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Regulation of Fructose Transport and Its Effect on Fructose Toxicity in *Anabaena* spp.▽†

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Anabaena variabilis **grows heterotrophically using fructose, while the close relative *Anabaena* sp. strain PCC 7120 does not. Introduction of a cluster of genes encoding a putative ABC transporter, herein named *frtRABC*, into *Anabaena* sp. strain PCC 7120 on a replicating plasmid allowed that strain to grow in the dark using fructose, indicating that these genes are necessary and sufficient for heterotrophic growth. FrtR, a putative LacI-like regulatory protein, was essential for heterotrophic growth of both cyanobacterial strains. Transcriptional analysis revealed that the transport system was induced by fructose and that in the absence of FrtR, *frtA* was very highly expressed, with or without fructose. In the *frtR* mutant, fructose uptake was immediate, in contrast to that in the wild-type strain, which required about 40 min for induction of transport. In the *frtR* mutant, high-level expression of the fructose transporter resulted in cells that were extremely sensitive to fructose. Even in the presence of the inducer, fructose, expression of *frtA* was low in the wild-type strain compared to that in the *frtR* mutant, indicating that FrtR repressed the transporter genes even in the presence of fructose. FrtR bound to the upstream region of *frtA*, but binding was not visibly altered by fructose, further supporting the hypothesis that fructose has only a modest effect in relieving repression of *frtA* by FrtR. *A. variabilis* grew better with increasing concentrations of fructose up to 50 mM, showing increased cell size and heterocyst frequency. *Anabaena* sp. strain PCC 7120 did not show any of these changes when it was grown with fructose. Thus, although *Anabaena* sp. strain PCC 7120 could take up fructose and use it in the dark, fructose did not improve growth in the light.**

Although the majority of cyanobacteria are obligate photoautotrophs, dependent on sunlight for ATP and on CO_2 for carbon, a few well-studied strains can take up and use sugars either only in the light, growing mixotrophically, or in the dark, growing heterotrophically (41). *Synechocystis* sp. strain PCC 6803 is one of the best-studied strains that can grow in the dark using glucose as the sole carbon source; however, it requires short, regular exposure to light for heterotrophic growth (3). Two well-studied filamentous heterocyst-forming cyanobacterial strains, namely, *Nostoc punctiforme* ATCC 29133 and *Anabaena variabilis* ATCC 29413, are capable of true heterotrophic growth in complete darkness (14, 43). The former strain grows on glucose or fructose, while *A. variabilis* ATCC 29413 can use only fructose (14). In these two heterocyst-forming strains, sugars support not only growth in the dark but also nitrogen fixation, an energetically expensive reaction. *N. punctiforme* ATCC 29133 is a symbiont of the bryophyte *Anthoceros punctatus*, and hence, it is likely that its ability to use sugars is essential for its role in symbiosis (49). *A. variabilis* is not known to be an endosymbiont; however, by morphology, phenotype, and genetics, it is virtually identical to many strains called *Anabaena azollae*, isolated from the symbiotic association of cyanobacteria with the water fern *Azolla* (6, 12, 28, 36, 37, 39, 52). Although it was first believed that *Anabaena azollae* was the symbiont, it was shown later that the true symbiont is different and is probably nonculturable (13, 28). Hence, there is some possibility that *A. variabilis* also once came from *Azolla* and that fructose utilization is associated with symbiosis in this strain.

Fructose dramatically affects the physiology of *A. variabilis*. The cells grow faster, are bigger, and in filaments that have differentiated heterocysts, produce more and larger heterocysts, fixing more nitrogen and producing more hydrogen than do cells grown photoautotrophically (14, 35, 42). [^{14}C]fructose, which is taken up almost immediately by vegetative cells in a filament, is quickly transported in some form to the heterocysts, where the ^{14}C compound accumulates and is metabolized to provide a reductant for nitrogen fixation (14). Although fructose supports nitrogen fixation in whole filaments, isolated heterocysts cannot use fructose as a source of reductant, suggesting either that fructose cannot be transported by heterocysts or that fructose is converted to another compound in the vegetative cell before it moves to the heterocyst (16). For *N. punctiforme*, a mutant deficient in glucose-6-phosphate dehydrogenase cannot fix nitrogen and cannot grow in the dark with fructose, indicating that the oxidative pentose phosphate pathway is the major pathway for fructose metabolism and is important in heterocysts for nitrogen fixation (43). In fructose-grown filaments, the heterocysts not only are bigger than those in cells grown photoautotrophically but also store more glycogen and are morphologically different (21).

Growth with fructose results in increased respiration and decreased chlorophyll (14, 33, 36, 46). In long-term, dark-grown, fructose-adapted cells, there is an increase in photosystem II, resulting in a decrease in the ratio of photosystem I to photosystem II (23). Cells grown with low CO_2 in the presence

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of fructose do not fix CO_2 well because of decreased carbonic anhydrase and decreased ribulose bis-phosphate carboxylase oxygenase (29). The decrease in oxygen production in fructose-grown cells is thought to contribute to a micro-oxic environment that better supports nitrogen fixation (14). Microarray analysis of RNA from the non-nitrogen-fixing unicellular cyanobacterium *Synechocystis* sp. strain PCC 6803 under conditions of nitrogen starvation shows increased expression of genes important in glycolysis, the oxidative pentose phosphate pathway, and glycogen catabolism and increased activities of glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, two key enzymes of the oxidative pentose phosphate pathway (31). In *Synechocystis*, transcription of the genes for sugar catabolism is regulated by Hik8 (40), a homolog of a protein (SasA) in *Synechococcus* that regulates *kaiC*, which is part of the central oscillator of circadian rhythm (15, 20). In addition, the sigma factor SigE positively regulates three glycolytic genes, four oxidative pentose phosphate genes, and two glycogen metabolism genes (32). Activation of sugar catabolic genes under conditions of nitrogen starvation requires the global nitrogen activator NtcA (30, 32). SigE in *Anabaena* sp. strain PCC 7120, a strain that cannot use fructose, is not essential for nitrogen fixation but is expressed late in heterocyst differentiation, suggesting that it has a role in heterocyst function (1, 19).

The transport of glucose in *Synechocystis* is known to occur via a glucose-fructose permease, the product of the *glcP* transport gene (11, 17, 50). Transport of fructose is toxic to the cells; inactivation of *glcP* relieves the toxicity but no longer allows the cells to grow using glucose (11, 17, 50). Expression of *glcP* in *Synechococcus* sp. strain PCC 7942 resulted in a strain that was capable of glucose transport but also died in the presence of glucose (51). Uptake of fructose in *A. variabilis* and *Nostoc* sp. strain ATCC 29150 is constitutive but increases after exposure to fructose (38, 46) and is energy dependent in *A. variabilis* (46). The K_m for fructose uptake is about 160 μM for cells that have not been grown with fructose and about 50 μM for cells pregrown with fructose, and it does not change in the light versus the dark (14, 16). We describe here the genes for fructose transport in *A. variabilis*, their regulation, and the effect of their expression on growth of the obligately photoautotrophic strain *Anabaena* sp. strain PCC 7120.

