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Characterization of the alternative σ-factors SigD and SigE in *Synechococcus* sp. strain PCC 7002. SigE is implicated in transcription of post-exponential-phase-specific genes

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Abstract The *sigD* and *sigE* genes, which encode two alternative σ-factors from the unicellular marine cyanobacterium *Synechococcus* sp. PCC 7002, were cloned and characterized. Strains in which the *sigD* and *sigE* genes were insertionally inactivated were viable under standard laboratory conditions, indicating that SigD and SigE are group 2 σ-factors. When stationary-phase cells were diluted into fresh growth medium, it was observed that the *sigE* mutant strain required longer times to re-establish exponential growth than the wild-type strain. By monitoring the growth rates in such dilution experiments, it was observed that the lag times for the mutant strain became progressively longer as the original cultures progressed towards stationary phase. Transcripts for the *sigE* gene initially increased and subsequently decreased as cells grew further into stationary phase. It was determined that a functional SigE protein is required for the expression of the starvation-induced protein DpsA/PexB. The results suggest that SigE is involved in the transcription of genes specifically expressed in the post-exponential phase.

Key words *Synechococcus* sp. PCC 7002 · Cyanobacterium · σ-Factor · RNA polymerase · RpoS · DpsA · PexB · Stationary phase

Abbreviations *IPTG* Isopropyl-β-D-thiogalactoside · *PCC* Pasteur Culture Collection

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Introduction

σ-Factors are dissociable subunits that confer promoter specificity on eubacterial core RNA polymerase and are required for transcription initiation. Two major families of σ-factors are known to occur in eubacteria: the σ^{70} (RpoD) family (Lonetto et al. 1992; Gruber and Bryant 1997) and the σ^{54} (RpoN) family (Kustu et al. 1989; Collado-Vides et al. 1991). The σ^{70} family has been divided into three groups based upon sequence comparisons and functional considerations (Lonetto et al. 1992). Group 1 comprises the primary σ-factors of organisms and are essential for cell viability. Group 2 includes nonessential, alternative σ-factors that are highly similar in sequence to the respective group 1 members; group 3 σ-factors are nonessential, alternative σ-factors that vary more significantly in sequence from the other two groups and include functional groupings such as heat-shock (Grossman et al. 1984) and sporulation σ-factors (Haldenwang 1995).

Most eubacteria have the ability to synthesize several different σ-factors (Helmann and Chamberlin 1988; Lonetto et al. 1992) that direct transcription from different promoters. Thus, multiple σ-factors provide cells with the means of regulating a large number of genes coordinately and of responding to changing environments. *Escherichia coli* utilizes at least seven σ-factors: the group 1 σ-factor of the σ^{70} family, RpoD (Lonetto et al. 1992); the group 2 member RpoS (Mulvey and Loewen 1989); the lone member, of the σ^{54} family, RpoN (Kustu et al. 1989); and the four group 3 σ-factors, RpoH (Grossman et al. 1984), FliA (Liu and Matsumura 1995), and RpoE and FecI (Lonetto et al. 1994). Another well-studied eubacterium, *Bacillus subtilis* is believed to contain ten σ-factors (Haldenwang 1995) including one member of the σ^{54} family, one group 1 member of the σ^{70} family, and eight members of group 3. In *B. subtilis*, sporulation is a starvation response in which the organism converts itself from an actively growing vegetative cell into a dormant spore. Four σ-factors in *B. subtilis* appear to be solely dedicated to spore formation, and cascades of σ-factors are partly re-

sponsible for the sequential pattern of gene expression that occurs during sporulation (Stragier and Losick 1990). However, the majority of eubacteria, including *E. coli*, do not undergo morphological development in response to nutritional stress; instead, they enter what is normally referred to as "stationary phase" or "post-exponential phase." Even though the cells do not noticeably differentiate, significant physiological changes take place that allow cells to survive a wide range of environmental stresses (Loewen and Hengge-Aronis 1994). The group 2 σ-factor RpoS has been shown to mediate the post-exponential-phase adaptive process in *E. coli* (Mulvey and Loewen 1989).

Synechococcus sp. strain PCC 7002 is a unicellular, marine cyanobacterium that does not differentiate heterocysts, akinetes (spore-like cells), or hormogonia. Transcription of a large number of genes in several species of cyanobacteria have been shown to be altered by environmental changes (Tandeau de Marsac and Houmard 1993). Alignment of sequences believed to encompass the –10 and –35 promoter regions of many *Synechococcus* sp. strain PCC 7002 genes has shown that these promoter regions for several genes contain sequences similar to the consensus promoter sequence of the *E. coli* group 1 σ-factor RpoD (Curtis and Martin 1994). This consensus promoter sequence is suspected to be largely conserved throughout all eubacteria (Gruber and Bryant 1997). Although multiple σ-factors have been found in all cyanobacteria studied to date (Tanaka et al. 1992; Brahamsha and Haselkorn 1992; Tsinoremas et al. 1996; Kaneko et al. 1996a, b), the roles of the alternative σ-factors has remained uncertain. Five σ-factors of the σ^{70} family, including one member of group 1 and four members of group 2, have been identified in *Synechococcus* sp. PCC 7002 (Caslake and Bryant 1996; Gruber and Bryant 1997). The principal σ-factor (SigA) of *Synechococcus* sp. strain PCC 7002 (Caslake and Bryant 1996) and two group 2 members (SigB and SigC) that appear to be involved in cellular responses to carbon and nitrogen stress have been characterized (Caslake et al. 1997). In this work, we describe the cloning and initial characterization of the remaining two group 2 σ-factors of this cyanobacterium.

