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Growth phase and metal-dependent regulation of the *dpsA* gene in *Synechococcus* sp. strain PCC 7942

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Abstract The *Synechococcus* sp. strain PCC 7942 *dpsA* gene encodes a stress-inducible DNA-binding protein whose transcription increases in the stationary phase. Such transcription is likely under the control of an alternative sigma factor. Our current work indicated that *dpsA* transcription is also important under metal-ion limitation, because *dpsA* mRNA levels increased 12-fold under low-iron conditions, and that *dpsA* function is essential for growth under iron-limiting conditions. Promoter activity of the *dpsA*–promoter–*lacZ* reporter gene constructs implied that a region of dyad symmetry centered 28 nucleotides from the transcription start is required for metal-dependent repression, as judged by the level of *lacZ* induction following treatment of cultures with the chelator 2,2′-dipyridyl. This potential operator sequence is distinct from the site recognized by the cyanobacterial Fur repressor homologue. No other nutrient stresses (nitrogen, sulfur, phosphorus) yielded the high level of induction seen following chelator treatment. These studies suggest that there may be more than one class of metal-dependent repressor in cyanobacteria.

Key words Sigma factor · Iron limitation · Fur repressor · Nutrient stress · Promoter · *Synechococcus*

Introduction

The Dps family of proteins are a diverse group of bacterial stress-inducible polypeptides that bind DNA and likely confer resistance to peroxide damage during periods of oxidative stress and long-term nutrient limitation (Almiron et al. 1992). Such stress-dependent DNA binding in *Escherichia coli* yields a rapid biocrystallization of the chromosome (Wolf et al. 1999).

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Expression of *dps* in *E. coli* is dependent on integration host factor (IHF) and the alternative sigma factor RpoS in stationary phase, and OxyR in the exponential phase (Altuvia et al. 1994). Previous work from this laboratory suggested that the Dps proteins are divergent members of the bacterioferritin/ferritin superfamily and that the *Synechococcus* sp. strain PCC 7942 Dps homologue, DpsA, is a DNA-binding hemoprotein having heme-dependent catalase activity (Peña and Bullerjahn 1995; Peña et al. 1995). More recent work from Hogle and colleagues has proven the structural similarity of *E. coli* Dps to the bacterioferritins, but the precise mechanism for DNA binding is still unknown (Grant et al. 1998).

We have speculated that this protein in *Synechococcus* sp. may yield a peroxide-consuming mechanism located on the chromosomal DNA, and we also have suggested that this activity may play a role in mitigating the endogenous oxidative stresses associated with oxygenic photosynthesis (Peña and Bullerjahn 1995). Indeed, the growth of *Synechococcus* sp. PCC 7942 null mutants lacking *dpsA* function is inhibited under high light, and the cells are supersensitive to paraquat treatment and exogenously added peroxide (Dwivedi et al. 1997). These are conditions that give rise to photooxidative stress. The *dpsA* null mutants demonstrate a distinctly severe peroxide-sensitive phenotype compared to the *E. coli dps* (Almiron et al. 1992) and *Bacillus subtilis mrgA* mutants (Chen et al. 1995) lacking Dps protein function.

Recent work has also examined the steady state levels of *dpsA* mRNA under both nutrient stress and during the growth phase. Whereas *dpsA* mRNA is detectable at low levels in the exponential phase, the transition to stationary phase yields a significant increase in steady state mRNA levels (Dwivedi et al. 1997). Work of Gruber and Bryant (1998) has also implicated an alternative sigma factor in *dpsA* expression in *Synechococcus* sp. strain PCC 7002, because mutants defective in *sigE* function do not yield high levels of DpsA after a transition to stationary phase. Although no RpoS homologue has been identified in cyanobacteria, SigE is a group 2 sigma factor (Gruber and Bryant 1998); thus the involvement of sigma factor

switching in cyanobacterial stationary phase gene expression represents a mechanism that is functionally similar to that seen in the regulation of *E. coli dps.*

