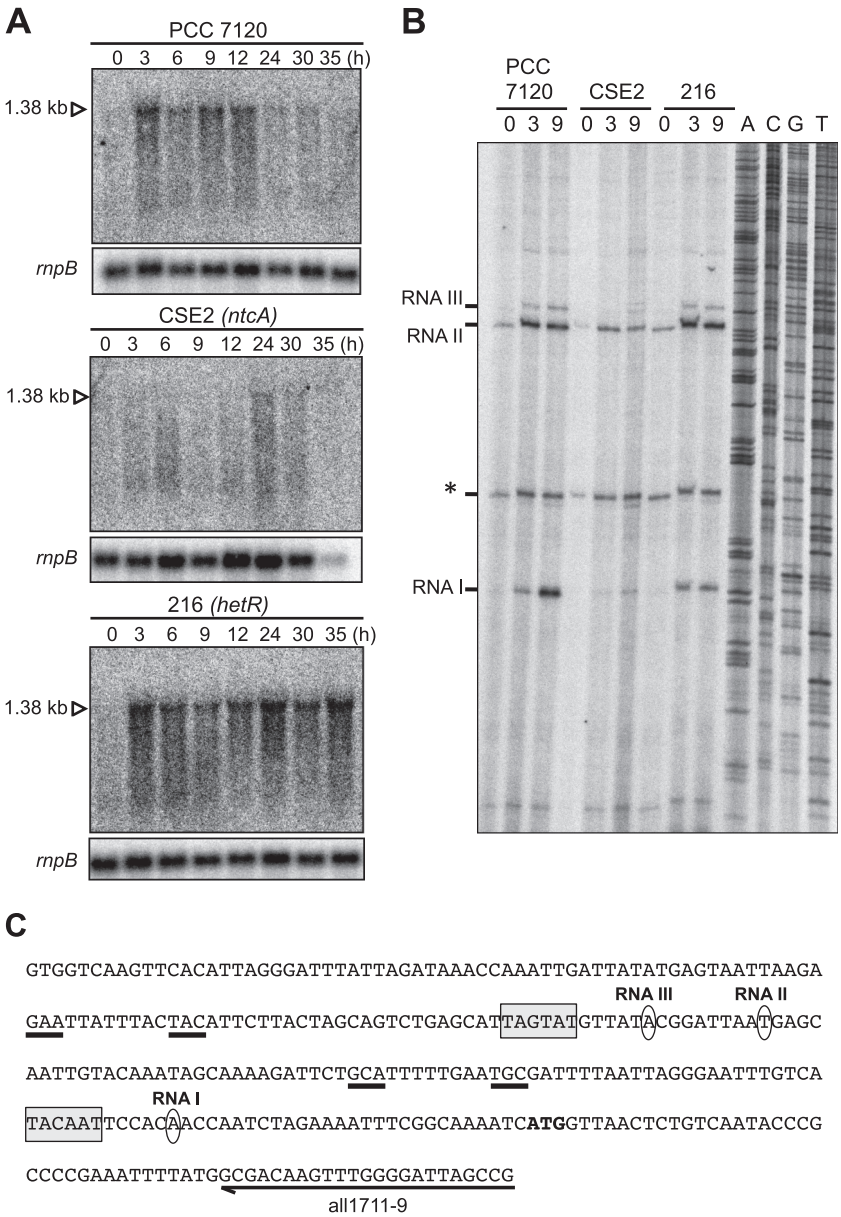

FIG 3 Expression of *all1711*. (A) Northern blot analysis performed with the *all1711* probe indicated in Fig. 1A and RNA from strains PCC 7120 (wild type), CSE2 (*ntcA*), and 216 (*hetR*). RNA was isolated from bubbled ammonium-grown cultures (with Sm and Sp for the CSE2 mutant) incubated for the indicated number of hours without combined nitrogen (and without antibiotics). The different images correspond to different parts of the same filter and therefore can be compared. The size of the identified *all1711* transcript is indicated. Hybridization with a probe of the *Anabaena rnpB* gene was used as a loading and transfer control (lower panels). (B) Primer extension analysis performed with oligodeoxynucleotide primer all1711-9 and the RNA samples from the induction times (indicated in hours) of the same preparations used for the Northern blot analysis (see panel A). The sequencing ladder shown at right corresponds to the *all0862* gene promoter (37). (C) Sequence of the *all1711* promoter region, with indication of primer all1711-9 used for the analysis shown in panel B, the detected RNA 5′ ends (nucleotides enclosed in ovals), two putative −10 promoter hexamers (shaded boxes), and two putative NtcA binding sites identified by the underlined triplets (GAA/TAC and GCA/TGC, respectively).