Materials and methods

Bacterial strains, plasmids, and growth conditions

The unicellular marine cyanobacterium *Synechococcus* sp. PCC 7002 (Rippka et al. 1979) was grown on 1.5% (w/v) agar plates or in liquid culture in Medium A (Stevens and Van Baalen 1973) supplemented with 1 g sodium nitrate l^{-1}. For standard growth conditions, liquid cultures were bubbled with 1% CO_2-99% air (v/v) at 39° C and with continuous illumination provided by cool-white fluorescent lamps at approximately 250 μE m^{-2} s^{-1}. For growth of kanamycin-resistant transformants, Medium A plus nitrate was supplemented with 200 mg kanamycin l^{-1}. The strain with the insertionally inactivated *sigD* gene is designated *Synechococcus* sp. strain PR6503, and the strain with the insertionally inactivated *sigE* gene has been designated *Synechococcus* sp. strain PR6504. The cyanobacterium *Synechococcus* sp. PCC 6301 was grown in liquid culture in Medium B-Hepes (Dubbs and Bryant 1991). *E. coli* strain DH5α (Bethesda Research Laboratories) was used for recombinant DNA manipulations. Plasmid vector pUC19 (Yanish-Perron et al. 1985) was used for all routine cloning and double-stranded sequencing procedures.

Recombinant DNA procedures

Small- and large-scale plasmid preparations from *E. coli* (Birnboim and Doly 1979) and chromosomal DNA from *Synechococcus* sp. PCC 7002 (De Lorimier et al. 1984) were isolated as previously described. Conditions for Southern hybridization analyses have been described (Bryant and Tandeau de Marsac 1988). Labeling of DNA probes with [α-^{32}P] dATP (New England Nuclear, Beverly, Mass., USA) was performed using a Random-primed DNA Labeling Kit (Boehringer Mannheim, Indianapolis, Ind., USA). DNA sequencing was performed by the chain-termination method (Sanger et al. 1977) with a Sequenase Version 2.0 DNA Sequencing Kit from United States Biochemical (Cleveland, Ohio, USA) according to the recommendations of the manufacturer. All nucleotide sequences were verified by completely sequencing both strands. Nucleotide sequence data were analyzed with MacVector Sequence Analysis Programs Versions 5.0 and 6.0 (Eastman Kodak, Rochester, N.Y., USA). Protein sequence alignments were generated with the CLUSTALW program (Thompson et al. 1994) within the MacVector software package.

Synechococcus sp. PCC 7002 RNA was extracted by a mechanical breakage protocol (Golden et al. 1987). RNA samples (5 μg) were separated by electrophoresis, transferred from formaldehyde-agarose gels to NytranPlus membranes (Schleicher and Schuell, Keene, N.H., USA) according to the manufacturer's instructions, and hybridized at 65° C under conditions described previously

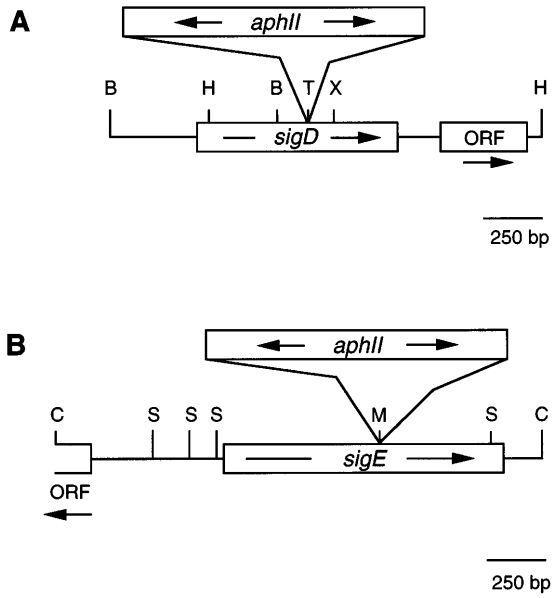


Fig. 1 Physical maps of the 1.84-kb *Bam*HI-*Hin*dIII fragment encoding the *sigD* gene (**A**) and the 2.10-kb *Hinc*II fragment containing the *sigE* gene (**B**) encoding alternate σ-factors of the RNA polymerase of *Synechococcus* sp. PCC 7002. *Arrows* indicate the direction of transcription. The ORF found downstream of *sigD* is homologous to *Synechocystis* sp. PCC 6803 slr1674 (accession no. D90913); the 5′-end of the ORF found upstream of *sigE* is homologous to *Synechocystis* sp. PCC 6803 sll1336 (accession no. D90916). The *aphII* gene, which encodes aminoglycoside 3′-phosphotransferase II and confers resistance to kanamycin, was inserted (in both orientations as indicated by the *arrows*) into the *Bst*XI site of the *sigD* gene and the *Sma*I site of the *sigE* gene in mutational studies (see text). Restriction enzyme sites are: *B* *Bam*HI, *C* *Hinc*II, *H* *Hin*dIII, *M* *Sma*I, *S* *Ssp*I, *T* = *Bst*XI and *X* *Xba*I

(Church and Gilbert 1984). The 5′-endpoint of the *sigE* mRNA was mapped using the primer-extension protocol of Ausubel et al. (1987), except that 100 µg of total RNA was used in the extension reaction. An oligonucleotide (5′-CTTGGCTCTTGGACTGCTTG-GCG-3′) corresponding to the complement of nucleotides 749–776 for the *sigE* sequence was synthesized on a Model 392 Applied Biosystems (Foster City, Calif., USA) automated DNA/RNA synthesizer for use in the primer-extension experiment.