The present study deals with the construction of reporter gene fusions with the *dpsA* promoter to assess both the environmental signals triggering *dpsA* transcription and the *cis*-acting regions responsible for transcriptional regulation. Firstly, primer extension of *dpsA* mRNA had identified a 5′ end preceded by a TAGAAT –10 sequence (Dwivedi et al. 1997). Thus, we sought to determine by promoter deletions the minimum promoter sequence necessary for *dpsA* transcription. Secondly, we examined the possibility that *dpsA* transcription is activated in the exponential phase upon the addition of metal chelators, analogous to the case of *mrgA* transcription in *B. subtilis* (Chen and Helmann 1995). *mrgA*, encoding a homologue of DpsA from *B. subtilis*, is induced during manganese and iron limitation (Chen et al. 1993) and controlled by a repressor sensing both transition metal ions and peroxide levels (Chen et al. 1995; Bsat et al. 1998). The connection between metal ion availability and expression of Dps proteins is further strengthened by the fact that Dps proteins are divergent bacterioferritins (Peña and Bullerjahn 1995; Grant et al. 1998) and thus may also participate in metal ion binding as has been suggested recently for *E. coli* Dps (Grant et al. 1998).

As a result of these studies, we show that *dpsA* function is essential for low-iron survival and that *dpsA* mRNA accumulates under iron limitation. Additionally, we identify a putative operator sequence important in conferring metal ion-mediated repression in the exponential phase.

Materials and methods

Strain and growth conditions

Synechococcus sp. strain PCC 7942 derivatives were grown in BG-11 medium (Allen1968) at 25 °C under constant illumination by fluorescent lights (General Electric Cool White). All promoter construct strains were generated in strain Δ*dpsA*6, in which the *dpsA* coding sequence plus 700 nucleotides flanking *dpsA* are replaced with a kanamycin resistance gene (Dwivedi et al. 1997). Low-iron BG-11 medium was prepared by omitting ferric ammonium citrate from BG-11 medium. The fluence rate was maintained at 150 μmol quanta m^{-2} s^{-1}. Where appropriate, kanamycin was added to the medium to a final concentration of 30 μg ml^{-1} and spectinomycin was added to a final concentration of 100 μg ml^{-1}. To induce rapid metal ion limitation in BG-11 medium, the chelator 2, 2′ dipyridyl was added to the mid-exponential phase cultures to a final concentration of 0.3 mM. For the peroxide treatment, H_2O_2 was added to 1 mM 30 min prior to assay. Growth was monitored by light scattering at 750 nm; mid-exponential cultures were defined as those having an optical density of 0.25. Stationary phase and long-term stationary phase cultures were defined as cultures having achieved saturation for 1 day and 5 days, respectively.

Standard procedures

Oligolabeling of DNA probes, restriction enzyme digestions, and agarose gel electrophoresis were performed as described in Sambrook et al. (1989). Nucleotide sequencing was carried out by the chain-termination method (Sanger et al. 1977) using the Sequenase kit (Amersham/USB) according to the manufacturer's instructions. PCR with *Taq* polymerase was as described in the manufacturer's instructions (GeneAmp, Perkin-Elmer Cetus), with 200 pmol primer, 50 ng genomic DNA for 35 cycles of the following temperatures: 94 °C, 1 min; 40 °C, 3 min; 40→72 °C, 2 min; 72 °C, 1 min. Custom PCR primers were obtained from Genosys (The Woodlands, Tex.).

Construction of reporter-gene–promoter fusions

Three different segments of the *dpsA* promoter region were amplified by PCR. These promoter fragments contained 200, 100 and 30 bp upstream from the transcription start (Fig. 1). The PCR products were synthesized having engineered *Sma*I and *Bgl*II restriction sites at the 5′ and 3′ ends respectively. These fragments were ligated to the 5′ *Sma*I and *Bgl*II sites upstream from the promoterless *lacZ* reporter gene in plasmid pAM990 (Li and Golden 1993). pAM990 is a promoter fusion vehicle capable of recombination into the chromosome at a phenotypically neutral site. Thus, promoter activity can be monitored in the chromosome without complications arising from plasmid copy number. Strain Δ*dpsA*6 (Dwivedi et al. 1997) was chosen as a host for such fusions because deletion of the *dpsA* region would prevent recombination between the native *dpsA* promoter and the *dpsA* promoter fused to *lacZ*. Genetic transformation (Golden and Sherman 1984) of Δ*dpsA*6 with the individual promoter fusion plasmids yielded 6–10 spectinomycin-resistant colonies per construct per 5×10^8 cells plated. These colonies were then extensively subcultured in BG-11 medium with spectinomycin prior to further characterization by the MUG plate assay (see below). Fluorescent clones (1-200, bearing a 200-bp promoter sequence; 2-100, bearing a 100-bp promoter; and 3-30, bearing a 30-bp promoter) from each construction were analyzed by PCR to confirm the presence of the cloned promoter inserted into the chromosome. As a negative control, Δ*dpsA*6 was transformed with pAM990 lacking a promoter insert, yielding construct 4-0.