mid pCSRL88 (not shown). Strain CSRL22 produces heterocysts and grows diazotrophically, implying that the All1711-GFP fusion protein provides All1711 function. Strain CSRL22 showed GFP fluorescence in all of the cells of the filament after 6 h of nitrogen deprivation, with no or very weakly detectable fluorescence at 0 or 3 h (Fig. 4B). At 9, 12, and 24 h after nitrogen step-down, fluorescence was also observed in all cells of the filament but at somewhat increased levels in presumptive proheterocysts (at 9 and 12 h) and heterocysts (at 24 h). Expression in all cells of the filament is consistent with a lack of dependence on HetR as shown above.

GFP fluorescence in strain CSRL22 originated from the periphery of the cells (Fig. 5), consistent with the idea that All1711 is an MFS protein localized in the CM. Determinations of the intensity of GFP fluorescence for several contiguous cells in filaments of strain CSRL22 incubated without combined nitrogen for 12 h (Fig. 5A) or 24 h (Fig. 5B) showed higher fluorescence from

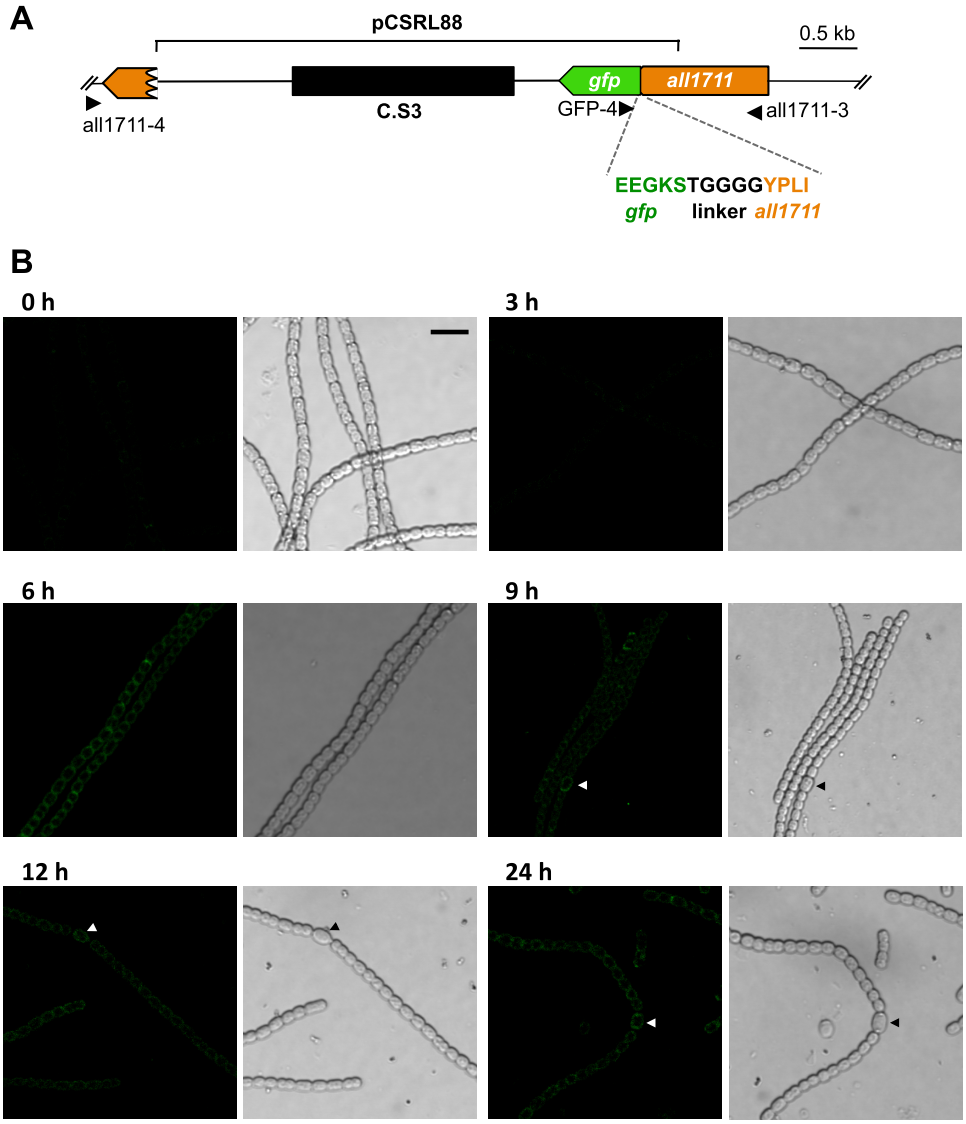

FIG 4 Expression of an All1711-GFP fusion protein. (A) Schematic of the *all1711* locus in strain CSRL22 carrying the *all1711-gfp-mut2* fusion. Note that the fusion gene was incorporated into the chromosome through a single-recombination event between the *Anabaena* sp. DNA cloned in plasmid pCSRL88 and the corresponding chromosomal region; therefore, no wild-type copy of *all1711* remained. The locations of the primers used to study the genetic structure of CSRL22 (see Materials and Methods) are shown. The wavy line denotes the junction of linearized pCSRL88 with the 3′ end of *all1711* that was interrupted by the insertion of pCSRL88. (B) GFP fluorescence (left panels) and bright-field (right panels) micrographs of filaments of strain CSRL22 grown with ammonium and incubated without combined nitrogen (in the presence of Sm and Sp for the mutant) for the times indicated (in hours). Arrowheads point to proheterocysts (at 9 and 12 h) or heterocysts (at 24 h). Bar, 10 μm. Identical microscope settings were used for the different time points.