The *sigD* gene was isolated by screening libraries of size-fractionated fragments derived from digests of *Synechococcus* sp. PCC 7002 total chromosomal DNA. A 1.4-kb *Hin*dIII fragment and a 0.7-kb *Bam*HI fragment were cloned into pUC19 to obtain the complete sequence of the *sigD* gene. The *sigE* gene was cloned as a 2.1-kb *Hin*cII fragment. The DNA sequences for the *sigD* and *sigE* genes of *Synechococcus* sp. PCC 7002 were deposited in GenBank under accession nos. U82484 and U82485, respectively. The *sigD* gene was insertionally inactivated by cloning a 1.32-kb *Hin*cII fragment, that carries the *aphII* gene encoding aminoglycoside 3′-phosphotransferase II and thus confers resistance to kanamycin, into the unique *Bst*XI site of the *sigD* gene (see Fig. 1A). The *sigE* gene was inactivated by inserting the *aphII* gene into the unique *Sma*I site within its coding sequence (see Fig. 1B). These constructions were introduced into *Synechococcus* sp. PCC 7002 by transformation (Buzby et al. 1983). Transformants were restreaked on selective media several times, and segregation of alleles was verified by Southern blot hybridization.

Polyacrylamide gel electrophoresis and immunoblot analysis

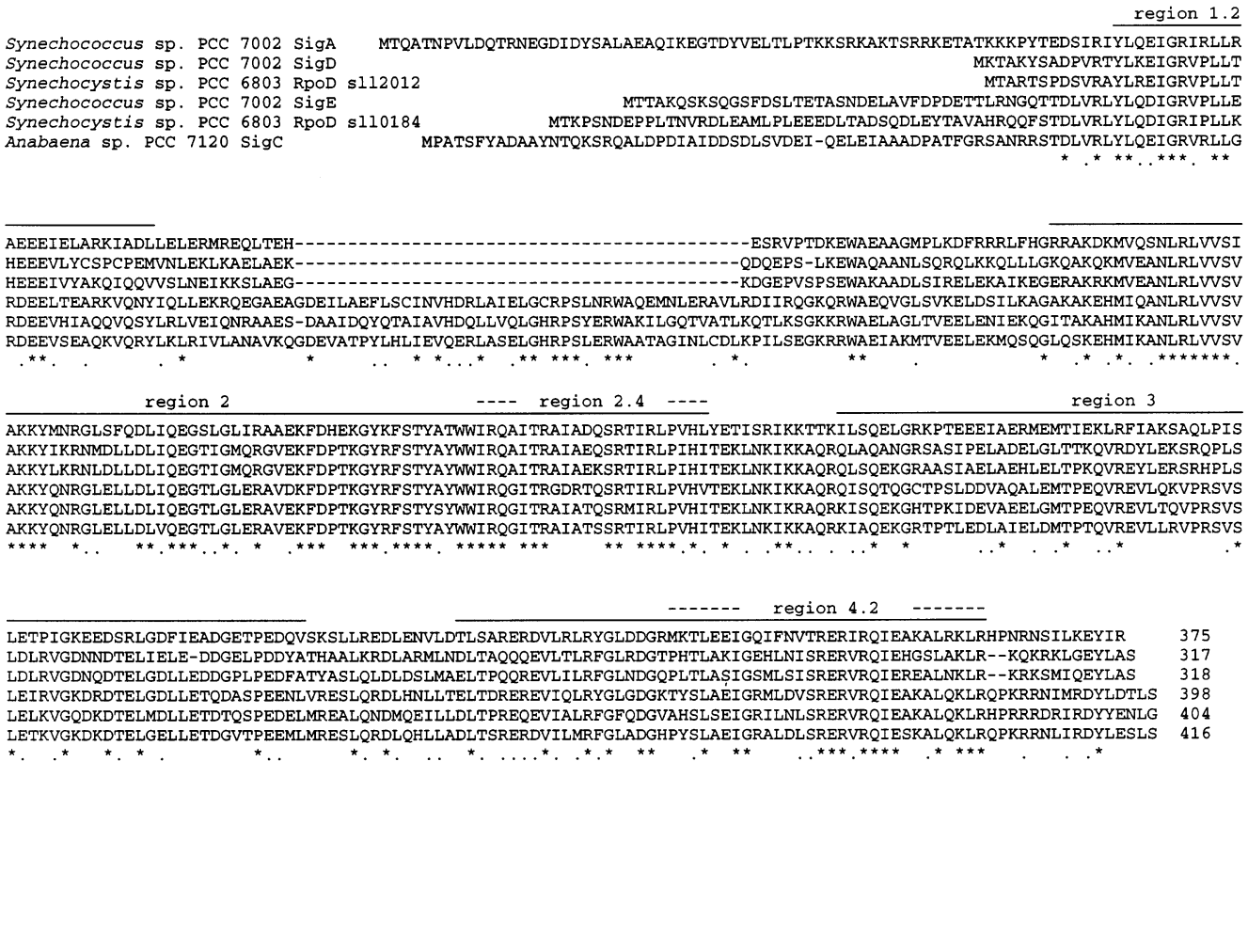
Polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecylsulfate (SDS) was performed as described (Laemmli 1970). Proteins were either stained with Coomassie Brilliant Blue or transferred electrophoretically onto nitrocellulose membranes (Schleicher and Schuell) for 400 mAmp-h. Rabbit antibodies against the *Synechococcus* sp. PCC 7942 DpsA/PexB protein were kindly provided by G. Bullerjahn (Bowling Green State University, Bowling Green, Ohio, USA). Protein extracts used as positive controls were derived from *Synechococcus* sp. PCC 6301, a strain that is closely related to *Synechococcus* sp. PCC 7942 (Golden et al. 1989). Immunodetection of polypeptides on blots was performed with alkaline phosphatase conjugated to goat anti-rabbit IgG as previously described (Harlow and Lane 1994).

