

Nitrogen-Regulated Group 2 Sigma Factor from *Synechocystis* sp. Strain PCC 6803 Involved in Survival under Nitrogen Stress

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The expression of *sll1689*, an open reading frame from the cyanobacterium *Synechocystis* sp. strain PCC 6803 putatively encoding a member of the σ^{70} family of sigma factors, appears to be regulated by the nitrogen control transcription factor NtcA. Disruption of *sll1689* had no noticeable effect on exponential growth, identifying its product as a member of the group 2, nonessential class of σ^{70} -like sigma factors; however, this disruption decreased the viability of the cells after long periods of nitrogen starvation. We have named this gene *rpoD2-V*. The expression of *glnN*, encoding a type III glutamine synthetase, was impaired in strains bearing an inactivated copy of the *rpoD2-V* gene.

Eubacterial RNA polymerase consists of a core enzyme and a sigma factor that directs the complex to a specific class of promoter sequences. Most eubacterial genomes encode a number of different sigma factors that recognize different subsets or groups of promoters. This variety of sigma factors allows basal expression of certain genes as well as regulated expression of other genes in response to environmental or developmental signals (44). Two families of sigma factors have been defined on the basis of sequence similarity, the σ^{70} and the σ^{54} families, named after the primary sigma factor and the factor involved in nitrogen regulation in *Escherichia coli*, respectively. All eubacteria contain one or more sigma factors belonging to the σ^{70} family (44). Sigma factors that are responsible for the transcription of most genes in exponentially growing cells and that are essential for survival constitute group 1 or primary sigma factors; group 2 includes “primary-like” sigma factors that are nonessential for exponential cell growth (secondary sigma factors); and group 3 includes the so-called alternative sigma factors (30). A single group 2 sigma factor, stationary-phase σ^{38} , has been found in *E. coli*, but multiple group 2 sigma factors are present in high-GC-content gram-positive bacteria (e.g., *Streptomyces* spp.), cyanobacteria, and *Chloroflexus aurantiacus* (26, 44).

Cyanobacteria are a widely distributed group of phototrophic bacteria that carry out oxygenic photosynthesis and are considered the precursors of chloroplasts. In cyanobacteria and chloroplasts, the core of RNA polymerase consists of five subunits ($\alpha_2\beta\beta'\gamma$ in cyanobacteria and $\alpha_2\beta\beta'\beta''$ in chloroplasts) (28, 39) and thus is slightly different from that in other eubacteria ($\alpha_2\beta\beta'$ tetramer) (9). The β' subunit of eubacterial RNA polymerase is considered to be split into two subunits in cyanobacteria and chloroplasts, $\beta'\gamma$ and $\beta'\beta''$, respectively (5). Multiple sigma factors of the σ^{70} family (groups 1 and 2) have been found in cyanobacteria of the genera *Anabaena* (7, 8), *Nostoc* (11), *Synechococcus* (12, 13, 27, 40, 41), *Microcystis* (2,

3) and *Synechocystis* (29). The chromosome of *Synechocystis* sp. strain PCC 6803 contains five open reading frames (ORFs) that would correspond to sequences encoding primary or primary-like sigma factors (groups 1 and 2) and three ORFs that would correspond to sequences encoding alternative sigma factors (29). The sequences of the genome of *Anabaena* sp. strain PCC 7120 available to date (<http://www.kazusa.or.jp/cyano/anabaena>) contain at least eight ORFs similar to sequences encoding primary or primary-like sigma factors. No sigma factor belonging to the σ^{54} family has yet been identified for cyanobacteria. Phylogenetic analysis of cyanobacterial sigma factor sequences known to date indicates that group 1 sigma factors are tightly clustered (cluster I) and that group 2 sigma factors form a separate, coherent clade (25, 26) which appears to be further divided into four clusters, II to V (25). Each cyanobacterial strain analyzed to date bears members belonging to each group 2 cluster (25).