MATERIALS AND METHODS

Strains and growth conditions. Strains of *A. variabilis* FD, a derivative of *A. variabilis* 29413 that can grow at 40°C and supports the growth of bacteriophages better than the parent strain does (9), and *Anabaena* sp. strain PCC 7120 were maintained on agar-solidified Allen and Arnon (AA) medium (2) supplemented, when appropriate, with 5 mM NH_4Cl, 10 mM *N*-tris (hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), pH 7.2, 25 to 40 μg ml^{-1} neomycin sulfate, or 3 μg ml^{-1} each of spectinomycin and streptomycin. Strains were grown photoautotrophically in liquid cultures in an eightfold dilution of AA medium (AA/8) or in AA/8 supplemented with 5 mM NH_4Cl and 10 mM TES, pH 7.2, at 30°C, with illumination at 50 to 80 microeinsteins m^{-2} s^{-1}. Antibiotics were included as follows (when required): neomycin (3 to 5 μg ml^{-1}) and spectinomycin (0.3 μg ml^{-1} for liquid). For experiments to measure the growth of strains with fructose, cells were harvested at an optical density at 720 nm (OD_{720}) of 0.2, washed once in AA/8, and resuspended in AA/8 with the indicated concentrations of fructose at an OD_{720} of 0.02. All growth experiments were performed three times, and a representative graph is presented.

Construction of plasmids and strains. A neomycin resistance (Nm^r) cassette containing a transcriptional terminator was PCR amplified from pRL648 (10), using primers nptTerm-3′and nptTerm-5′, digested with EcoRI, and cloned into the EcoRI site of pUC1819RI to create pBP285. Primer sequences are provided in Table S1 in the supplemental material. The Nm^r cassette was used to create pBP299, a plasmid with an insertional mutation in *frtR*. The bom site of pRL1075, required for conjugation, was inserted into pBP299 to create pBP301. Replacement of the wild-type *frtR* gene in the chromosome of strain FD with the mutant *frtR* allele in pBP301 was accomplished by conjugation followed by double recombination (45). The mutant was segregated as described previously and tested by PCR to verify that no wild-type copies of the gene remained (22).

Plasmid pBP289 was created to contain the ava2169 to ava2173 genes from genomic library clone pAAWY3009. This plasmid was used to construct the replicating plasmids pBP291 (containing *frtRABC*) and pBP292 (containing *frtABC* without *frtR*). Plasmids were constructed as described in Table 1. Replicating plasmids pBP291 (containing *frtRABC*) and pBP292 (containing *frtABC* without *frtR*) were conjugated into *Anabaena* sp. strain PCC 7120, selecting for the antibiotic resistance on the plasmid, and the presence of the plasmid was verified by PCR.

The BglII and BamHI Nm^r fragment in pPE20 was replaced with a Sp^r Sm^r cassette containing a transcriptional terminator (amplified from pBP288, using the Sm Ω L and Sm Ω R primers, engineered with BglII and BamHI sites at the 5′ and 3′ ends, respectively, for cloning) to create pBP350. The Sp^r Sm^r version of pPE20, pBP350, was used to create *lacZ* transcriptional fusions of *frtA* (pBP352) and *frtR* (pBP353) at the EcoRV and ClaI sites, respectively. Integration of the transcriptional fusions, pBP352 and pBP353, into the chromosome of FD was accomplished by conjugation of the nonreplicative plasmids, selecting for single recombinants containing the entire plasmid in the chromosome.

Plasmid pBP288 is a 16.7-kb pBR322-based vector that contains (i) a promoterless *lacZ* gene for assaying promoter activity in vivo; (ii) a 6.5-kb *ntcA* region of *A. variabilis* that allows for good homologous recombination; (iii) a 1.1-kb *npt* gene from pRL648 interrupting *ntcA*, ensuring only one functional copy of *ntcA* after recombination; (iv) a 1.0-kb Ω Sp^r Sm^r cassette with a transcriptional terminator from pRL277 upstream of and directed away from the *lacZ* gene; and (v) a Tet^r cassette between the BglII and SmaI cloning sites to allow for easy cloning of promoter fragments upstream of the *lacZ* gene.

Plasmid pBP313 contained the *psbA* promoter in the BglII/SmaI sites of pBP288. A Tet^r gene (PCR amplified from pBR322 by use of primers pBR322-L2 and pBR322-R2) was inserted into the SmaI/SacI sites of pBP313 to create pBP328, a plasmid that destroyed the *lacZ* gene but gave selection for inserting fragments under the control of the *psbA* promoter in vivo. The plasmid used to overexpress FrtR in *A. variabilis* was constructed by PCR amplifying the *frtR* gene, using primers psbAFrtR-5psbAFruR-5 and psbAFrtR-3′psbAFruR-3′, and inserting it into the SmaI/SacI sites of pBP328 to create pBP356. Additionally, the *frtABC* coding region (PCR amplified using primers frtABC-L and frtABC-R) was cloned downstream of the *psbA* promoter on pBP313 to generate pJU377. These plasmids were conjugated into FD by single recombination to yield BP356 and JU377.

A 500-bp *frtA* promoter fragment (amplified from FD by use of the frtA498A-L and frtA-R10 primers) and a 400-bp *frtR* promoter fragment (amplified from FD by use of the frtR397-L and frtR-R10 primers) were cloned into the BglII/SmaI sites upstream of *lacZ* on pBP288 to generate pJU338 and pJU336, respectively. These plasmids were then conjugated, with selection for single recombinants, into FD to generate strains JU338 and JU336, into *Anabaena* sp. strain PCC 7120 to generate strains JU357 and JU356, and into BP301 to produce strains JU355 and JU353.

The plasmid pBP354, used to overexpress FrtR in *Escherichia coli*, was constructed by PCR amplification of *frtR* with NdeI and BamHI sites at the 5′ and 3′ ends, respectively, using primers FrtR-L3 and FrtR-R3, and insertion into the same sites of pBP314. pBP314 was constructed by inserting a Tet^r gene [PCR amplified from pBR322 by use of primers Tet(NdeI)-L and Tet(BamHI)-R] into the NdeI/BamHI sites of pET22b (Invitrogen), therefore making it easier to select for insertion of a DNA fragment encoding protein into the vector.

FrtR overexpression and purification and electrophoretic mobility shift assay. The FrtR protein was purified from *E. coli*/pBP354, overexpressing FrtR, as inclusion bodies as described by Campbell et al. (8), with the following modifications: cells were lysed by four 30-s rounds of sonication and the protein concentration was adjusted to 1.0 mg ml^{-1} before renaturation. Electrophoretic mobility shift assay binding reaction mixtures contained 4 mM Tris, pH 8.0, 12 mM HEPES, 12% glycerol, 100 mM KCl, 0.5 mM EDTA, 1 mM dithiothreitol, 5 mM $MgCl_2$, 1.0 μg poly(dI-dC), and 10,000 cpm ^{32}P-end-labeled probe. FrtR was added (100 to 700 ng of protein), and the mixture was incubated for 20 min at 30°C. After the binding reaction, the reaction mixtures were loaded into a 4% polyacrylamide gel with a Tris-glycine buffer (pH 8.0) and were electrophoresed at 40 mA for 25 min. Bands were visualized using a phosphorimager.