Results

Sequence analysis of the *sigD* and *sigE* genes

Southern blot hybridization experiments (Caslake and Bryant 1996) had previously shown that *Synechococcus* sp. PCC 7002 contains at least five σ^{70}-type σ-factor homologs. The group 1 σ-factor (denoted SigA; Caslake and Bryant 1996) and two group 2 members (denoted SigB and SigC; Caslake et al. 1997) have been described previously. The *sigD* gene was cloned on overlapping *Hin*dIII and *Bam*HI fragments, and the complete nucleotide sequence (data not shown) was deposited in GenBank under accession no. U82484. The *sigD* gene is predicted to encode a

Fig. 2 Alignment of the predicted amino acid sequences of RNA polymerase σ-factors SigA, SigD, and SigE from *Synechococcus* sp. PCC 7002 (Caslake and Bryant 1996; this work), *Anabaena* sp. PCC 7120 SigC (Brahamsha and Haselkorn 1992), and *Synechocystis* sp. PCC 6803 RpoD sll2012 (Genbank accession no. D90908), and RpoD sll0184 (Genbank accession no. D64002) (Kaneko et al. 1996a, b). The *overlined regions* correspond to conserved regions 1, 2, 3, and 4 of σ-factors as defined by Lonetto et al. (1992). Amino acid identities are indicated with a star, and conserved amino acids are indicated by a *period*; insertions/deletions introduced to align the protein sequences optimally are indicated by *dashes*. The number of amino acids of each protein is indicated at the C-terminus of the sequence



protein of 317 amino acids with a mol. mass of 36.3 kDa (Fig. 2). No discernable ribosome binding sequence at the 5′-end of the gene nor inverted repeat at the 3′-end of the gene was detected. Downstream from the *sigD* gene (see Fig. 1A), an additional open reading frame was identified through searches of the databases. This open reading frame has significant sequence similarity to two hypothetical proteins, the products of open reading frames slr1674 and slr1638, of *Synechocystis* sp. PCC 6803 (Kaneko et al. 1996a, b). These open reading frames are not found near the *sigD* sequence (sll2012) of *Synechocystis* sp. PCC 6803, and they are not associated with other genes that suggest possible functional roles for them.

The *sigE* gene was cloned on a 2.1-kb *Hin*cII fragment, and the complete nucleotide sequence (data not shown) of *sigE* was deposited in GenBank under accession no. U82485. The *sigE* gene predicts a polypeptide of 398 amino acids with a mol. mass of 45.7 kDa (Fig. 2). A consensus ribosome binding site (5′-AGGAGA-3′) precedes the ATG start codon of the *sigE* gene, and an inverted repeat with the potential to form a stable stem-loop structure followed by a run of T residues is found 34 bases downstream of the stop codon (data not shown). This sequence resembles rho-independent transcription termination signals in *E. coli* (Richardson and Greenblatt 1996) and could play a role in transcription termination, mRNA stabilization, or both processes. Upstream from the *sigE* gene (see Fig. 1A), an additional open reading frame was identified through searches of the databases. This open reading frame has strong sequence similarity to a hypothetical protein (sll1336) of *Synechocystis* sp. PCC 6803. This open reading frame is not found near the *sigE* sequence (sll0184) of *Synechocystis* sp PCC 6803, nor is it associated with other genes that suggest a possible functional role (Kaneko et al. 1996a, b).

An alignment of the SigA, SigD, and SigE σ-factors of *Synechococcus* sp. PCC 7002 is shown in Fig. 2. The group 2 σ-factors (see below), SigD and SigE, were 45 and 44% identical to the group 1 σ-factor, SigA, of this organism. The identity values (~ 80%) are significantly higher in the region implicated in binding the –10 hexamer; the identity values are ~ 60% in region 4.2, which binds the –35 promoter motif. The SigA, SigD, and SigE proteins of *Synechococcus* sp. PCC 7002 are also compared to selected σ-factors from other cyanobacteria including *Synechocystis* sp. PCC 6803 (Kaneko et al. 1996a, b) and *Anabaena* sp. PCC 7120 (Brahamsha and Haselkorn 1992). *Anabaena* sp. PCC 7120 SigC and *Synechocystis* sp. PCC 6803 RpoD (sll0184) appear to be homologs of *Synechococcus* sp. PCC 7002 SigE. *Synechococcus* sp. PCC 7002 SigD is probably homologous to the *Synechocystis* sp. PCC 6803 RpoD (sll2012) protein. It is interesting to note that the *Synechococcus* sp. PCC 7002 SigE protein and its closest homologs share a 42-amino-acid insertion following region 1.2 (Fig. 2) that does not occur in SigA, in SigD and its homologs, or in the other group 1 or 2 σ-factors (SigA, SigB, or SigC) of *Synechococcus* sp. PCC 7002. A search of the databases revealed no additional proteins that contain regions of significant similarity to this insertion.

Transcript analyses of *sigD* and *sigE*

Total RNA was isolated from *Synechococcus* sp. PCC 7002 cells that had been grown to late exponential phase in replete media under standard conditions (see Materials and methods). These RNA samples were separated by electrophoresis, transferred to a nylon membrane, and hybridized with either the 0.5-kb *Xba*I/*Hin*dIII fragment of the *sigD* gene or the 1.2-kb *Ssp*I fragment of the *sigE* gene (see Fig. 1). No hybridization signal was detected for the *sigD* gene (data not shown); likewise, hybridization ex-

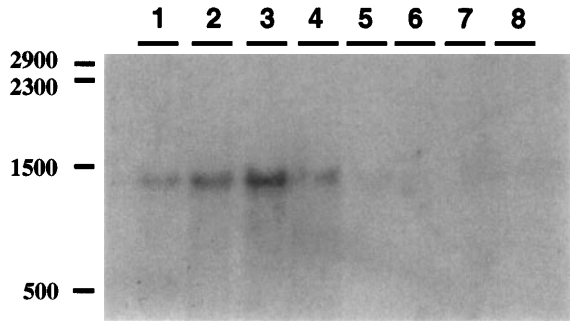


Fig. 3 Northern blot hybridization analysis of the *Synechococcus* sp. 7002 *sigE* transcript during growth from mid-exponential to stationary phase. The Northern blot was probed with a radiolabeled 1.1-kb *Ssp*I fragment encoding the *sigE* gene (see Fig. 1A). *Lane 1* RNA extracted from *Synechococcus* sp. strain PR6000 cells in mid-exponential phase. *Lanes 2–8* RNA isolated from cells grown increasing lengths of time beyond mid-exponential phase: *2* 24 h, *3* 48 h, *4* 77 h, *5* 99 h, *6* 126 h, *7* 152 h and *8* 171 h. Transcript sizes were estimated by comparison with 23 S (Kumano et al. 1983) and 16 S (Tomioka and Sugiura 1983) rRNAs and their breakdown products (Doolittle 1972)