(pro)heterocysts than from vegetative cells. Because the amount of recorded fluorescence can be affected by cell size, heterocysts being larger than vegetative cells, determinations of fluorescence levels in different zones of the cell periphery were performed. Determinations in lateral walls of (pro)heterocysts and vegetative cells showed a ratio of 1.58:1 in filaments incubated 12 h without combined nitrogen and of 1.69:1 in filaments incubated 24 h without combined nitrogen. These ratios were larger (2.58:1 and 3.01:1 for 12-h and 24-h filaments, respectively) for the GFP fluorescence from the heterocyst poles than for that from vegetative cell lateral walls. These data indicate that All1711-GFP is present at higher concentrations in the CM of proheterocysts and heterocysts than in the CM of vegetative cells. Additionally, determinations of fluorescence in the septa between vegetative cells and in lateral walls of vegetative cells (where a single membrane is present) showed a ratio of 2.01:1 in filaments incubated 12 h without combined nitrogen and of 1.92:1 in filaments incubated 24 h without combined nitrogen, indicating that in the septa there was not greater accumulation of All1711-GFP than that expected from the juxtaposition of two cytoplasmic membranes. (The standard error of the mean was approximately 3% to 5% of the mean values for ratios involving heterocysts and was close to 1% of the mean values for ratios involving only vegetative cells, for which more numerous measurements were obtained.)

Complementation of the *all1711* mutants. To attempt complementation of *all1711* mutant FQ163, we cloned a wild-type