TABLE 1. Strains and plasmids used for this study

Strain or plasmid	Relevant characteristics	Reference
Strains		
A. variabilis FD	*Anabaena variabilis* parent strain	9
Anabaena sp. strain PCC 7120	Wild-type strain	
Anabaena sp. strain PCC 7120 BP291	*frtRABC* expressed from plasmid pBP291	This work
Anabaena sp. strain PCC 7120 BP292	*frtABC* expressed from plasmid pBP292	This work
A. variabilis FD BP301	*frtR* mutated with insert of Nm^r cassette at NaeI sites	This work
A. variabilis BP352	pBP352 integrated into the chromosome	This work
A. variabilis BP353	pBP353 integrated into the chromosome	This work
A. variabilis BP356	pBP356 integrated into the chromosome	This work
A. variabilis JU336	pJU336 integrated into the chromosome	This work
A. variabilis JU338	pJU338 integrated into the chromosome	
A. variabilis JU377	pJU377 integrated into the chromosome	This work
Anabaena sp. strain PCC 7120 JU356	pJU336 integrated into the chromosome	This work
Anabaena sp. strain PCC 7120 JU357	pJU338 integrated into the chromosome	This work
Anabaena FD JU353	pJU336 integrated into the chromosome of *A. variabilis* FD BP301	This work
Anabaena FD JU355	pJU338 integrated into the chromosome of *A. variabilis* FD BP301	This work
Plasmids		
pAAWY3009	9.3-kb clone of *A. variabilis* DNA containing *frtRABC*; Cm^r	JGI sequencing clone
pBP285	Km^r Nm^r cassette in a polylinker [C.K.3 (10)] with a transcriptional terminator	This work
pBP288	Cloning vector for integration of transcriptional fusions into the chromosome; Tc^r Km^r Nm^r Sp^r Sm^r Ap^r	This work
pBP289	9.8-kb EcoRI fragment of pAAWY3009 (containing Ava2169 to Ava2173 but lacking Ava4074), self-ligated	This work
pBP291	8.4-kb ScaI/SmaI fragment from pBP289 (containing *frtRABC*) inserted into the SmaI sites of pRL57	This work
pBP292	4.9-kb NaeI/SmaI fragment from pBP289 (containing *frtABC*) ligated into the SmaI sites of pRL57	This work
pBP299	1.1-kb SmaI fragment containing the Nm^r cassette of pBP285 inserted into NaeI site of *frtR* in pHL110	This work
pBP301	5-kb BglII fragment of pRL1075 ligated into the BamHI site of pBP299	This work
pBP313	Cloning vector to overexpress genes from *psbA* promoter; Km^r Nm^r Sp^r Sm^r Ap^r	This work
pBP314	PCR-amplified Tc^r cassette of pBR322 inserted into the NdeI/BamHI sites of pET22b	This work
pBP328	PCR-amplified Tc^r cassette of pBR322 inserted into the SmaI/SacI sites of pBP313	This work
pBP350	Replaced BglII/BamHI fragment containing Nm^r cassette of pPE20 with PCR-amplified Ω Sp^r Sm^r cassette from pBP288	This work
pBP351	4.2-kb EcoRI/ScaI fragment of pHL110 (containing Ava2169 to -71) cloned into EcoRV/EcoRI fragment of pBR322	This work
pBP352	5-kb SmaI fragment (containing a *lacZ* Sp^r Sm^r cassette) of pBP350 inserted into EcoRV site of pBP351	This work
pBP353	5-kb SmaI fragment (containing a *lacZ* Sp^r Sm^r cassette) of pBP350 inserted into ClaI site (blunted) of pBP351	This work
pBP354	PCR-amplified *frtR* gene inserted into NdeI/BamHI sites of pBP314 for overexpression of native FrtR	This work
pBP356	PCR-amplified *frtR* gene inserted into SmaI/SacI sites of pBP313 under the control of *psbA* promoter	This work
pBR322	Mobilizable plasmid; Ap^r Tc^r	5
pET22b	T7 expression vector; expression induced by isopropyl-β-D-thiogalactopyranoside	Invitrogen
pHL110	4.5-kb HindIII *frt* region of pAAWY3009 inserted into HindIII site of pUC18	This work
pJU336	PCR-amplified 400-bp *frtR* promoter fragment inserted into BglII/SmaI sites of pBP288	This work
pJU338	PCR-amplified 500-bp *frtA* promoter fragment inserted into BglII/SmaI sites of pBP288	This work
pJU377	PCR-amplified *frtA* gene inserted into SmaI/SacI sites of pBP313 under the control of *psbA* promoter	This work
pPE20	Source of *lacZ* for transcriptional fusions; Ap^r Km^r Nm^r	44
pRL1075	Source of mobilization site, *oriT*, and *sacB* gene, which confers sucrose sensitivity; Cm^r Em^r	4
pRL57	S.K5 + L.HEH2 + C.S3; positive selection shuttle (pDU1) cloning vector	10
pRL648	Km^r Nm^r cassette in a polylinker (C.K.3)	10
pUC18	Cloning vector; Ap^r	47
pUC1819RI	Cloning vector; Ap^r	7

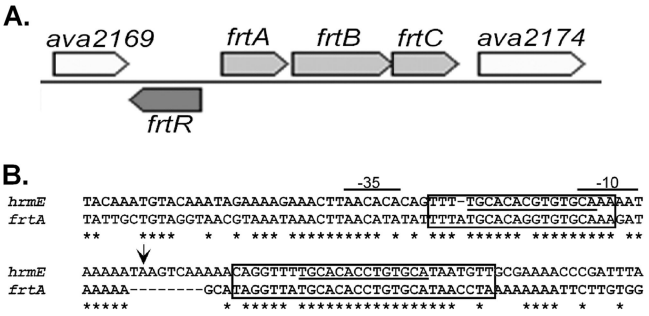

FIG. 1. Fructose transport genes. (A) The region of the chromosome of *A. variabilis* with the fructose transport genes, namely, *frtR* (ava2170), encoding a putative *lacI*-like transcriptional regulator, and *frtABC* (ava2171 to -2173), encoding a putative periplasmic binding component, ATPase component, and transmembrane component, respectively. (B) Alignment of the promoter region of *hrmE* of *N. punctiforme* with a conserved region of the *frtA* promoter region, beginning about 300 bp upstream from the start codon of *frtA*. The transcription start site of *hrmE* is indicated by an arrow, and the −10 and −35 regions of the *hrmE* promoter are labeled. The HrmR binding sites, which are underlined, are shown within boxes that indicate longer conserved palindromic sequences.

RNA isolation and RT-PCR. RNAs were isolated from 50-ml cultures grown in AA/8, with or without fructose, and subjected to DNase digestion using a Turbo DNA-free kit (Ambion). Reverse transcription-PCR (RT-PCR) was performed as previously described (34), with the following change: 2.5 ng of RNA and 2.5 U of Superscript III (Invitrogen) were added per reaction. Primers were annealed at 58°C. The primers frtA-L/R and frtR-L/R were used to amplify the *frtA* and *frtR* transcripts, respectively. RNA from the housekeeping gene *rnpB* was amplified using rnpB-L/R primers as a control (48).

Microtiter β-galactosidase assays. Cultures were grown as described above to an OD_{720} of 0.1 and divided into two equal portions, and 5 mM fructose was added to one portion to induce expression of the *frt* genes. Two hours after induction, the cultures were adjusted to an OD_{720} of 0.05, and 700 μl of culture was added to 700 μl of 2× LacZ buffer (120 mM Na_2HPO_4, 80 mM NaH_2PO_4, 20 mM KCl, 2 mM $MgSO_4$, 100 mM β-mercaptoethanol). The samples were vortexed for 60 s with 30 μl 0.1% sodium dodecyl sulfate and 60 μl chloroform. The chloroform was removed, and 250 μl of sample was placed in microtiter wells. Eighty microliters of *o*-nitrophenyl-β-D-galactopyranoside (4 mg ml^{-1}) was added to the wells, and a microtiter plate reader measured the OD_{420} every 90 s for 1 h. Eight replicates were done for each sample. Excel was used to process the raw data, yielding the rate of the reaction, which was normalized to the OD_{720} of the culture.