Cyanobacteria are able to use a number of different nitrogen sources (17). The regulation of the use of these sources is mediated by the nitrogen control, CAP (catabolite activator protein)-family transcription factor NtcA. In the absence of ammonium, NtcA activates the expression of genes required for the assimilation of nitrogen sources alternative to ammonium (42). A consensus sequence needed for NtcA binding to DNA (GTAN₈TAC) has been defined (31). The NtcA-activated promoters bear this sequence, located at about position –40.5, and a –10 sequence of the form TAN₃T (18, 31). The *ntcA* gene is widely distributed in cyanobacteria (21). In all strains analyzed to date, the sequence of the NtcA protein is highly conserved, as is the sequence of the promoters activated by NtcA in those strains. Insertional mutants of *ntcA* have been obtained for *Synechococcus* sp. strain PCC 7942 and *Anabaena* sp. strain PCC 7120. However, attempts to isolate a completely segregated *ntcA* mutant of *Synechocystis* sp. strain PCC 6803 have been unsuccessful (23).

The complete sequence of the chromosome of *Synechocystis* sp. strain PCC 6803 is available (29) (<http://www.kazusa.or.jp/cyano/cyano.html>). In the context of our attempts to identify genes controlled by the global nitrogen regulator NtcA, we have carried out a search for putative NtcA-activated promot-

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ers in the genomic sequence of strain PCC 6803. In this report, the identification of *sll1689*, encoding a protein homologous to σ^{70} -like sigma factors, as a nitrogen-regulated gene as well as the effects of disruption of *sll1689* are described.

Search for putative NtcA-activated promoters. A computer search for sequences of the genome of *Synechocystis* sp. strain PCC 6803 that could correspond to putative NtcA-activated promoters was carried out using overlapping 30-kb segments of the genome sequence and MatInspector software (34). The consensus sequence needed for NtcA binding, GTAN₈TAC, was found in a total of 367 positions, 44 of which were followed, at a distance of 20 to 23 nucleotides, by a sequence matching the consensus for a putative -10 box, TAN₃T. Only in 31 of those cases was the NtcA box located less than 2 kb upstream from an ORF, that is, in a position that might be considered compatible with a role in transcription activation. Although some of the identified sequences were located upstream from ORFs encoding hypothetical proteins of unknown function, 22 of them were located upstream from either known genes or ORFs whose predicted products were similar to some proteins in the databases. Among those, as expected, we could identify the NtcA-regulated promoter of the *glnA* gene, encoding glutamine synthetase (35), and the region upstream from *nirA*, encoding nitrite reductase, a gene whose expression has been shown to be NtcA dependent in *Synechococcus* sp. strain PCC 7942 (31) and *Anabaena* sp. strain PCC 7120 (20). A sequence, GTAN₈TACN₂₁TAN₃T, matching the structure of NtcA-regulated promoters was found 270 nucleotides upstream from *sll1689*, one of the five ORFs identified in the genome of strain PCC 6803 as *rpoD*-like genes encoding members of the σ^{70} family of sigma factors (29). We refer to this ORF as *rpoD2-V* because its product belongs to cluster V of the phylogenetic tree of cyanobacterial primary or primary-like sigma factors (25).

Regulation of expression of *rpoD2-V*. In order to analyze whether the expression of *rpoD2-V* was in fact under nitrogen control, Northern hybridization analysis was carried out with RNA isolated from *Synechocystis* sp. strain PCC 6803 cells that had been subjected to nitrogen starvation. Standard molecular biology procedures were performed by published methods (4, 37). *Synechocystis* cells were grown in BG11₀ medium (medium BG11 [36] without NaNO₃) supplemented with 0.84 g of NaHCO₃ liter⁻¹ (BG11₀C medium) and 15 mM NH₄Cl and bubbled with a mixture of CO₂ (1% [vol/vol]) and air. Twice as much *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)-NaOH buffer (pH 7.5) as NH₄Cl was added to all NH₄Cl-containing media. For RNA isolation, cells growing exponentially in NH₄Cl-containing BG11₀C medium were harvested at room temperature and either used directly or washed with and resuspended in BG11₀C medium (nitrogen free) and further incubated under culture conditions for various times. RNA was isolated as previously described (22). Northern hybridization was carried out using Hybond N⁺ membranes according to manufacturer recommendations. Northern blot experiments performed using the insert of pCSAM82 (a plasmid containing *sll1689* and flanking sequences; see below) as a probe indicated that the expression of *rpoD2-V* was indeed induced in cells subjected to nitrogen deprivation (Fig. 1). As has also been observed for the *sigC* transcript of *Synechococcus* sp. strain PCC 7002 (13), the transcript hybridizing to the