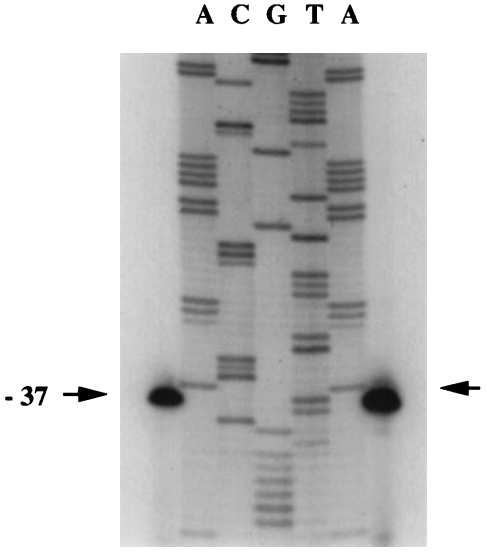


Fig. 4 Primer extension analysis of the *Synechococcus* sp. PCC 7002 *sigE* transcript. The RNA was extracted from *Synechococcus* sp. PCC 7002 cells grown under standard conditions to late exponential phase. The primer-extension product was denatured and subjected to electrophoresis along both sides of a DNA sequencing ladder generated using the same primer on a plasmid DNA template encoding *sigE*. The mapped 5′-endpoint occurs 37 nucleotides upstream from the translation start codon for the *sigE* gene

periments performed with RNA isolated from cells subjected to a variety of environmental stresses failed to detect any transcript for the *sigD* gene. The abundance of *sigE* transcripts was monitored in cells growing in batch culture for a period extending 171 h beyond mid-exponential growth phase (Fig. 3). During the initial 48-h period after mid-exponential phase, *sigE* mRNA levels increased; however, *sigE* transcript levels then decreased, and transcripts were no longer detectable in cells incubated 99 h or more beyond mid-exponential phase (Fig. 3).

To map the 5′-endpoint of the *sigE* mRNA, primer-extension analysis (Fig. 4) was performed with RNA extracted from *Synechococcus* sp. PCC 7002 cells grown under standard conditions to late exponential phase. As shown in Fig. 4, the major 5′-endpoint of the *sigE* transcript corresponds to an A residue that occurs 37 nucleotides upstream from the translational start codon (ATG). Upstream from this A residue, the sequence motif 5′-TTG-AAA–17 bp–AAATTT–6 bp-3′, with strong similarity to the consensus σ^{70}-type promoter, is present. The observed transcript length of approximately 1,300 nucleotides (Fig. 3) is in excellent agreement with the size that is predicted from the mapped 5′-endpoint and the predicted 3′-endpoint if transcription terminates at the putative rho-independent terminator. Several additional, very minor primer-extension products were detected (data not shown); these included endpoints that occur 217 and 220 nucleotides upstream from the translational start codon.

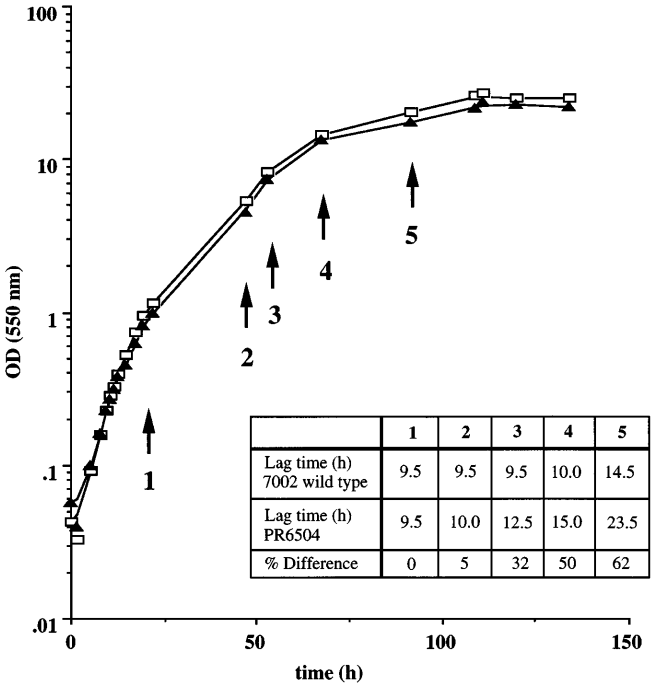
Interposon mutagenesis of the *sigD* and *sigE* genes

To determine whether the *sigD* and *sigE* gene products are required for viability of *Synechococcus* sp. PCC 7002 and to learn more about the roles of these σ-factors, each gene was insertionally inactivated by interposon mutagenesis. The *aphII* gene, which encodes aminoglycoside 3′-phosphotransferase and confers resistance to kanamycin, was inserted into a unique restriction site within the coding sequence of each gene (see Fig. 1). These constructions were transformed into *Synechococcus* sp. PCC 7002, and the resultant kanamycin-resistant transformants were repeatedly subcultured in selective medium and plated to obtain isolated colonies and to allow segregation of the mutant and wild-type alleles. Southern blots of chromosomal DNA isolated from several kanamycin-resistant transformants for each construction were hybridized under highly stringent conditions. The hybridization experiments showed that complete segregation of the wild-type and mutant alleles had occurred (data not shown). These results clearly demonstrate that neither the *sigD* gene nor the *sigE* gene is essential for viability of *Synechococcus* sp. PCC 7002 under standard laboratory growth conditions. Thus, both SigD and SigE can be classified as group 2 σ-factors (Lonetto et al. 1992; Gruber and Bryant 1997).