Light micrographs. Filaments were viewed with a Zeiss epifluorescence microscope and imaged using a Retiga EXi (QImaging) cooled charge-coupled device camera with IP Labs 4.0 software (BD Biosciences). The exposure time was about 0.05 s for bright-field images.

RESULTS

Identification of fructose transport genes. Fructose transport genes were identified as putative ABC-type sugar transport genes present in the genomes of *A. variabilis* and *N. punctiforme*, both capable of heterotrophic growth in the dark with fructose as the sole carbon source, but not in the genome of *Anabaena* sp. strain PCC 7120, an obligate photoautotroph. The organization of the ABC-type transport genes, *frtABC*, and a *lacI*-like regulatory gene, *frtR*, is shown in Fig. 1A. The flanking ava2169 gene is tentatively identified as encoding dihydrouridine synthase and has close homologs in the genomes of *Anabaena* sp. strain PCC 7120, *N. punctiforme*, and *Nodularia spumigena* CCY9414, while the ava2174 gene encodes a putative porin-like protein. There is a close homolog of the ava2174 gene in the genome of *N. punctiforme* but not in the genomes of *Anabaena* sp. strain PCC 7120 and *N. spumigena*. The homolog of FrtR in *N. punctiforme* (73% identical) is HrmR (Npun02008536), a repressor of its own gene, *hrmR*, and of *hrmE* (Npun02008530), which are genes involved in the differentiation of hormogonia in *N. punctiforme* (8). There is no homolog of *hrmE* in *A. variabilis* or in the genomes of any other sequenced cyanobacteria. The homologs of FrtABC in *N. punctiforme* (Npun02008528, Npun02008527, and Npun02008526 to Npun02006538), with 72 to 85% identities to the proteins in *A. variabilis*, are downstream of *hrmE* in the *N. punctiforme* genome. Downstream of the *frtABC* homologs in *N. punctiforme* is a gene (Npun02006539) that appears to encode a glucose transporter. This gene is absent from *A. variabilis*, consistent with the fact that this strain cannot use glucose as a carbon source; however, there is a homolog (sll0771; named *glcP* for glucose permease) with 71% identity in *Synechocystis* sp. strain PCC 6803, a strain that can grow heterotrophically in the dark with glucose.

The *hrmE* gene in *N. punctiforme* is regulated by HrmR, a homolog of FrtR (8). Although there is no similarity between HrmE in *N. punctiforme* and FrtA in *A. variabilis*, there is striking similarity in the regulatory regions of these genes. The region of *hrmE* that contains the transcription start site and the two binding sites for HrmR is well conserved in the region upstream of *frtA* in *A. variabilis* (Fig. 1B), and the two binding sites for HrmR are nearly identical between *frtR* and *hrmE*. In the regulatory region of *frtA*, the second HrmR-like binding site is within a larger, 28-bp, almost perfect palindrome (Fig. 1B); however, that larger palindrome is not as well conserved in *hrmE*. In contrast, in *hrmE*, the first HrmR binding site is within a 20-bp perfect palindrome (Fig. 1B) that is not as well conserved in *frtA*. The striking similarity in these regions suggests that *hrmE* and *frtA* have similar modes of regulation, which is supported by the similarity of FrtR and HrmR. There are two HrmR binding sites in the promoter region of *hrmR*, and HrmR binds to this region; thus, it is autoregulatory (8). Since *frtA* and *frtR* in *A. variabilis* are divergent genes (Fig. 1A), they share a regulatory region and therefore share the two HrmR binding sites shown in Fig. 1B.

Function of *frtRABC*. In order to determine whether the *frtRABC* genes function to transport fructose, we transferred *frtRABC* or *frtABC*, lacking *frtR*, to *Anabaena* sp. strain PCC 7120, a strain that lacks the *frtRABC* genes and cannot grow heterotrophically in the dark with sugars. An *frtR* mutant of *A. variabilis* was also constructed. The wild-type strain of *A. variabilis* grew well in the light with or without fructose but grew in the dark only in the presence of fructose (Fig. 2, lane 5). In contrast, *Anabaena* sp. strain PCC 7120 grew only in the light and could not grow in the dark with fructose (Fig. 2, lane 4) unless the strain also contained the *frtRABC* genes of *A. variabilis* (Fig. 2, lane 1). Thus, the *frtRABC* genes in *Anabaena* sp. strain PCC 7120 were sufficient to allow the strain to transport fructose. In *Anabaena* sp. strain PCC 7120, the only barrier to the utilization of fructose in the dark is the inability of the strain to transport the sugar. The *frtR* gene was essential for growth in the dark with fructose; neither the *frtR* mutant of *A. variabilis* (Fig. 2, lane 3) nor a mutant of *Anabaena* sp. strain PCC 7120 containing only *frtABC*, without *frtR*, was able to grow in the dark in the presence of fructose. These results suggested that FrtR is essential for expression of *frtABC* and

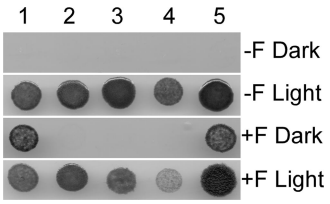

FIG. 2. Growth of strains with or without fructose transport genes. Cells of *A. variabilis* strain FD or *Anabaena* sp. strain PCC 7120 with or without *frtRABC* genes were grown on BG-11 agar medium with or without 5 mM fructose (F) for 4 days in the light or 7 days in the dark. Lane 1, *Anabaena* sp. strain PCC 7120 BP291, containing the *frtRABC* genes; lane 2, *Anabaena* sp. strain PCC 7120 BP292, containing the *frtABC* genes; lane 3, *A. variabilis* BP301 (*frtR* mutant); lane 4, *Anabaena* sp. strain PCC 7120; lane 5, *A. variabilis* strain FD.

might be an activator; however, this was not consistent with its similarity to the LacI repressor and to HrmR, which is also a repressor, so we explored this further.

Transcription of *frtA* and *frtR*. If the *frtRABC* genes encode a fructose transport system, then they would likely be regulated by fructose. Analysis of transcripts of *frtA* and *frtR* in cells grown with or without fructose indicated that in both *A. variabilis* and *Anabaena* sp. strain PCC 7120 with *frtRABC*, transcription of *frtA* and *frtR* was induced by fructose, although low levels of transcript were detected for both genes in the absence of fructose. *frtA* was more strongly induced by fructose than *frtR* was (see Fig. 4). If FrtR were an activator, then transcription of *frtABC* would depend on FrtA. However, it is a repressor, since in both the *frtR* mutant of *A. variabilis* and in *Anabaena* sp. strain PCC 7120 with *frtABC* but not *frtR*, expression of *frtA* was constitutive (Fig. 3A, lanes 5 to 8). Further supporting the role of FrtR as a repressor was the finding that in a strain of *A. variabilis* that overexpressed *frtR*, there was no expression of *frtA*, with or without fructose (Fig. 3A, lanes 9 and 10).

Regulation of *hrmR* by fructose. Since the homologue of *frtR* in *N. punctiforme* is *hrmR*, which has been shown to regulate itself and another gene of unknown function, *hrmE* (8), we used RT-PCR to determine whether these genes were also induced by growth with fructose. Both genes were repressed by fructose (Fig. 3B). Since the results were the reverse of those expected, the entire experiment was done a second time, beginning with new cultures of *N. punctiforme* grown with or without fructose, but the results were the same. Thus, despite the similarity in the encoded proteins, *frtR* and *hrmE* respond differently to fructose.