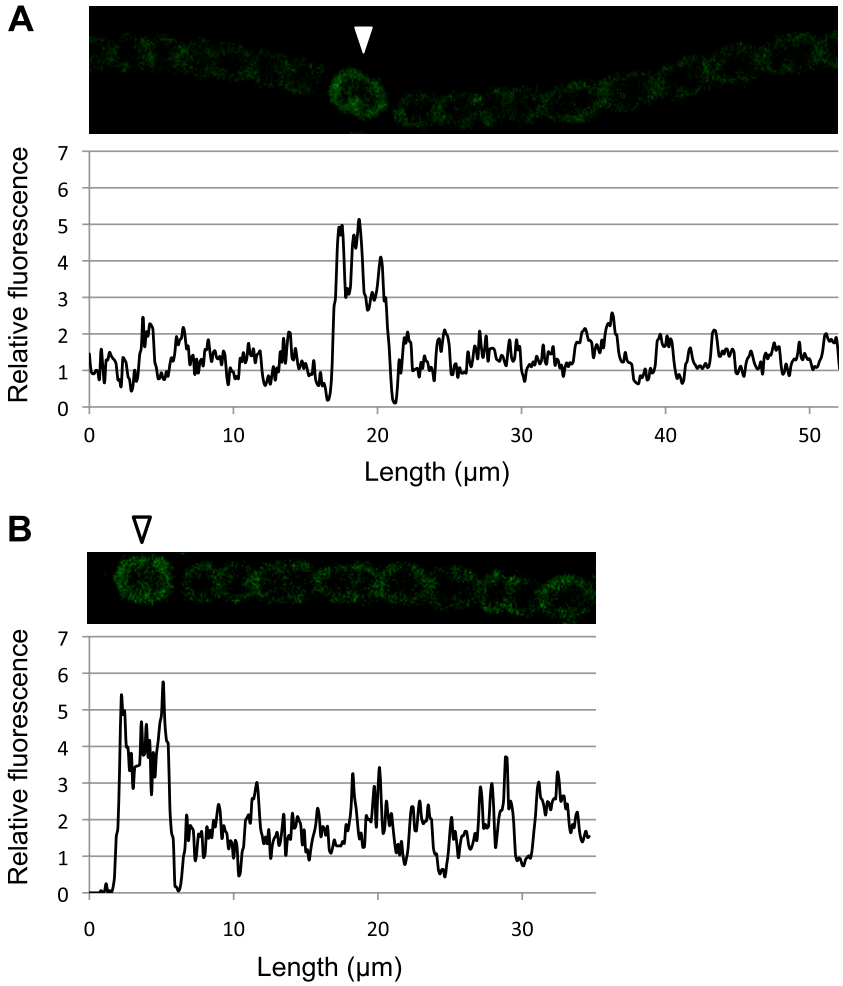

FIG 5 GFP fluorescence along examples of filaments of strain CSRL22 incubated without combined nitrogen for 12 h (A) or 24 h (B). The lower panels present the quantification of the fluorescence from each whole cell along the filaments shown in the upper panels. Relative levels of GFP fluorescence are presented in arbitrary units that are comparable for panels A and B. Arrowheads point to a proheterocyst (A) or a heterocyst (B).

copy of *all1711* downstream from the promoter of the *Anabaena* sp. *glnA* gene in a pDU1-based plasmid (63), yielding pRL3161. The strain bearing this plasmid, FQ163-C1, showed increased nitrogenase activity under oxic conditions compared to its parental strain. However, nitrogenase activity in FQ163-C1 was relatively low compared to that of the wild type (Table 1), indicating incomplete complementation. Given the possibility that incomplete complementation resulted from impaired transcription of the downstream gene, *all1710*, we constructed and tested strain FQ163-C2. FQ163-C2 contains, in addition to pRL3161, *all1710* in an RSF1010-based plasmid, pRL2880. However, FQ163-C2 showed no further increase in nitrogenase activity (Table 1).

Because an antisense RNA originating from the P_{cat} promoter in pRL3161 might interfere with expression of *all1711*, Cm^r Em^r plasmid pRL3845 was prepared in which the *cat* gene was oppositely oriented and a T7 transcriptional terminator was inserted between *all1711* and the *erm* gene (see Materials and Methods). Mutant strains FQ163-C3, FQ296-C, and FQ880-C, all bearing pRL3845, produced heterocysts that were stained by alcian blue (Fig. 2) and showed increased nitrogenase activity in the presence of O_2 compared to their respective parental strains but were still incompletely complemented (Table 1). These activities permitted only limited growth in medium lacking any source of combined nitrogen (Fig. 6: $BG11_0$ medium). Adding Em to the medium to increase the selection pressure for the plasmid resulted in modestly increased growth (Fig. 6).