RNA isolation and Northern blotting

RNA was extracted by TRIzol reagent method (Chomczynski and Sacchi 1987; Gibco BRL Life Technologies). Aliquots (35 μg) of

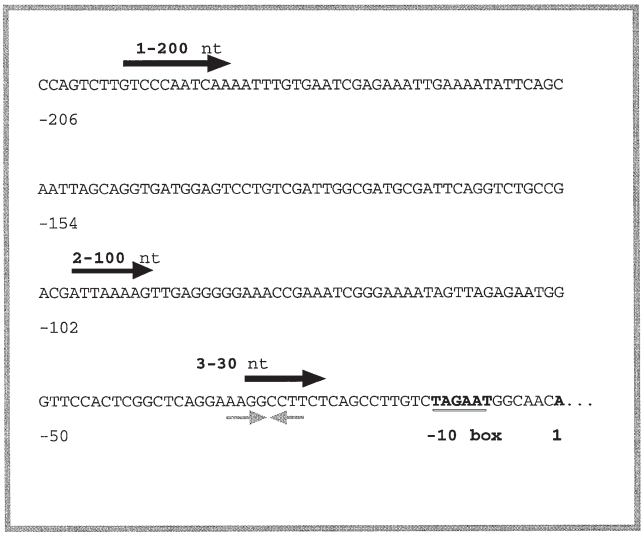

Fig. 1 Nucleotide sequence analysis of the upstream region of the *dpsA* gene. The *solid arrows* indicate the direction and start points of the different constructs. The –10 box and the transcription start site (*A*) are indicated in *bold*. A putative repressor-operator region is indicated by *lighter arrows*

total RNA were denatured with formamide-formaldehyde and separated on formaldehyde-agarose gels. Northern blotting and hybridization were performed according to standard methods (Ausubel et al. 1989); blots were probed with a ^{32}P-end-labeled, PCR-derived 528-bp internal fragment of *dpsA* (Dwivedi et al. 1997). The hybridization signals on the autoradiograms were quantitated by densitometry.

Quantitative fluorometric assay for β-galactosidase activity and MUG plate assay

The LacZ activity of the constructs was recorded by quantifying fluorescence emitted from the cultures after 4-methylumbelliferyl-β-D-galactoside (MUG) addition (Youngman 1987). One ml cell culture from each construct was collected and centrifuged. The pellet was then resuspended in 1 ml assay buffer (60 mM K_2HPO_4, 40 mM KH_2PO_4, 100 mM NaCl). The resuspended cells were lysed by two cycles of freeze-thaw in liquid nitrogen. The lysates were treated with 10 µl of 0.4 mg MUG (ml dimethyl sulfoxide)$^{-1}$. Fluorescence of three replicate lysates was recorded with a Fluorometer TKO 100 (Hoefer), at time intervals of 30, 100, and 200 min. Fluorescence values were corrected by subtraction of the low background fluorescence emitted from the 4-0 negative control. This correction eliminated the contribution of the fluorescent endogenous cellular pigments [corresponding to <3 pmol product (mg protein)$^{-1}$ h^{-1}]. For quantitative analysis, a standard curve was constructed using several concentrations of 4-methylumbelliferone (Sigma), the fluorescent product of MUG hydrolysis. The protein content of the samples was calculated by the Bradford (1976) assay.

The MUG plate assay was performed by spraying MUG solution (10 mg ml^{-1} in dimethyl sulfoxide) on transformant colonies on BG-11 plates and incubating the plates at room temperature for 30–40 min. The colonies were then examined by eye under ultraviolet light excitation for fluorescence from methylumbelliferone generated by MUG hydrolysis.