Expression of *frtA-lacZ* and *frtR-lacZ* fusions. The *frtA* and *frtR* promoters were fused to *lacZ* to measure changes in expression of these genes in response to fructose in the presence and absence of FrtR. Two fusions were created for each gene. In the first type, a promoterless *lacZ* gene was inserted into *frtA* and *frtR*, thus providing not only a normal promoter but also a normal context for the promoter in the chromosome. The second type of fusion placed a 400-bp *frtR* or 500-bp *frtA* promoter fragment in front of *lacZ*, and then the entire construct was integrated into the chromosome. For the first type of fusion, expression of *frtA* (strain BP352), as measured by β-galactosidase activity, increased about 30-fold with fructose, while expression of *frtR* (strain BP353) increased about 7-fold with fructose (Fig. 4A). For the second type of fusion, expression of *frtA* (strain JU338) increased about fourfold with fructose, while expression of *frtR* (strain JU336) increased about fivefold (Fig. 4A). Even though the promoter fragments used were large and should have had all the necessary *cis*-acting elements, expression of *frtA* and *ftrR* in the BP352 and BP353 strains, in which the fusions were in the normal chromosomal locations, was more stringently controlled than that in strains JU338 and JU336, which had the promoter-*lacZ* fusion integrated at a different site by single-crossover recombination. This suggests that control of expression of *frtA* and *frtR* may depend on additional *cis*-acting sites that are not within the 400- to 500-bp promoter fragments used in the second type of fusion. Consistent with the results from RT-PCR (Fig. 3), in the presence of fructose the expression of *frtA* was higher than the expression of *frtR*, and the expression of *frtA* was more strongly induced by fructose than the expression of *frtR*.

To determine the effect of FrtR on expression, the promoter fragment fusions were also constructed in a *frtR* mutant of *A. variabilis* (BP301) and in *Anabaena* sp. strain PCC 7120, which naturally lacks *frtR*. In the absence of FrtR, *frtA* expression, as

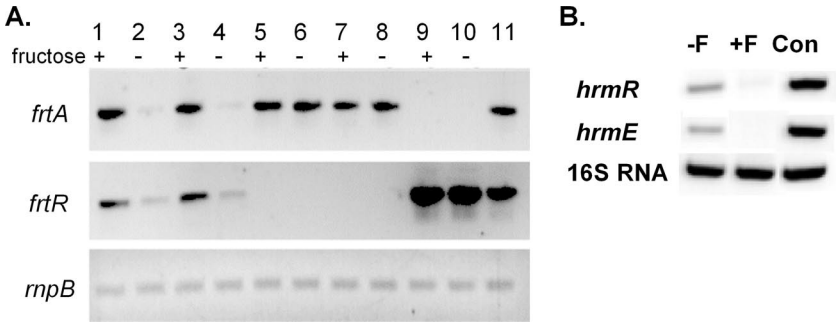

FIG. 3. Transcription of *frtA*, *frtR*, *hrmR*, and *hrmE*. (A) Transcription of *frtA* and *frtR* was determined by RT-PCR, using RNAs extracted from *A. variabilis* strains grown with or without 5 mM fructose for 24 h. Lanes 1 and 2, wild-type *A. variabilis*; lanes 3 and 4, BP291 (*Anabaena* sp. strain PCC 7120 with *frtRABC*); lanes 5 and 6, BP292 (*Anabaena* sp. strain PCC 7120 with only *frtABC*); lanes 7 and 8, BP301 (*A. variabilis frtR* mutant); lanes 9 and 10, BP356 (*A. variabilis* strain overexpressing *frtR*); lane 11, positive control (FD DNA). Transcription of *rnpB* was the control for equal amounts of RNA in each reaction. (B) Transcription of *hrmR* and *hrmE* was determined by RT-PCR, using RNAs extracted from *N. punctiforme* grown in AA/8 without (−F) or with (+F) 5 mM fructose for 24 h. Con, positive control using chromosomal DNA from *N. punctiforme.* 16S rRNA was the control for equal amounts of RNA in each reaction.

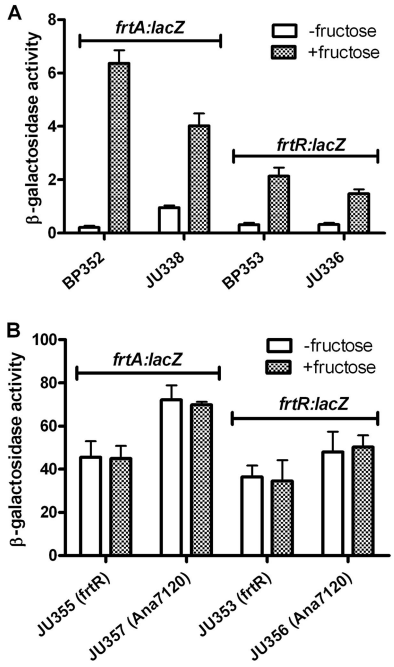

FIG. 4. Expression of *frtA-lacZ* and *frtR-lacZ* fusions. (A) Relative rates of β-galactosidase activity were measured in strains BP352 (*lacZ* inserted within the *frtA* gene), JU338 (containing a 500-bp *frtA* promoter fragment fused to *lacZ*), BP353 (*lacZ* inserted within the *frtR* gene), and JU336 (containing a 400-bp *frtR* promoter fragment fused to *lacZ*), grown in AA/8 with or without 5 mM fructose. (B) Relative rates of β-galactosidase activity were measured in strains JU355 (containing a 500-bp *frtA* promoter fragment fused to *lacZ* in strain BP301, the *frtR* mutant), JU357 (containing a 500-bp *frtA* promoter fragment fused to *lacZ* in *Anabaena* sp. strain PCC 7120), JU353 (containing a 400-bp *frtR* promoter fragment fused to *lacZ* in strain BP301, the *frtR* mutant), and JU356 (containing a 400-bp *frtR* promoter fragment fused to *lacZ* in *Anabaena* sp. strain PCC 7120), grown with or without 5 mM fructose.

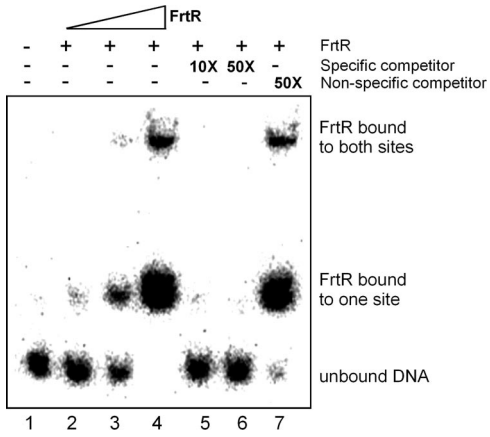

FIG. 5. Binding of FrtR to the promoter region of *frtA*. A ^{32}P-labeled 131-bp DNA fragment upstream of *frtA* was incubated with or without recombinant FrtR protein. Samples in lanes 2 and 3 contained 100 ng and 300 ng of FrtR protein extract, respectively. Samples in lanes 4 to 7 contained 700 ng of FrtR protein extract.

measured by β-galactosidase activity, was about 10-fold higher than that in the wild-type strain (compare strains JU355 and JU338) (note the difference in scale of the *y* axes in Fig. 4A and B) and was unaffected by growth with fructose (Fig. 4B). In the absence of FrtR, *frtR* expression, as measured by β-galactosidase activity, was about 25-fold higher than that in strains with FrtR (compare strains JU353 and JU336) and was also unaffected by growth with fructose (Fig. 4B). This indicates that in the presence of fructose, FrtR represses itself more strongly than it represses *frtA*. For both *frtA* and *frtR*, constitutive expression in the absence of FrtR was about 1.5-fold higher in *Anabaena* sp. strain PCC 7120 than in the *frtR* mutant of *A. variabilis* (BP301) (Fig. 4B). The constitutive expression of *frtA* and *frtR* in the absence of FrtR provides further evidence that FrtR is a repressor.