Because, under anoxic conditions, the nitrogenase activity of the complemented strains was even lower than that of the noncomplemented strains (Table 1), the plasmids appeared to diminish nitrogenase activity. Figure 6 compares the growth of the mutants and complemented strains on $BG11_0$ and BG11 solid media. The transposon provides resistance to Nm, and the complementing plasmid, pRL3845, provides resistance to Em. Whereas, as mentioned above, diazotrophic growth of the complemented strains was poor, growth in the presence of nitrate was substantial in every case. However, whereas the spots of the wild type or the mutants were lusterless, those of the complemented strains were shiny. The latter were also difficult to handle with inoculating loops, consistent with the presence of sheath material.

Alcian blue has been reported to stain insoluble polysaccharides as well as bacterial capsules (42). As is shown in Fig. 7 (top panel), alcian blue stained the edge of a colony of the comple-

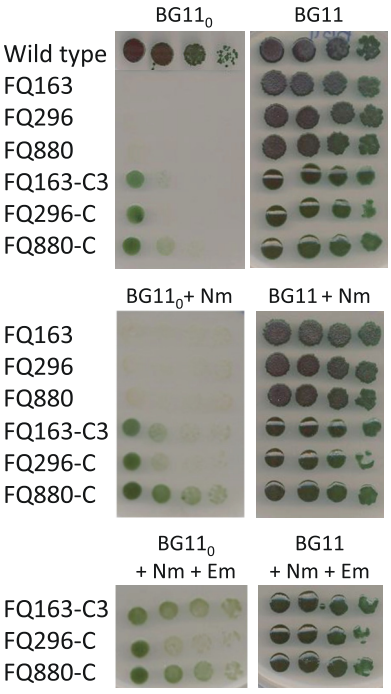

FIG 6 Growth of *Anabaena* sp. PCC 7120 wild type, *all1711* mutants, and complemented mutant strains with nitrate as the nitrogen source (BG11 medium) or without combined nitrogen (BG11$_0$ medium). Spots of each indicated strain (see genotypes in Table 1) were inoculated with an amount of nitrate-grown cells resuspended in BG11$_0$ medium containing (from left to right) 100, 10, 1, or 0.1 ng Chl. The plates were then incubated under culture conditions for 15 days and photographed. Where present, Nm was at 30 μg ml^{-1} and Em at 5 μg ml^{-1}. Complemented strains were pregrown with Nm with or without Em as appropriate.