Results

Analysis of the *dpsA* promoter region

When the promoter region of *dpsA* was analyzed, the sequence revealed an eight nucleotide potential operator sequence starting at the position of –32 nucleotides from the transcription start site (Fig. 1). Sequence analysis of the *dpsA*-promoter–*lacZ* constructs revealed that fusion 3–30 lacks this complete sequence. Furthermore, 3-30 represents a construct in which a –35 region, if functional, would be destroyed by deletion.

Behavior of the Δ*dpsA*6 mutant under low-iron conditions

When the wild type and the mutant Δ*dpsA*6 were each subcultured in low-iron medium, the wild-type cell culture demonstrated slow growth, while the mutant culture exhibited bleaching accompanied by cell death after several days (Fig. 2). Thus, the DpsA protein is likely essential under low-iron growth conditions. This is the only growth condition tested so far in which the presence of a functional *dpsA* gene is required for survival, as the survival of N-, P-, or S-limited Δ*dpsA* 6 cultures is similar to that of the wild type (data not shown).

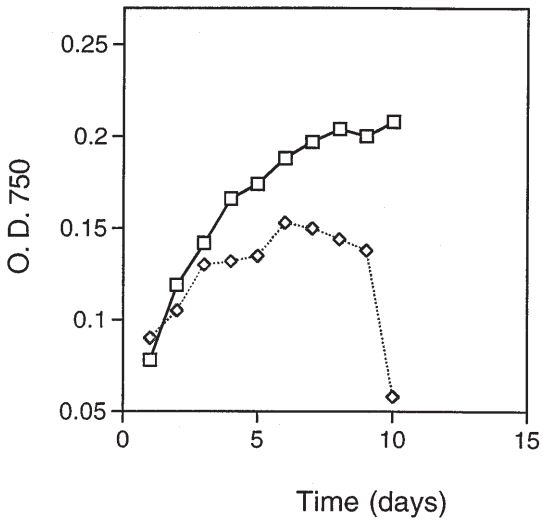

Fig. 2 Growth of the wild type and *dpsA* mutant under low-iron conditions. The wild type (*solid lines*) and the mutant Δ*dpsA*6 (*broken lines*) were cultured in low-iron BG-11 medium. Growth was measured by light scattering at 750 nm

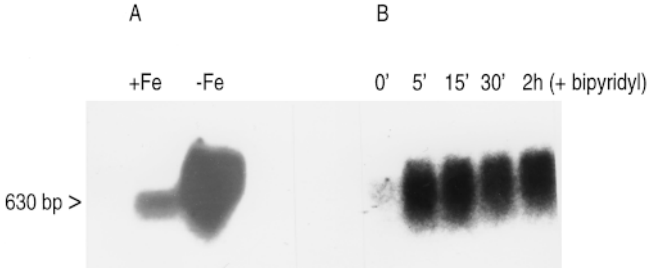

Fig. 3 **A** Northern blot analysis of total RNA from wild-type *Synechococcus* PCC 7942 grown in iron replete (*+Fe*) low-iron (*–Fe*) BG-11 medium. **B** Northern blots of RNA samples taken 0, 5, 15, and 30 min as well as 2 h after dipyridyl addition. All blots were probed with a ^{32}P-labeled PCR-derived 528 bp integral fragment of the *dpsA* gene (Dwivedi et al 1997)

Northern analysis of *dpsA* expression under low-iron conditions

Northern blots of total RNA extracted from iron-replete and low-iron stationary phase cultures showed a 12-fold increase in steady state *dpsA* mRNA under low-iron conditions (Fig. 3A). Additionally, wild-type mid-exponential phase cultures were treated with the chelator 2, 2′-dipyridyl. Northern blots revealed a 40-fold increase in *dpsA* mRNA beginning as early as 5 min following dipyridyl treatment (Fig. 3B), indicating that *dpsA* transcription is influenced by metal ion availability.