In strains with FrtR, expression of *frtA* and *frtR* was much more strongly repressed, even in the presence of fructose, than that in strains lacking FrtR, indicating that there was a repression of *frtA* and *frtR* by FrtR under all growth conditions tested. In the strains with FrtR in which *frtA* and *frtR* were expressed with fructose, *frtA* was more strongly expressed than *frtR*. However, in the absence of FrtR, the difference in expression between *frtA* and *frtR* was much smaller, suggesting that the lower level of *frtR* expression in the strains with FrtR was the result of stronger repression of *frtR* than of *frtA* in the presence of fructose and not the result of a much stronger promoter for *frtA*. Together, these results indicated that FrtR repressed expression of both *frtA* and *frtR* in the presence or absence of fructose, but the repression was much weaker in the presence of fructose. Furthermore, FrtR repressed *frtR* more than it repressed *frtA* in cells grown with fructose.

Binding of FrtR to the *frtR-frtA* promoter region. Recombinant FrtR was purified from *E. coli* as inclusion bodies, and the protein was renatured. The protein bound to two sites on a DNA fragment that included the intergenic region between *frtR* and *frtA* (Fig. 5). This region includes the two HrmR-like binding sites shown in Fig. 1. The binding was competed using the same cold DNA fragment but was not competed using an unrelated DNA fragment from the *rnpB* gene. The addition of fructose to the binding reaction mix had no effect on the mobility shift. This may be due to binding of FrtR to this region even in the presence of fructose. This is evident from the repression of *frtA* and *frtR* by FrtR even in the presence of fructose, as shown by the much higher levels of expression of *frtA-lacZ* and *frtR-lacZ* in an *frtR* mutant than in the wild-type strain in the presence of fructose (Fig. 4A and B).

Growth of strains with fructose. As shown in Fig. 2, neither the *frtR* mutant of *A. variabilis* nor the mutant of *Anabaena* sp. strain PCC 7120 with *frtABC* but without *frtR* grew in the dark with fructose, suggesting that FrtR might be an activator. However, the transcription data shown in Fig. 3 and 4 demonstrate that FrtR is a repressor. Furthermore, FrtR repressed *frtABC* even in the presence of fructose, suggesting that overexpression of these genes may be detrimental to cells. We grew the wild-type strain of *A. variabilis* and the *frtR* mutant with various concentrations of fructose in the light. The wild-type strain grew much better with fructose than without it, whereas the *frtR* mutant did not (Fig. 6A). For the wild-type strain, increasing concentrations of fructose, from 1 to 50 mM, supported increased growth rates, but 200 mM fructose decreased the growth rate to about the same rate as that with 1 mM fructose

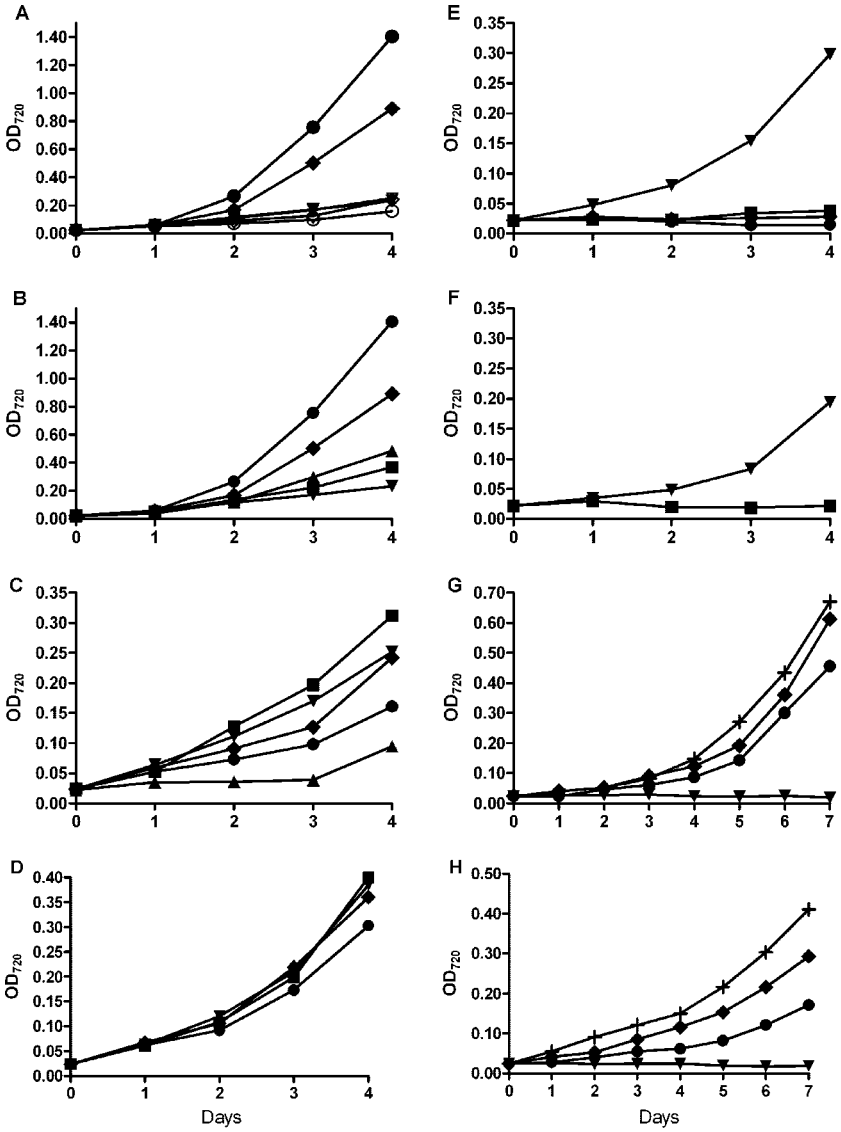

FIG. 6. Growth of strains with fructose. The strains indicated for each panel were grown in AA/8 without fructose and then diluted in medium containing the concentrations of fructose indicated by the symbols on day 0. (A) Strains FD (*A. variabilis* wild type) (solid symbols) and BP301 (*frtR* mutant) (open symbols). (B) Wild-type strain FD. (C) *A. variabilis* BP301 (*frtR* mutant). (D) *Anabaena* sp. strain PCC 7120 with the *frtRABC* genes (strain BP291). (E) *Anabaena* sp. strain PCC 7120 with the *frtABC* genes (lacking *frtR*) (strain BP292). (F) Strain JU377, a strain of *A. variabilis* in which the *frtABC* genes are constitutively expressed from the strong *psbA* promoter. (G) Wild-type strain FD grown in the dark. (H) *Anabaena* sp. strain PCC 7120 with the *frtRABC* genes (strain BP291) grown in the dark. Fructose concentrations were as follows: ▼, 0 mM; ■, 1 mM; ◆, 5 mM; +, 10 mM; ●, 50 mM; and ▲, 200 mM.