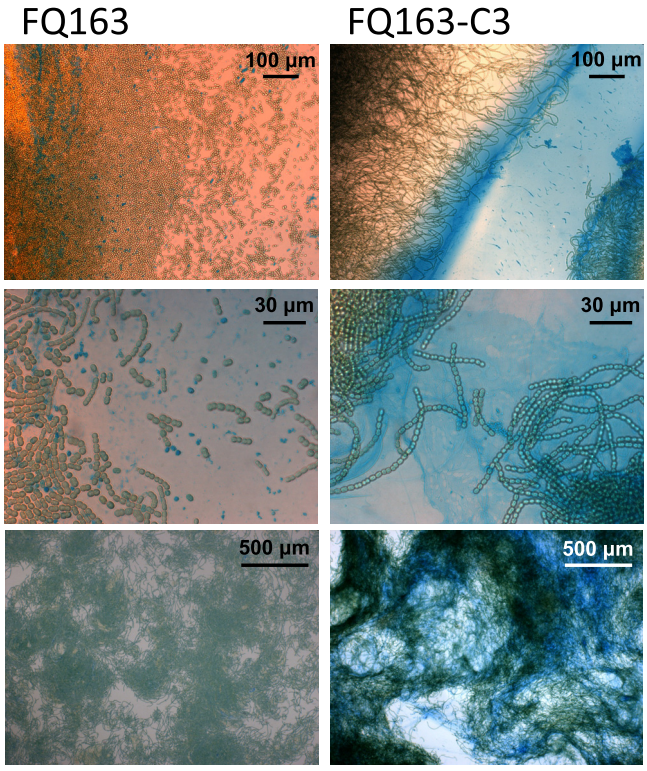

FIG 7 Extracellular polysaccharides in strain FQ163-C3. (Top panels) Sample colonies of strains FQ163 and FQ163-C3 (from BG11-agar plates containing 30 μg Nm ml^{-1} without and with 5 μg Em ml^{-1}, respectively) were supplemented with a solution of 1% alcian blue 8GX (Sigma) and visualized by light microscopy. (Middle panels) Filaments from spots atop BG11-agar plates (with antibiotics as described above) were suspended in 150 μl of BG11 medium, supplemented with 10 μl of 1% alcian blue 8GX, and visualized by light microscopy. (Bottom panels) Biomass from the surface of BG11-agar plates (with antibiotics as described above) was harvested and suspended in 9 ml of BG11$_0$ medium. These filament suspensions were supplemented with 1 ml of 0.02% alcian blue–0.06% acetic acid and, after 5 min at room temperature, harvested by centrifugation (4,000 × *g*, 5 min). Samples from the sediment were visualized by light microscopy.

mented strain FQ163-C3 but not of a colony of mutant FQ163. When filaments from such colonies were suspended in a small volume of medium and supplemented with alcian blue, some material that was stained could be observed in the suspension of the complemented strain but not in that of the mutant (Fig. 7, middle panel). Notably, this material was not bound to the cells. A different staining protocol (based on that of Sohm et al. [52]) also showed alcian blue-staining material in the biomass of the complemented strain but not in that of the mutant (Fig. 7, bottom panel).

Because nitrate-grown filaments are composed almost exclusively of vegetative cells, these results suggest that complementation induces the vegetative cells to produce increased amounts of the sheath material. We therefore used Northern blot analysis to check expression of *all1711* in nitrate-grown cells of the wild type, FQ163, FQ296, FQ163-C3, and FQ296-C (Fig. 8). As expected for cells grown in the presence of combined nitrogen, very low expression was detected in the wild type. In mutant FQ296, expression was also very low, and greater expression was not expected because the transposon was inserted upstream from the region of the gene to which the hybridization probe corresponds (see Fig. 1A). However, appreciable expression of RNA hybridizing with the *all1711* probe in FQ163 was detected, corresponding to transcription of the region of the gene 5′ from the site of insertion of the transposon (see Fig. 1A). Perhaps the transcript of *all1711* is produced at a low level in nitrate-grown FQ163 cells and is stabilized upon reaching the transposon, thereby becoming detectable by the *all1711* probe used (see Fig. 1A). In any case, a high level of expression was detected in the two complemented strains, presumably corresponding in part to transcription from P_{glnA} of *all1711* cloned in pRL3845. This observation confirms that *all1711* is overexpressed in the complemented strains FQ163-C3 and FQ296-C.

Sucrose uptake. We previously found that the relatively sucrose-impermeable outer membrane can be bypassed by subjecting filaments of the *Anabaena* sp. to mechanical fragmentation (47). Use of [U-^{14}C]sucrose thereupon showed that filaments grown in BG11 medium have a sucrose transport system (47). In fragmented filaments from ammonium-grown cultures, uptake of [U-^{14}C]sucrose occurred at similar levels for the wild type and strains CSE2, 216, and FQ163-C1 and was about 20% lower for FQ163 (Table 2). In fragmented filaments from cultures that had been incubated for 16 h without any source of combined nitrogen, uptake of [U-^{14}C]sucrose increased differentially (i.e., uptake of −N less uptake of $+NH_4^+$) in the wild type. A much smaller differential was found in strain CSE2 and no differential was observed in strain 216 (Table 2), suggesting that the differential cor-

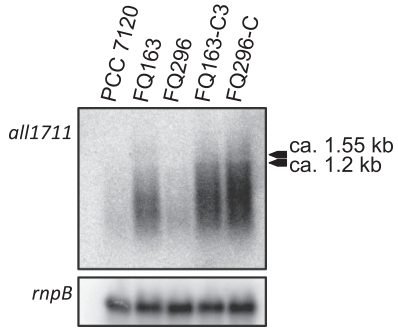

FIG 8 Expression of *all1711* in the wild type, two *all1711* mutants, and corresponding complemented *all1711* mutant strains. Northern blot analysis was performed with the *all1711* probe indicated in Fig. 1A and RNA from cells grown in bubbled BG11C medium containing Nm for FQ163 and FQ296 and Nm plus Em for the complemented mutants (see genotypes in Table 1). The approximate sizes of different *all1711* transcripts are indicated. Because the gene is expressed from a set of promoters in the plasmid complementing the mutants that differ from the promoter in the wild-type chromosome, the observed sizes differ from that in Fig. 3. Hybridization with a probe of the *Anabaena rnpB* gene was used as a loading and transfer control (lower panel).