Growth properties of the *sigD* and *sigE* mutant strains

Under nutrient-replete, standard growth conditions, the growth rates of the *sigD* and *sigE* mutant strains were identical to the growth rate of the wild-type strain. Although a variety of growth conditions and physiological stresses have been tested, no significant difference between the growth characteristics of the wild-type strain and the *sigD* mutant strain PR6503 has been identified. Since no transcripts have yet been detected for this gene (see above), this result is perhaps not surprising.

It was repeatedly observed that strain PR6504, in which the *sigE* gene is insertionally inactivated, displayed longer times to re-establish exponential growth than the wild-type PR6000 strain when diluted into fresh medium from a stationary-phase culture. This behavior was observed both for cells grown in liquid medium and for cells grown on agar plates. Figure 5 shows the results of a growth experiment that demonstrates this effect. To insure that all cells were in exponential growth phase, both the wild-type strain and strain PR6504 were repeatedly diluted back while still growing in early exponential phase (OD_{550} less than 1.0). Subsequently, batch-culture growth characteristics were monitored for both strains for 135 h (Fig. 5). As can be seen, the growth rates under these optimal growth conditions were practically identical for both strains. As indicated by timepoints 1–5 in Fig. 5, aliquots of cells were withdrawn for both strains at different stages of



	1	2	3	4	5
Lag time (h) 7002 wild type	9.5	9.5	9.5	10.0	14.5
Lag time (h) PR6504	9.5	10.0	12.5	15.0	23.5
% Difference	0	5	32	50	62

Fig. 5 Growth studies of *Synechococcus* sp. PCC 7002 and *Synechococcus* sp. strain PR6504, which lacks a functional *sigE* product. The optical density (OD) was measured at 550 nm. The growth rates of both strains were monitored for 135 h. At selected times throughout the time course and representing different growth stages, aliquots of cells were withdrawn for both strains as indicated by timepoints 1–5. These aliquots were diluted with fresh growth medium to OD_{550} = 0.05, and their growth under optimal conditions was again monitored. The lag times (arbitrarily defined as the time for cells to grow from OD_{550} = 0.05 to an OD_{550} = 0.2) and the percentage differences of the lag times between the two strains are shown in the *inserted table*. □ *Synechococcus* sp. PCC 7002 wild-type strain, ▲ *Synechococcus* sp. strain PR6504

growth at selected times throughout the time course of this growth experiment. These aliquots were diluted into fresh growth medium to OD_{550} = 0.05, and their growth under optimal conditions was again monitored. As the cells of the original batch cultures progressed towards stationary phase, the lag times for strain PR6504 became progressively longer than those for the wild-type cells. However, once the cells began to grow again, the growth rates were identical for the two strains (data not shown). The inserted table in Fig. 5 presents these results in a quantitative manner. The length of the lag time was arbitrarily defined as the time for cells to grow from OD_{550} = 0.05 to OD_{550} = 0.2. No difference in lag times was observed for the two strains when cells of the batch cultures were in early exponential phase. However, as the batch cultures of the two strains progressed through mid-exponential phase towards stationary phase, the lag times increased to be as much as 62% longer for strain PR6504 than for the wild-type strain PR6000. Viable cell counts demonstrated that these effects were not due to differences in cell viability. This behavior was exclusively characteristic of the *sigE* strain and was not observed for strains PR6503 (*sigD*), PR6501 (*sigB*), and PR6502 (*sigC*) (data not shown). These results indicate that cells lacking a functional *sigE* gene product cannot easily re-establish exponential growth after entering into stationary phase; however, once growth is re-established, their growth properties closely resemble those of the wild-type strain.

Involvement of the *sigE* gene in the expression of the DpsA/PexB protein

The starvation-induced DpsA/PexB protein of *E. coli* is a 19.7-kDa, DNA-binding protein that forms high-molecular-weight complexes implicated in the protection of DNA from oxidative damage during stationary phase (Almiron et al. 1992). A homolog of the DpsA/PexB protein has been identified in nitrate-limited cells of *Synechococcus* sp. PCC 7942 (Pena et al. 1995; Pena and Bullerjahn 1995). The cyanobacterial protein has been shown to form hexamers of approximately 150 kDa that are relatively stable even in the presence of SDS. Since the expression of this protein is specific to stationary-phase (or nutrient-limited) cells in *E. coli* (Matin 1991; Almiron et al. 1992) and its transcription in stationary phase was shown to be dependent on RpoS (Altuvia et al. 1994), it was reasoned that expression of this protein in *Synechococcus* sp. PCC 7002 might be under the control of the SigE σ-factor. Immunoblot analyses (Fig. 6) were performed to determine the relative levels of the DpsA/PexB protein in the wild-type and *sigE* mutant strain PR6504 cells that had been grown into stationary phase for varying times (1, 4, or 10 days). The immunoblots were probed with antibodies raised against the DpsA/PexB protein of *Synechococcus* sp. PCC 7942 (Pena et al. 1995). No DpsA/PexB protein was detected in wild-type cells after 1 day of growth, but after 4 or 10 days of growth, a protein of the expected size cross-reacted with the anti-DpsA/PexB antibodies. However, no DpsA/PexB was detected in cells of the *sigE* mutant strain PR6504 even after 10 days of growth. These results strongly imply that a functional *sigE* gene product is necessary for the expression of the *dpsA/pexB* gene during stationary phase in *Synechococcus* sp. PCC 7002.