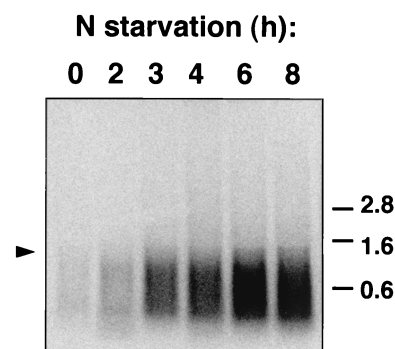


FIG. 1. Northern blot analysis of the expression of *rpoD2-V* in *Synechocystis* sp. strain PCC 6803. RNA was isolated from ammonium-grown cells (lane 0) or from ammonium-grown cells incubated in nitrogen-free medium for 2, 3, 4, 6, or 8 h. Hybridization to a probe for *rpoD2-V* was carried out as described in the text. Samples contained 40 μ g of RNA. Sizes of standards (in kilobases) are indicated on the right. Arrowhead indicates transcripts of about 1.5 kb.

rpoD2-V probe appeared as a smear. Control hybridization experiments with different probes showed that the RNA in the filters was not degraded (data not shown).

Primer extension experiments with ³²P-labeled primers were carried out in order to determine the possible transcription start point(s) (tsp) from which the observed transcription originated. These experiments were performed as previously described (33) using the above-mentioned RNA samples and oligonucleotide RD5 (5'-TTA TTC AAT CGG CCA GAG C-3', complementary to positions -89 to -107 with respect to the putative translational start site of *rpoD2-V*) (Fig. 2). In addition to a constitutive putative tsp located at position -202 with respect to the translational start site of *rpoD2-V*, an inducible transcript which corresponded to a putative tsp located at position -264 was observed. The sequences located upstream from the inducible tsp corresponded to the consensus NtcA-activated promoter sequence (GTAN₈TACN₂₁TAN₃TN₅tsp) identified in the computer search described above. The identification of these two putative tsp was confirmed by using oligonucleotides RD4 (5'-AGA GCA TCA TCC AGA TAG ACC-3', complementary to positions -103 to -123 with respect to the putative translational start site of *rpoD2-V*) and RD6 (5'-TCA GCG AGG CCA TCC AAA GCC-3', complementary to positions $+77$ to $+57$ with respect to the putative translational start site of *rpoD2-V*) (data not shown). The apparent inconsistency between the Northern and primer extension results could be due to different stabilities of the constitutive and inducible transcripts. In any case, the tsp detected at -264 is the one which reproduces the regulatory pattern observed by Northern analysis for the *rpoD2-V* gene.

Band shift assays were carried out with a 402-bp DNA fragment containing the putative inducible promoter of *rpoD2-V*. This fragment was amplified by PCR using oligonucleotides RD1 (5'-ACG CTT GGA ATG GCA ACA GG-3', corresponding to positions -545 to -526 with respect to the putative translational start site of *rpoD2-V*) and RD2 (5'-CCA CTT TCA GCT ATG CGC ACT GCG G-3', complementary to positions -144 to -168 with respect to the putative translational start site of *rpoD2-V*) and plasmid pCSAM82 (see below) as a template. Binding assays with purified NtcA protein