responded to uptake by (pro)heterocysts. The increase in uptake observed in strain FQ163 was about 17% that in the wild type, and the increase observed in complemented strain FQ163-C1 was about 55% that in the wild type, again representing an incomplete complementation. These results are consistent with the idea that All1711, although not the only mediator of sucrose uptake, can mediate sucrose uptake, representing a main mediator of sucrose uptake into (pro)heterocysts. However, the possibility cannot be ruled out that sucrose uptake in strain FQ163 is indirectly affected because (pro)heterocysts are altered.

DISCUSSION

The results described above indicate that ORF *all1711* of the genome of *Anabaena* sp. strain PCC 7120 encodes a protein that is needed for production of the polysaccharide layer of the heterocyst envelope (Hep). Consistently, transposon insertion mutants of *all1711* exhibit a Fox^- Fix^+ phenotype. We have shown that expression of *all1711* is transiently induced strongly during the initial differentiation of heterocysts and then diminishes (Fig. 3). Although RNAseq analysis showed that *all1711* was induced in response to deprivation of combined nitrogen (18), microarray analysis indicated that expression was, overall, low (13; reevaluated by Xu et al. [64]). As seen with an All1711-GFP fusion, induction takes place in vegetative cells and, at somewhat higher levels, in cells that are differentiating into heterocysts. A marked increase in expression of the *all1711* transcript was visualized by Northern blot analysis as of 3 h of nitrogen step-down (Fig. 3A) but was visualized by fluorescence of All1711-GFP only as of 6 h (Fig. 4B), suggesting that appreciable fluorescence requires an accumulation of fusion protein. Increased expression of All1711-GFP after combined-nitrogen deprivation is consistent with dependence on NtcA, the nitrogen-control transcription factor of cyanobacteria, as observed by Northern analysis (Fig. 3A). Three 5′ RNA ends that are influenced by NtcA are detected by primer extension analysis upstream from *all1711*. Whereas RNA III originates from a class II NtcA-activated promoter and RNA I may originate from a suboptimal NtcA-activated promoter (Fig. 3C), no obvious putative promoter is found upstream of RNA II, which might be related to RNA III. The presence of multiple promoters upstream from genes that are related to the differentiation and function of heterocysts, first described for the *glnA* gene (54), is widely found (19).

Expression of *all1711* does not require HetR, and the three RNA species detected by primer extension (RNA I, RNA II, and RNA III) were observed with RNA isolated from a *hetR* mutant (Fig. 3B). Expression of *all1711* was shown by Northern analysis to take place at a higher level and to persist longer in the *hetR* mutant than in the wild type (see Fig. 3A). This observation suggests that HetR may negatively affect expression of *all1711* in vegetative cells. Negative effects of HetR on expression of other genes have been noted previously (30, 36, 48). HetR is a transcription factor with DNA binding activity (27, 31) that, although induced after nitrogen step-down (5), is found at substantial levels in ammonium-grown cells (66) and so may directly repress some genes. Because we observed increased expression of *all1711* in a *hetR* mutant, repression takes place in vegetative cells. Nonetheless, as seen in Fig. 4 and 5, some expression of *all1711* takes place in vegetative cells during heterocyst differentiation.

All1711 is a predicted MFS protein that is similar to glycoside transporters and, as observed with an All1711-GFP fusion protein, appears to reside in the CM. Although similarity to proteins of known function in the entire database is too low to indicate the precise substrate(s) for All1711, BLASTp analysis of All1711 against the *Arabidopsis thaliana* proteome shows that 7 of the top 8 targets represent four different sucrose transporters, which are sucrose:proton symporters. At least some plant sucrose:proton symporters can function bidirectionally, acting as importers or exporters, depending on the membrane potential and proton gradient (the proton-motive force) and the sucrose gradient (11). As is the case for some plant sucrose transporters (32), All1711 might transport glycosides, including sucrose. Although assays of transport of [^{14}C]sucrose (Table 2) suggest that a sucrose transporter is present in proheterocysts of *Anabaena* sp. strain PCC 7120 and is missing from an *all1711* mutant, it remains possible that uptake of sucrose is affected only indirectly in that mutant.