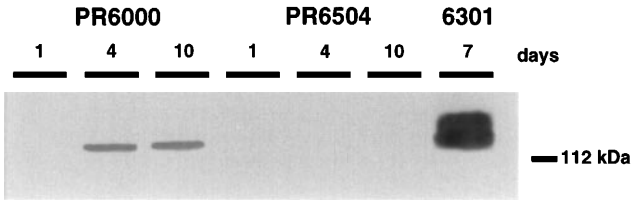


Fig. 6 Immunoblot analysis of DpsA/PexB production in *Synechococcus* sp. PCC 7002 wild-type cells and *Synechococcus* sp. strain PR6504, which lacks a functional SigE protein. The cells were grown for 1, 4, and 10 days, and protein extracts of the cells were electrophoresed and transferred to nitrocellulose. The immunoblot was probed with antibodies raised against the DpsA/PexB protein of *Synechococcus* sp. PCC 7942. Protein extracts of a stationary-phase culture (grown for 7 days) of *Synechococcus* sp. PCC 6301 were loaded as a positive control. The migration position of a 112-kDa molecular mass marker is indicated on *the right*

Discussion

In this study, the *Synechococcus* sp. PCC 7002 *sigD* and *sigE* genes encoding two σ^{70}-type σ-factors were cloned and characterized. Growth experiments indicated that a *sigE* mutant strain had more difficulty escaping stationary-phase and resuming exponential growth than the wild-type strain. To examine further the role of SigE in stationary-phase cells, the production of a stationary phase-specific DpsA/PexB-like protein was monitored in both *Synechococcus* sp. PCC 7002 wild-type cells and in *sigE* mutant strain PR6504. The DpsA/PexB-like protein was detected in wild-type cells in stationary phase, but was not detected in strain PR6504 cells. These results strongly imply that a functional SigE protein is required for the production of the DpsA/PexB-like protein.

The occurrence of multiple group 2 σ-factors as observed in cyanobacteria is generally uncommon in eubacteria, although it has also been demonstrated in the *Streptomyces* sp. (Buttner et al. 1990) and in *Chloroflexus aurantiacus* (Gruber and Bryant 1997). Analysis of the complete genome of *Synechocystis* sp. PCC 6803 (Kaneko et al. 1996a, b) has revealed the presence of at least eight σ-factors: one group 1 σ-factor; four group 2 σ-factors; one group 3 σ-factor with strong sequence similarity to SigB of *B. subtilis* (Binnie et al. 1986); and two members of the group 3-RpoE subfamily (Lonetto et al. 1994). Close homologs of each of these eight σ-factors have been identified in the genome of *Synechococcus* sp. PCC 7002 (Caslake and Bryant 1996; Gruber and Bryant 1997; T. Gruber and D. A. Bryant, unpublished results).

The roles of the group 2 σ-factors in gene regulation and cellular physiology are just beginning to be investi-

gated in cyanobacteria. Both nonheterocystous (Tanaka et al. 1992; Gruber and Bryant 1997) and heterocyst-forming (Brahamsha and Haselkorn 1992) cyanobacteria contain multiple group 2 σ-factors, which suggests that these σ-factors are probably not involved in heterocyst differentiation. This was confirmed by Brahamsha and Haselkorn (1992), who have shown that *sigB* and *sigC* mutants of *Anabaena* sp. PCC 7120 are still able to differentiate heterocysts and fix nitrogen. In that study, both the *sigB* and the *sigC* genes were induced under nitrogen-limiting conditions, but mutations in these genes produced no obvious phenotypic change. It is interesting to note that the homolog of *Anabaena* sp. PCC 7120 *sigC* in *Synechococcus* sp. PCC 7002, *sigE*, is expressed under nitrogen-replete conditions (Fig. 4). This suggests that despite the close evolutionary relationship between these two gene products (Gruber and Bryant 1997), their functions in the respective organisms might be different. Recent studies by Tsinoremas et al. (1996) have shown that the RpoD2 protein of *Synechococcus* sp. PCC 7942 plays a role in controlling gene expression during circadian responses of that organism. Caslake et al. (1997) have recently found that SigB and SigC of *Synechococcus* sp. PCC 7002 probably play roles in modifying transcription in response to changes in carbon and nitrogen availability.

The RpoS proteins of *E. coli* and other proteobacteria are the best-characterized members of the group 2 σ-factors of the σ^{70} family (Loewen and Hengge-Aronis 1994). RpoS homologs have not yet been found in any other bacterial family. RpoS has been found to be the key transcription regulator of a group of stress-response proteins induced during the transition to stationary phase [reviewed in Loewen and Hengge-Aronis (1994)], even though it is clearly not the only regulatory molecule involved in the general starvation response (Siegele and Kolter 1992). As many as 32 proteins that are believed to be under the transcriptional control of RpoS (McCann et al. 1991) have been identified on two-dimensional gels. These proteins encompass a diverse group of functions including prevention of and repair of DNA damage, modification of cell morphology, osmoprotection, thermotolerance, and glycogen synthesis (Loewen and Hengge-Aronis 1994). RpoS mutants have reduced viability under several starvation conditions (Lange and Hengge-Aronis 1991; McCann et al. 1991).

No RpoS homolog has been identified in *B. subtilis*, and the majority of general stress genes are induced at SigB-dependent promoters [reviewed in Hecker et al. (1996)]. SigB is a member of the group 3 family of σ^{70}-type σ-factors [reviewed in Haldenwang (1995)]. There is evidence that SigB is required for the induction of more than 40 general stress proteins in *B. subtilis* and that it controls the expression of different genes during entry into stationary phase (Boylan et al. 1993). It is interesting that SigB does not appear to be essential to the survival of *B. subtilis* under several different environmental stresses (Haldenwang 1995). Additionally, *sigB* mutants are not impaired in vegetative growth or in sporulation (Duncan et al. 1987).