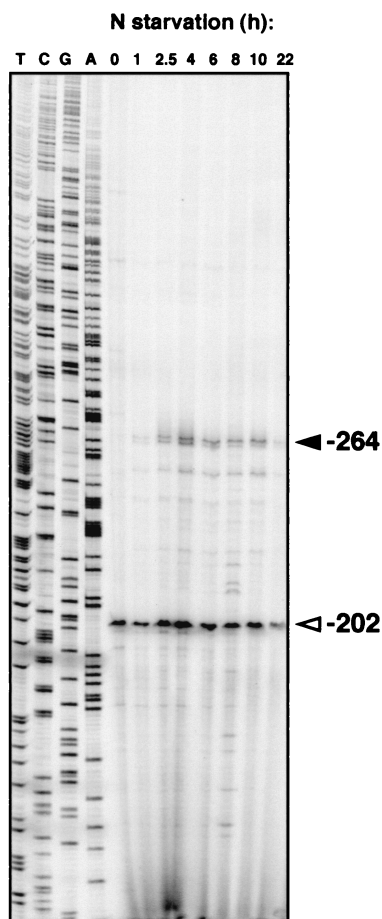


FIG. 2. Primer extension analysis of the expression of the *rpoD2-V* gene in *Synechocystis* sp. strain PCC 6803. Primer extension assays were carried out with RNA isolated from cells grown on ammonium (lane 0) or grown on ammonium and incubated in nitrogen-free medium for 1, 2.5, 4, 6, 8, 10, or 22 h. Assays were carried out using oligonucleotide RD5 (see the text). The sequencing ladders shown were generated with the same oligonucleotide and plasmid pCSAM82. Arrowheads indicate the putative tsp identified at positions -264 and -202 .

were carried out as described previously (33). The results shown in Fig. 3 indicate that NtcA specifically bound to the fragment containing the putative inducible promoter of *rpoD2-V*. The binding could be competed by the addition of the same, unlabeled fragment or a fragment containing the NtcA-regulated promoter of the *glnA* gene from *Anabaena* sp. strain PCC 7120 (19; see the fragment used in reference 33).

The expression of *rpoD2-V* in an *ntcA* background could not be tested, since no completely segregated *ntcA* mutant of strain PCC 6803 is currently available. However, the two pieces of evidence presented here, the location of sequences corresponding to an NtcA-activated promoter sequence in front of the inducible tsp and the specific binding of purified NtcA to those sequences, suggest that the expression of *rpoD2-V* is directly regulated by the global nitrogen regulator NtcA. Thus, induction of the expression of *rpoD2-V* appears to be part of the transcriptional changes that are induced by NtcA and take place in response to nitrogen starvation in *Synechocystis* sp.

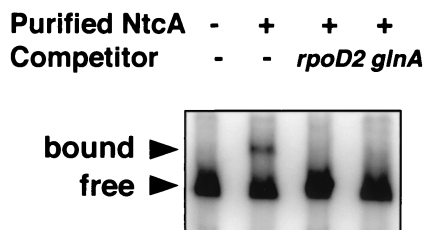


FIG. 3. Band shift assays of a DNA fragment from the *rpoD2-V* promoter with purified histidine-tagged NtcA. Assays were carried out as described in the text using a fragment from the region upstream of *rpoD2-V*. Assay mixtures contained 5 pmol of purified histidine-tagged *Anabaena* NtcA without competitor DNA or with a 25-fold molar excess of the same unlabeled fragment (*rpoD2*) or with an unlabeled fragment from the NtcA-regulated promoter of the *glnA* gene of *Anabaena* sp. strain PCC 7120 (*glnA*).

strain PCC 6803. Interestingly, the expression of *Synechococcus* sp. strain PCC 7002 SigC (a sigma factor also belonging to cluster V) has also been found to increase under nitrogen limitation (13). One of the three possible *tsp* defined for strain PCC 7002 *sigC*, located at position -255 (13), could in fact correspond to an NtcA-regulated promoter, since it exhibits a sequence (GTAN₈AA) that resembles that of the NtcA box, separated by 22 nucleotides from a putative -10 box of the form TAN₃T.