Quantitative analysis of *dpsA* promoter activity in nutrient-replete medium

To quantify the level of β-galactosidase reporter activity, the promoter construct cultures were collected, the cells

Table 1 β-Galactosidase specific activity of promoter fusion constructs from cultures grown in BG-11 medium (metal-replete conditions), plus effect of 1 mM peroxide treatment on construct 1. Units expressed as pmol methylumbelliferone (mg protein)$^{-1}$ h^{-1}. Values are calculated following subtraction of background fluorescence yielded from the 4-0 promoterless *lacZ* construct, which was equivalent to <3 pmol methylumbelliferone (mg protein)$^{-1}$ h^{-1}

Construct	β-Galactosidase specific activity		
	Exponential phase	Stationary phase	Long-term stationary
1-200	15	20	310
2-100	20	45	205
3-30	1645	1510	23,210
1-200 (H_20_2)	12	35	280

were lysed by freeze-thaw and treated with MUG, and the 4-methylumbelliferone fluorescence was recorded over time. The experiment was conducted with exponential phase, early stationary phase and long-term stationary phase cultures. Under these conditions, all constructs exhibited higher promoter activity during long-term stationary phase (Table 1), consistent with the observation that steady state *dpsA* mRNA levels increase during the stationary phase (Dwivedi et al. 1997) and that sigma factor switching in the stationary phase plays a role in increased *dpsA* expression in *Synechococcus* sp. strain PCC 7002 (Gruber and Bryant 1998). Notably, the 3-30 construct, lacking a potential –35 promoter sequence, exhibited high levels of promoter activity throughout the exponential phase and maximum levels during long-term stationary phase. This indicates that a –35 sequence is likely not required for *dpsA* transcription and that the 3-30 promoter deletion may lack a *cis*-acting sequence conferring repression on the other promoter constructs.

Quantitative analysis of *dpsA* promoter activity under metal limitation

MUG hydrolysis by the *dpsA*-promoter–*lacZ* reporter constructs under low-metal-ion concentration were recorded following the addition of dipyridyl. Samples of exponential phase, early stationary phase and long-term stationary phase cells were collected 30 min after addition of the chelator and assayed for β-galactosidase activity as described above (Table 2). All the constructs demonstrated high fluorescence upon addition of the chelator compared to the control strain. Thus, under metal-depleted conditions, the expression of the 100- and 200-nucleotide *dpsA* promoter constructs was two to three orders of magnitude higher than recorded under metal-replete growth conditions. The promoter-fusion experiments together suggest that the 200- and 100-nucleotide promoter sequences may interact with a repressor whose action is blocked by chelator addition. The activation of transcription is specific for metal ion depletion, as 1-200 mid-exponential cultures transferred to N-limited or S-limited medium (data not shown) do not yield an increase in *dpsA* transcription appreciably higher than that seen in stationary phase cultures (Table 1). Additionally, addition of peroxide to exponential phase cultures does not yield high-level *dpsA* transcription (Table 1), in contrast to what has been documented for *B. subtilis mrgA* transcription, which is induced by both iron/manganese limitation and peroxide treatment (Chen et al. 1995; Bsat et al. 1998).

Table 2 β-Galactosidase specific activity of promoter fusion constructs from cultures grown in BG-11 medium following bipyridyl treatment to induce starvation for transition metals. Units expressed as pmol methylumbelliferone (mg protein)$^{-1}$ h^{-1}

Construct	β-Galactosidase specific activity		
	Exponential phase	Stationary phase	Long-term stationary
1-200	9720	13,240	28,500
2-100	4060	5020	12,140
3-30	17,680	18,010	23,100

Discussion

In this paper, we demonstrated the pattern of *dpsA* transcription during growth and under metal limitation. Northern blots and growth curves of *dpsA* null mutants imply that DpsA function plays an important role under low-iron conditions (Figs. 2, 3), prompting a more detailed analysis of *dpsA* transcription under conditions of metal starvation and global nutrient stress in stationary phase.