(Fig. 6B). For the *frtR* mutant, increasing concentrations of fructose did not increase the growth rate (Fig. 6C; note the difference in scale of the *y* axis). In fact, concentrations of fructose of >1 mM decreased growth, and 200 mM fructose almost completely inhibited growth. Strain JU377, in which the *frtABC* genes were overexpressed from the strong *psbA* promoter in a wild-type $frtR^+$ background, grew poorly even in the absence of fructose and died after exposure to even 1 mM fructose (Fig. 6F). Thus, it appears that overexpression of *frtABC* in the *frtR* mutant leads to transport of fructose at levels that are toxic to the cells. In the light, the *frtR* mutant was apparently able to overcome this toxicity when the concentration of fructose was low, but in the dark, when the cells were dependent on fructose as a carbon source, even 5 mM fructose was toxic (Fig. 2, lane 3), suggesting that metabolism of fructose was involved in the toxic effect of high intracellular concentrations of fructose. Even without fructose, the poor growth of strain JU377, which overexpressed *frtABC*, suggested that excessive amounts of the transporter itself are deleterious to cell growth.

Anabaena sp. strain PCC 7120 mutant BP291 (with *frtRABC*) grew in the dark with fructose (Fig. 6H); however, it grew more slowly than *A. variabilis* FD (Fig. 6G). However, unlike the growth of *A. variabilis* FD in the light, the growth of BP291 in the light was not enhanced by fructose (Fig. 6D), and 50 mM fructose, which greatly stimulated growth of FD (Fig. 6A),

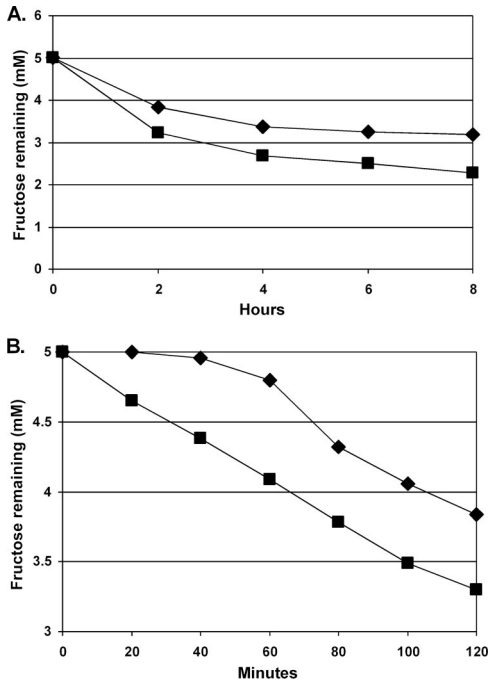

FIG. 7. Fructose transport in wild-type (◆) and BP301 (*frtR* mutant) (■) strains. Cells were grown in AA/8 to an OD_{720} of 0.250, fructose was added at time zero, and transport was measured as the disappearance of fructose from the medium over time (hours [A] or minutes [B]). Fructose was measured using a fructose assay kit (Sigma-Aldrich).

slightly inhibited growth of BP291 (Fig. 6D). *Anabaena* sp. strain PCC 7120 with *frtRABC* grew in the light with 5 mM fructose and DCMU [3-(3,4-dichlorophenyl)-1,1-dimethylurea], but growth was slow, particularly under nitrogen-fixing conditions, and increasing the fructose concentration above 5 mM did not help the growth (data not shown). Thus, although *Anabaena* sp. strain PCC 7120 mutant BP291 was able to transport and use fructose in the dark, in the light the strain did not use the fructose and grew photoautotrophically. *Anabaena* sp. strain PCC 7120 mutant BP292 (with *frtABC* but lacking *frtR*) did not grow in the dark with 5 mM fructose (Fig. 2, lane 2) and died in the light with as little as 1 mM fructose (Fig. 6E), showing even greater sensitivity to fructose than that of BP301, the *A. variabilis* strain lacking *frtR* (Fig. 6C). Unregulated expression of *frtABC* led to a complete inhibition of growth, suggesting that fructose is toxic to this strain in the light.

Uptake of fructose in the *frtR* mutant. Overexpression of *frtABC* in BP301 (*frtR* mutant) might be expected to affect the rate of fructose uptake. We measured fructose uptake by the disappearance of fructose from the medium in the wild-type strain and in BP301 (*frtR* mutant). In the first 2 hours after the addition of fructose, the rate of uptake was greater in the BP301 mutant, but the initial high rate slowed for both strains about 2 hours after the addition of fructose (Fig. 7A). BP301 continued to take up fructose slightly faster than the wild-type strain for even up to 8 h. In the absence of the repressor, FrtR, high levels of expression of *frtABC* allowed the uptake of fructose to begin immediately upon its addition, while the wild-type strain showed a lag in uptake of about 40 min (Fig. 7B).

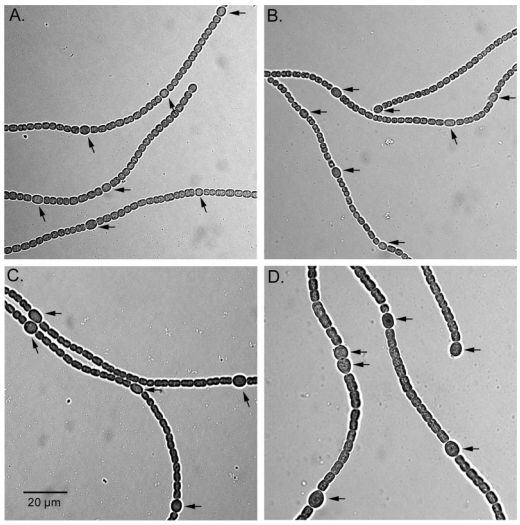

FIG. 8. Light micrographs of filaments of *Anabaena* sp. strain PCC 7120 with the *frtABC* genes (strain BP291) grown without (A) or with (B) 5 mM fructose and of filaments of *A. variabilis* FD grown without (C) or with (D) 5 mM fructose. The size scale is the same for all panels. Heterocysts are indicated by arrows.

This suggests that while *frtABC* is transcribed at low levels even in the absence of fructose, fructose is required for synthesis of sufficient FrtABC to efficiently transport fructose. In the absence of the repressor (strain BP301), sufficient FrtABC is made to allow the immediate transport of fructose.

Filaments and heterocysts of fructose-grown cells. Fructose has been reported to stimulate both the number and size of heterocysts of *A. variabilis*, consistent with the strain's ability to use fructose as a carbon source in the light (14, 21). Forty-eight hours after nitrogen stepdown, *A. variabilis* strain FD grown with 50 mM fructose had 11.9% heterocysts, compared to only 7.6% in filaments grown without fructose. In contrast, under the same growth conditions, BP291, the *Anabaena* sp. strain PCC 7120 mutant with *frtRABC*, produced 7.1% heterocysts with fructose and 6.6% heterocysts without fructose. Also, in contrast to the increased size of cells in filaments of *A. variabilis* FD grown with 5 mM fructose, filaments of BP291 showed no increase in size with 5 mM fructose (Fig. 8), and in contrast to the case for *A. variabilis*, they showed no increase in the rate of nitrogen fixation when grown with fructose (data not shown). These results support the growth data indicating that *Anabaena* sp. strain PCC 7120 mutant BP291 grown in the light does not use fructose as a carbon source.