These two examples demonstrate that various eubacterial species employ different regulatory mechanisms to respond to changing environments. The composition of σ-factor families within cyanobacteria is quite different from both proteobacteria and gram-positive bacteria. In *E. coli* and *B. subtilis*, the various σ-factors recognize and bind promoters that have distinct nucleotide motifs differing significantly from those recognized and bound by the major vegetative (group 1) σ-factor (Gross et al. 1992; Haldenwang 1995; Hecker et al. 1996). This may not be the case for the group 2 σ-factors of cyanobacteria. The amino acid sequences of regions 2.4 and 4.2, which are expected to contact the –10 and –35 promoter motifs, respectively, are very highly conserved and are nearly identical to the critical promoter-contact sequences, of the same regions of the group 1 σ-factors (see Fig. 2). We propose that changes in patterns of transcription do not result from the recognition of different promoter sequences but primarily result from protein-protein interactions between the group 2 σ-factors and specific transcriptional activators or repressors. Changes in the specific cellular composition of group 1 + 2 σ-factors could still lead to significant shifts in the expression patterns of genes, although the DNA sequences recognized and bound by all might be similar.

The results of this study are consistent with the notion that the participants in the regulatory pathways of cyanobacteria may be different from either of the examples described above. SigE has been implicated in the expression of genes in post-exponential-phase cells, although SigE is not a close homolog of either *E. coli* RpoS or *B. subtilis* SigB. *Synechococcus* sp. PCC 7002 does contain a *B. subtilis* SigB homolog, and characterization of a mutant lacking this gene product is in progress (T. Gruber and D. Bryant, unpublished data). It is very likely that this σ-factor and others are also involved in regulatory pathways functional during stationary phase and nutrient stress. The phenotypic characteristics of the *sigE* mutant strain – difficulty in escaping stationary phase and re-establishing exponential growth – have been observed in certain *E. coli* mutants to an even more drastic extent (i.e., the mutants failed to exit stationary phase; Siegele and Kolter 1992). This behavior could suggest that SigE is involved in the pathway of the synthesis of immediate upshift proteins, which are involved in the recovery from starvation (Marouga and Kjelleberg 1996).

An analysis of the temporal abundance of *sigE* transcripts (Fig. 3) showed that these transcripts are most abundant approximately 48 h after mid-exponential-phase growth. The maximum level of *sigE* transcription (Fig. 3) coincides with the entry of the cells into early stationary phase (also see Fig. 5). Subsequently, the levels decline until no *sigE* mRNA is detectable approximately 100 h after mid-exponential phase. When *Synechococcus* sp. PCC 7002 cells were grown for 10 days and diluted into fresh culture, it was observed that *sigE* transcript levels again became detectable in cultures in early exponential phase (data not shown). These observations suggest that the regulation of SigE levels may be similar to the regulation of RpoS levels in *E. coli*. The cellular concentration of the RpoS protein is controlled at the levels of transcription, translation, and protein stability, and it appears that post-transcriptional events exert the largest effects on protein

levels (Lange and Hengge-Aronis 1994). Translation of *rpoS* mRNA is stimulated as cells enter stationary phase and it is believed that secondary structure of the *rpoS* mRNA probably plays a role in this regulation (Hengge-Aronis 1996). The rate of RpoS synthesis decreases rapidly as cells enter stationary phase, although the concentration of RpoS continues to increase. This apparent discrepancy has been explained by determining the stability of the protein. RpoS is very unstable in exponentially growing cells, and its half-life increases approximately sevenfold in stationary-phase cells (Lange and Hengge-Aronis 1994; Muffler et al. 1996). This regulation pattern is very similar to the one found for the heat-shock σ-factor, RpoH, of *E. coli*. The increase in RpoH levels following the heat-shock induction is controlled by increased translation of *rpoH* transcripts and increased stability of the RpoH σ-factor (Straus et al. 1987). Further studies that involve the monitoring of SigE protein levels will be required to determine the mechanism of regulation in *Synechococcus* sp. PCC 7002. Towards this goal, the SigE protein has been overproduced and is being used to raise polyclonal antibodies (S. Zheng, T. Gruber, D. Bryant, unpublished results).

In *Synechococcus* sp. PCC 7002 strain PR6504, in which the *sigE* gene is insertionally inactivated, production of the DpsA/PexB-like protein was not detectable, indicating that SigE is directly or indirectly involved in its expression. Pena et al. (1995) have isolated a DpsA/PexB-like protein in *Synechococcus* sp. PCC 7942 that is believed to be structurally and functionally similar to the *E. coli* protein. Primer-extension mapping of the *dpsA* transcript of *Synechococcus* sp. PCC 7942 was recently performed (K. Dwivedi and G. Bullerjahn, personal communication). The putative *dpsA* promoter contains a –10 motif (5′-TAGAAT-3′) that is quite similar to the consensus motif for group 1 σ-factors such as the σ^{70} subunit of *E. coli*, but this promoter lacks a –35 sequence element completely. Assuming that transcription of the *dpsA* gene of *Synechococcus* sp. PCC 7942 is also dependent upon a SigE homolog, this result supports the hypothesis that promoters recognized by group 2 σ-factors in cyanobacteria are similar to those recognized by group 1 σ-factors; moreover, transcription from such a promoter could be minimal in the absence of a transcriptional activator.

In conclusion, these studies support the idea that SigE is involved in the regulation of stationary-phase-specific genes. The gene expression patterns and regulatory pathways in stationary phase that have been investigated so far in several bacterial species reveal highly complex systems. It appears that the general stress and starvation responses of bacteria exert nonspecific, protective functions to insure the survival of cells. However, null mutants lacking the cyanobacterial stress response σ-factors of group 2 are still generally viable under laboratory conditions (Brahamsha and Haselkorn 1992; Tsinoremas et al. 1996; Caslake et al. 1997; this work). A remaining challenge is to identify natural environmental conditions under which the specific products of various general stress responses are critical for cell survival and to simulate these conditions in the laboratory.

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