From the quantitative analysis of promoter constructs, it was evident that construct 3-30 was the most active of all the constructs both in exponential and in stationary phase. Since this construct contained a promoter fragment of only 30 nucleotides in length, this fact strongly suggests that the promoter of *dpsA* does not require a –35 region. The mapped *dpsA* promoter indicated a potential –10 sequence of TAGAAT (Dwivedi et al. 1997) which is very similar to a consensus –10 region found in cyanobacteria and *E. coli* (Tanaka et al. 1992). Since *dpsA* transcription occurs at low levels in exponential phase (Dwivedi et al. 1997; see also Fig. 3B, lane 1), our data imply that a –10 sequence is the only sequence required for both the vegetative and post-exponential phase sigma factors to recognize the *dpsA* promoter. Indeed, a thorough analysis of cyanobacterial promoters reveals considerable variability among –35 sequences (Curtis and Martin 1994), suggesting they may be dispensable in some or most promoters.

The breakpoint of the 3-30 promoter deletion was also found to interrupt a region of dyad symmetry of AAGGCCTT centered 28 nucleotides from the transcription start (Fig. 1). This construct yielded constitutively high levels of *lacZ* expression (Table 1); thus we propose that the 3–30 promoter construct lacks a *cis*-acting region recognized by a repressor. This symmetrical sequence may be a potential operator involved in *dpsA* exponential phase repression, and we propose that a repressor-operator might

be an alternate means of controlling *dpsA* regulation, in addition to the σ switching previously demonstrated by Gruber and Bryant (1998). This sequence is dissimilar to the *fur* box operator consensus in both enteric bacteria and cyanobacteria; thus the sequence may be recognized by a repressor distinct from the Fur protein controlling bacterial iron-regulated genes (Bagg and Nielands 1987; Crosa 1989; Straus 1994). Analysis of the region between the –30 and –100 breakpoints also fails to reveal a *fur* box sequence (Straus 1994). Finally, the comparatively low levels of promoter activity in the 1-200 and 2-100 constructs in long-term stationary phase (Table 1) implies that the sigma factor switching in stationary phase may yield only a fraction of the maximum promoter activity. This is in agreement with the data of Fig. 3A, in which stationary phase iron-limited cultures exhibit several fold higher levels of *dpsA* mRNA than found in stationary phase iron-replete cultures.

While our promoter fusion data do not yet rule out the possible role of a transcriptional activator in *dpsA* expression, we propose that the *dpsA* promoter is under the control of a repressor protein linked in some way to the intracellular concentration of iron or other metals. This mechanism is similar in some respects to what is seen in *B. subtilis*, in which metal limitation or peroxide treatment activate *mrgA*, encoding a Dps homologue (Chen et al. 1995). *mrgA* is under negative control by the PerR repressor, which appears to be a divergent Fur homologue distinct from the *B. subtilis* YqkL protein that is structurally and functionally most closely related to *E. coli* Fur (Bsat et al. 1998). However, *dpsA* transcription differs from *mrgA* regulation in that repression is not sensitive to peroxide treatment. Nonetheless, the fact that the *Synechocystis* sp. strain PCC 6803 genome contains two *fur* homologues (Cyanobase accession numbers sll0567 and sll1937; Kaneko et al. 1996) raises the possibility that *Synechococcus* sp. strain PCC 7942 *dpsA* may be under the control of a divergent Fur-like repressor. Future work aimed at characterizing the repressor will reveal whether a divergent Fur homologue is responsible for metal-dependent repression.

Prior work has affirmed the role of DpsA in protecting the chromosome from oxidative damage, in agreement with the initial observations on *E. coli* Dps (Almiron et al. 1992; Dwivedi et al. 1997). However, the control of *dpsA* transcription by metal ions and the requirement for DpsA function in low-iron medium together imply that DpsA may serve an additional important role related to iron acquisition and storage. Future studies on the DpsA complex may reveal whether this divergent bacterioferritin is capable of accumulating and storing ferric ions. Additionally, it is worth noting that the *dpsA* gene maps adjacent to a cluster of iron-regulated genes in *Synechococcus* sp. and that *dpsA* function likely is involved in the activation of the *idiA* gene within this cluster (Michel et al. 1999). Furthermore, IdiA is likely involved in protecting photosystem II under iron limitation (Michel et al. 1999; E.K. Pistorius, personal communication). Thus, it is likely that this region of the chromosome is dedicated to protective functions under metal ion limitation and that a hierarchy of regulation exists within this region.

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