Disruption of *rpoD2-V*. A 2-kb DNA fragment comprising *rpoD2-V* and flanking sequences was amplified by PCR using oligonucleotides RD1 (see above) and RD3 (5'-GAT GCG AGC GAA GAT TTC TG-3', complementary to positions $+342$ to $+323$ with respect to the putative translational stop site of *rpoD2-V*) and total DNA (isolated as described in reference 10) from strain PCC 6803 as a template. The PCR product was cloned in vector pGEM-T (Promega), generating plasmid pCSAM82. This plasmid was digested with *Bam*HI and *Bgl*II (both sites are internal to the *rpoD2-V* gene), and the 1.3-kb Km^r gene cassette C.K1 excised from pRL161 (S.A1/L.HEH1/C.K1; nomenclature as in reference 16) with *Bam*HI was inserted between the *Bam*HI and *Bgl*II sites of *rpoD2-V*, rendering plasmid pCSAM89 (a or b, depending on the orientation of C.K1 with respect to the *rpoD2-V* gene).

After transformation of *Synechocystis* sp. strain PCC 6803 with plasmids pCSAM89a and pCSAM89b (14), Km^r transformants were selected and maintained on solid BG11₀C medium supplemented with 4 mM NH₄Cl and 50 μg of kanamycin ml⁻¹. To test whether the resulting mutant strains were homozygous for the mutant chromosomes, PCR amplification with primers RD1 and RD3 and genomic DNA from the mutants as templates and Southern hybridization analysis were carried out. Clones homozygous for the mutated chromosomes were chosen and named CSAM4 (*rpoD2-V*::C.K1, which carries the gene cassette in the same orientation as *rpoD2-V*) and CSAM5 (*rpoD2-V*::C.K1, which carries the gene cassette in the orientation opposite that in *rpoD2-V*).

No noticeable effect on cell growth was detected in the mutant strains with respect to the wild-type strain in nitrate- or ammonium-containing media. The growth rate constant was determined for single cultures of the wild-type and mutant strains in 4 mM NH₄Cl-containing medium, and values of 0.62 day⁻¹ (wild type), 0.57 day⁻¹ (CSAM4), and 0.64 day⁻¹

(CSAM5) were obtained. Thus, *rpoD2-V* is not essential for cell viability under nutrient-replete conditions and can be classified as a group 2 sigma factor gene.

Since the expression of *rpoD2-V* increased under nitrogen-limiting conditions, a number of different phenotypes related to the assimilation of nitrogen sources in the mutant strains were tested. The induction of nitrate reductase and glutamine synthetase activities (measured as described previously [19]) took place in a similar fashion in the wild type and in the mutants (data not shown). Also, the decrease in phycobiliproteins that takes place in response to nitrogen deprivation (1, 15) was not altered in the mutants. The effect of *rpoD2-V* inactivation on the induction of nitrogen-regulated genes was also tested by Northern hybridization (Fig. 4). The genes whose expression was analyzed were *amt1* (ammonium/methylammonium permease) (32), *glnB* (P_{II} signaling protein) (22), and *glnN* (type III glutamine synthetase) (35). The probes used for *amt1*, *glnB*, and *glnN* were those indicated in the corresponding references. Quantification of the signals in the Northern blots shown in Fig. 4, normalized to the signal for the *rnpB* probe (43), used as a loading and transfer control, indicated that the expression of *amt1* or *glnB* was not significantly altered in the *rpoD2-V* mutants. Activation of the expression of *amt1* or *glnB* upon nitrogen deprivation took place earlier than the expression of the *rpoD2-V* gene itself (Fig. 1 and 4), making the participation of RpoD2-V in their expression unlikely. However, activation of the expression of *glnN*, which under our experimental conditions took place later than induction of the expression of *amt1* and *glnB* in response to nitrogen starvation, was impaired in the mutants; a decrease of about 30% was observed after 4 h of nitrogen starvation (Fig. 4). Interestingly, *glnN* recently has been shown to influence the recovery of *Synechococcus* sp. strain PCC 7942 cells from long periods of nitrogen starvation (38).