DISCUSSION

The normal Frt$^-$ phenotype of *Anabaena* sp. strain PCC 7120 was complemented by the addition of the *A. variabilis* fructose transport genes, *frtRABC*. Therefore, the lack of a fructose transport system in *Anabaena* sp. strain PCC 7120 was the only barrier to heterotrophic growth on fructose in the dark. However, both *Anabaena* sp. strain PCC 7120 BP292, which constitutively expressed *frtABC* due to a lack of the repressor FrtR, and *A. variabilis* strain BP301, a *frtR* mutant, were unable to grow on fructose in the dark even though they expressed *frtABC* and transported fructose. These results indi-

cate that the repressor, FrtR, is required for heterotrophic growth on fructose.

The evidence presented here suggests that the lack of FrtR caused excessive fructose uptake via the high-level constitutive expression of the transport genes and that this led to toxicity. Fructose is toxic in two cyanobacterial strains that have glucose transporters, i.e., *Synechocystis* sp. strain PCC 6714 and *Synechocystis* sp. strain PCC 6803, and expression of *glcP* from *Synechocystis* sp. strain PCC 6803 in the obligate photoautotroph *Synechococcus* sp. strain PCC 7942 results in glucose sensitivity (17, 51). Our results support this explanation by the requirement for the repressor, FrtR. With the inducer, fructose, expression of *frtABC* in a wild-type *frtR*$^+$ background increased about 30-fold. However, in an *frtR* mutant background, expression was 400-fold higher than that in the wild-type strain. This indicates that in the wild-type strain, under inducing conditions with fructose, *frtABC* was still highly repressed by FrtR. Furthermore, *A. variabilis* strain JU377, which overexpressed *frtABC* in a wild-type *frtR*$^+$ background, was extremely sensitive to fructose. This indicated that overexpression of the fructose transport genes in the presence of FrtR was sufficient to produce a fructose-sensitive phenotype. Finally, fructose toxicity resulted in impaired phototrophic growth as a function of fructose concentration in strains lacking a functional repressor but not in strains in which fructose uptake was regulated. Together, these findings indicate that fructose uptake must be tightly regulated in order to prevent toxic levels of fructose uptake. The fact that simply overexpressing the fructose transport proteins, even in the absence of fructose, greatly decreased growth suggests that at least part of the problem was the excessive amount of transporters made. However, the addition of fructose to the strains overexpressing the transport proteins resulted in much greater toxicity, which was proportional to the amount of fructose added, indicating that fructose or a metabolic product of fructose was toxic when present in large concentrations in the cell.

For *Anabaena* sp. strain PCC 7120, which normally cannot take up fructose, the addition of the *frtRABC* genes of *A. variabilis* allowed this strain to use fructose, but only in the dark. In contrast to the case for *A. variabilis*, fructose did not stimulate growth, increase heterocyst frequency, increase cell size, or stimulate nitrogen fixation in *Anabaena* sp. strain PCC 7120 with the *frtRABC* genes. Excessive entry of fructose into the *Anabaena* sp. strain PCC 7120 mutant with the *frtABC* genes but lacking *frtR* resulted in death. Expression of *glcP* in *Synechococcus* sp. strain PCC 7942 resulted in a glucose sensitivity in that strain (51). Analysis of the carbon catabolic pathways of the two *Anabaena* strains by use of KEGG, which is based on genome sequences, revealed no obvious differences (18); hence, a more detailed metabolomic analysis of fructose metabolism in the two strains may be necessary to understand the mechanism of fructose toxicity.

Although we demonstrated that the expression of *frtABC* is induced by fructose, we were unable to show that fructose directly affected the binding activity of FrtR to DNA in vitro. Our data suggest that FrtR remained bound to its target sequence irrespective of the presence or absence of fructose. Thus, either fructose has a low affinity for FrtR or binding of fructose to FrtR has little effect on the affinity of FrtR for DNA. Either condition would make it difficult to detect an FrtR-fructose interaction by our methods. It is also possible that the binding activity of FrtR is modulated by a secondary metabolite of fructose.

In *N. punctiforme*, specialized motile filaments called hormogonia are important in symbiosis (25, 26, 27). The *hrm* locus plays an important role in repressing further hormogonium differentiation after a functional symbiosis has been established between *N. punctiforme* and its host (8). The homologue of *frtR* in *N. punctiforme*, *hrmR*, has been shown to regulate itself and another gene of unknown function, *hrmE*. The activity of *hrmR* is modulated by an unidentified hormogonium repressing factor that is present in plant extracts (8). Immediately downstream of *hrmE* are the homologs of *frtABC*, namely, *hrmB1*, *hrmB2*, *hrmT*, and *hrmP* (25). It appears likely that *hrmB1-hrmB2-hrmTP*, like *frtABC*, is responsible for fructose transport in *N. punctiforme*. These genes are induced by the hormogonium repressing factor and are thus thought to be part of the *hrm* locus (25). The close similarity between *frtABC* and *hrmB1-hrmB2-hrmTP* (71 to 85% identity) and the proximity of *hrmB1-hrmB2-hrmTP* to other genes known to be involved in hormogonium formation suggest that fructose or a metabolite thereof might also be involved in regulating hormogonium differentiation. The fructose could be converted to a signaling metabolite that would then provide the signal to repress hormogonium differentiation and establish a lasting relationship with the plant. HrmR is the regulator of *hrmR* and *hrmE* (8), and both of these genes are negatively regulated by fructose (Fig. 3). It seems unlikely that HrmR directly regulates *hrmB1-hrmB2-hrmTP* because there is not a putative HrmR binding site upstream of *hrmB1-hrmB2-hrmTP.* A conserved 15-bp regulatory sequence upstream of *hrmB1-hrmB2-hrmTP* that is not bound by HrmR (26) and is absent in the intergenic region between *frtR* and *frtA* in *A. variabilis* might be the regulatory site for another regulatory protein controlling expression of *hrmB1-hrmB2-hrmTP* (26).

These data and other reports of sugar toxicity in other cyanobacteria (17, 51), combined with the apparent inability of the photoautotrophic strain *Anabaena* sp. strain PCC 7120 to use fructose when growing in the light, suggest that strains that are naturally capable of sugar transport and utilization have evolved mechanisms that allow them both to use sugars efficiently and to overcome sugar toxicity. These are of course likely to be metabolically linked processes. *N. punctiforme* and the free-living organism *Anabaena azollae*, which is genetically and morphologically very similar to *A. variabilis*, depend on sugar supplies from a plant when they are in a symbiotic association (26, 39). In the free-living state, these cyanobacteria retain the ability to use sugars and even show, in modified form, some of the characteristics of symbiosis (36, 37), including larger cells, more heterocysts, increased respiration, and increased nitrogen fixation, suggesting that some of the important changes associated with symbiosis are controlled by sugar metabolism in the cyanobacterium rather than by plant-derived factors. *A. variabilis* and *Anabaena* sp. strain PCC 7120 are very similar genetically, sharing about 95% nucleotide identity between homologous genes. They share about 5,000 homologous genes, but *A. variabilis* has about 650 genes that are not present in *Anabaena* sp. strain PCC 7120, and of these, about 240 have homologs in *N. punctiforme* (data calculated from information available at the Joint Genome Institute

(JGI) integrated microbial genome website) (24). Among these 240 genes, which include the *frtRABC* genes and their homologs in *N. punctiforme*, are likely to be other genes that will provide answers to questions concerning how sugars are used by and may modify important physiological characteristics of true heterotrophic strains. Further system-level analysis, comparing transcriptomes, proteomes, and metabolomes for photoautotrophic versus heterotrophic strain growth with and without sugars, should help to provide answers to these interesting questions.

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