When *all1711* is overexpressed, as in strains FQ163-C3 and FQ296-C complemented with pRL3845 (Fig. 8), the *Anabaena* sp. cells produce a substantial amount of a sheath material that can be stained with alcian blue, tentatively identifying it as exopolysaccharide. Overexpression of *all1711* may result from the relatively high copy number of pDU1-based plasmids, which can be about 17 copies of plasmid per chromosome copy (35), and (or) from expression from the relatively strong *glnA* promoter. That pro-

TABLE 2 Sucrose uptake in *Anabaena* sp. strain PCC 7120 and some mutant strains

Strain	Genotype	[U-^{14}C]sucrose uptake (nmol [mg Chl]$^{-1}$ min^{-1} ± SD) (no. of independent assays)[a]		
		NH_4^+ grown	−N (16 h)	Δ[(−N) − (NH_4^+)]
PCC 7120	Wild type	9.28 ± 0.48 (4)	16.69 ± 0.64 (6)	7.41 ± 0.80
CSE2	*ntcA*::C.S3	9.10 ± 0.50 (2)	10.77 ± 0.57 (3)	1.67 ± 0.76
216	*hetR*	9.78 ± 0.46 (2)	9.80 ± 1.03 (3)	0.02 ± 1.13
FQ163	*all1711*::Tn*5*-1063	7.35 ± 0.63 (2)	8.60 ± 0.21 (3)	1.25 ± 0.66
FQ163-C1	FQ163 (pRL3161)	9.40 ± 1.10 (2)	13.45 ± 0.34 (3)	4.05 ± 1.15

[a] Data represent the means and standard deviations of the results from the number of independent assays (indicated in parentheses) carried out with cells from independent cultures as described in Materials and Methods.

moter is known to be active under different nitrogen regimens and in vegetative cells as well as in heterocysts (15, 54, 56). Production of sheath material is evident in nitrate-grown filaments and, therefore, can take place from vegetative cells. Because All1711 is a possible glycoside transporter, it may promote, when overexpressed in the vegetative cells, the export through the CM of a substrate such as a glycoside for production of exopolysaccharide. Abnormal loss of a glycoside in response to overproduction of All1711 might explain the poor growth of the complemented *all1711* mutant strains in the absence of fixed nitrogen.

The identified *hep* and Hep region genes encode, in addition to regulatory proteins, cytoplasmic and CM enzymes, including glycosyl transferases, CM transporters, and envelope translocators (see, e.g., references 28, 34, 39, 59, and 65). HepA may be a Hep-related CM lipid A exporter (23, 28). The only other CM transporter previously identified as involved in Hep deposition is Alr2841, which shows homology to proteins of the WzyC superfamily involved in the synthesis of O-antigen (40). Nearly all hitherto-identified *hep* mutants have been identified by the phenotypes resulting from transposon-based knockout mutations that led to complete loss of Hep. The phenotype of the *all1711* transposon insertion mutants, which are conspicuously affected only in deposition of Hep, suggests that All1711 has a role in the process of Hep production, perhaps as an exporter of glycoside precursors of Hep. We therefore propose to name *all1711 hepP* (*P* for *p*ermease). Strong induction of this gene from 3 to 12 h after nitrogen step-down is consistent with induction of other *hep* genes, which peaks at 8 h (13, 64) to 12 h (18) after nitrogen step-down. Because All1711 is also expressed in vegetative cells, one can imagine that All1711 may export through the CM of the vegetative cells one or more precursors of exopolysaccharide that diffuse through the continuous periplasm of the filament to support Hep biosynthesis in the differentiating heterocysts. When overexpressed in the absence of heterocysts, All1711 seems to increase, by exporting such precursor(s), the production of vegetative cell exopolysaccharide.

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