The survival of wild-type and mutant cells after different periods of nitrogen starvation was studied. For determination of survival after nitrogen deficiency, cells growing exponentially in liquid BG11₀C medium supplemented with 15 mM NH₄Cl were harvested at room temperature, resuspended in BG11₀C medium (nitrogen free) at approximately 10 μg of chlorophyll ml⁻¹, and further incubated under culture conditions. For the determination of CFU, appropriate dilutions of liquid cultures were plated as soft-agar overlays on solid BG11₀C medium supplemented with 4 mM NH₄Cl. The viability of mutant cells after long periods of nitrogen starvation was severely altered with respect to that of wild-type cells. The survival of wild-type cells subjected to 25 days of nitrogen starvation was about 0.15, a value similar to that described for *Synechococcus* sp. strain PCC 7942 (24); however, survival dropped to 7.3×10^{-4} and 9.1×10^{-4} for mutant strains CSAM4 and CSAM5, respectively. Although differences were more dramatic after this long period of starvation, some differences in survival between the wild-type and mutant strains could be observed as soon as after 2 days of nitrogen deficiency.

In order to test whether the lower survival of the mutant strains was specific for nitrogen stress, we tested the survival of the cells after starvation for phosphorus, carbon, or sulfur. For this experiment, cells grown in 15 mM NH₄Cl-containing medium were washed and resuspended in the corresponding nu-

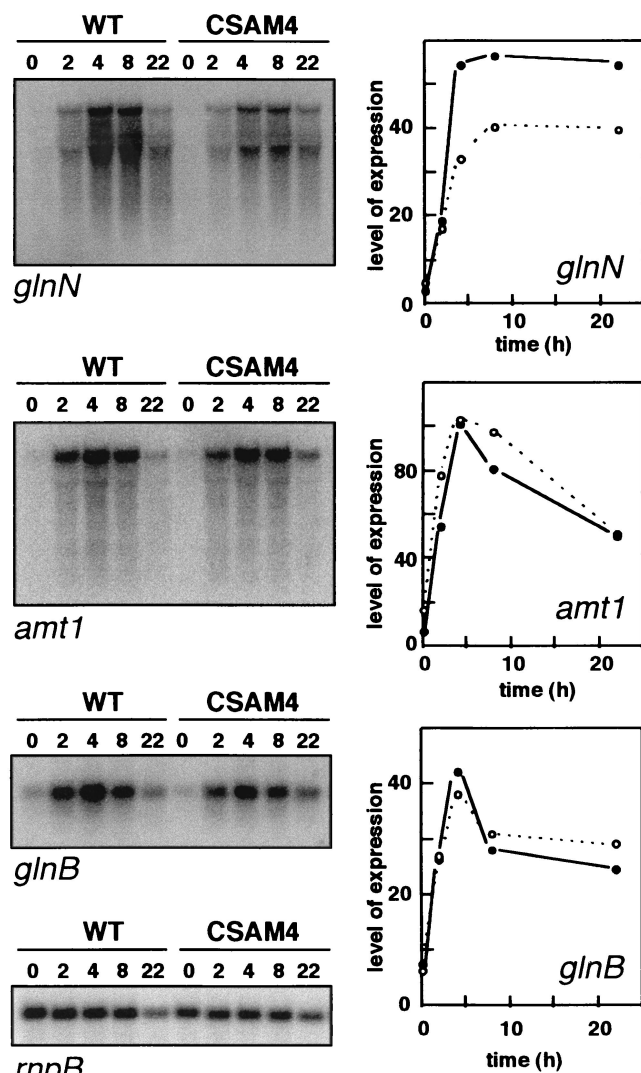


FIG. 4. Effect of the disruption of *rpoD2-V* on the expression of *amt1*, *glnB*, and *glnN*. RNA was isolated from ammonium-grown cells (lane 0) or from ammonium-grown cells incubated in nitrogen-free medium for 2, 4, 8, or 22 h. The filter was sequentially hybridized with probes for *glnN*, *glnB*, *amt1*, and *rnpB*. Samples contained 30 μg of RNA. Images of radioactive gels were obtained and quantified using a Cyclone storage phosphor system (Packard). The level of expression of each gene (defined as the ratio of the signal in each lane to the signal in the corresponding *rnpB* hybridization lane and expressed in arbitrary units) in the wild-type (WT) strain (solid circles) and in mutant strain CSAM4 (open circles) is depicted in the graphs on the right. Similar results were obtained with mutant strain CSAM5 (data not shown).

trient-deficient medium (10 mM NH₄Cl-containing BG11₀C medium lacking phosphorus in the case of phosphorus starvation, 10 mM NH₄Cl-containing BG11₀C medium lacking sulfur in the case of sulfur starvation, and 4 mM NH₄Cl-containing BG11₀ medium in the case of carbon starvation). All cultures except for the carbon-starved ones were bubbled with a mixture of CO₂ (1% [vol/vol]) and air. N₂ was bubbled through the carbon-starved cultures. The survival of wild-type PCC 6803 cells under phosphorus and carbon stress was similar to that

under nitrogen stress, i.e., about 0.25 after 10 days of starvation. However, whereas the survival of *rpoD2-V* mutant cells after nitrogen starvation was decreased with respect to that of wild-type cells (0.01 after 10 days of nitrogen starvation), the mutant cells behaved like the wild-type cells under phosphorus and carbon stress. Survival under sulfur stress was much lower for both the wild type and the mutants, about 0.1 after 2 days of starvation. These experiments indicate that the sigma factor encoded by *rpoD2-V* is specifically involved in survival under severe nitrogen stress.

Concluding remarks. Functions for cyanobacterial nonessential sigma factors have been assigned or suggested only in a few cases. The expression of *sigB* and *sigC* of *Anabaena* sp. strain PCC 7120 was found to be increased in the absence of nitrogen (8). However, single- and double-mutant strains bearing inactivated *sigB* and/or *sigC* genes were able to grow fixing nitrogen (8). The expression of *sigB* and *sigC* of *Synechococcus* sp. strain PCC 7002 is also induced under nutrient-limiting conditions (13), and *sigE* of this strain seems to be implicated in transcription in the stationary phase of growth (27). Mutations in *rpoD2* of *Synechococcus* sp. strain PCC 7942 affect the circadian rhythm of this organism (41), whereas mutations in *sigH* of *Nostoc punctiforme* affect the symbiosis of this cyanobacterium with a plant host (11). On the other hand, mutations in *sigF* of *Synechocystis* sp. strain PCC 6803, the only cyanobacterial alternative (group 3) sigma factor gene that has been characterized so far, result in a pleiotropic phenotype that includes alterations in pilus formation and motility (6).

The low survival of the *Synechocystis* sp. strain PCC 6803 *rpoD2-V* mutants after nitrogen stress could have been due to decreased *glnN* expression. Unfortunately, we have been unable to analyze the expression of *glnN* after long periods of nitrogen starvation because of the poor quality of mRNA samples isolated from the starved cells. *RpoD2-V* could also be involved in the expression of other genes required for survival under conditions of severe nitrogen deficiency. The fact that NtcA-activated promoters bear a -10 box of the form TAN₃T suggests that NtcA activates transcription mediated by RNA polymerase containing a σ^{70} -like sigma factor. Therefore, NtcA-dependent *rpoD2-V* expression might have the effect of increasing the levels of an appropriate, σ^{70} -like sigma factor to assist NtcA-activated gene expression under conditions of severe nitrogen starvation.

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