A differential effect of $\sigma^S$ on the expression of the PHO regulon genes of *Escherichia coli*

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The RNA polymerase core associated with $\sigma^S$ transcribes many genes related to stress or to the stationary phase. When cells enter a phase of phosphate starvation, the transcription of several genes and operons, collectively known as the PHO regulon, is strongly induced. The promoters of the PHO genes hitherto analysed are recognized by $\sigma^D$-associated RNA polymerase. A mutation in the gene that encodes $\sigma^S$, rpoS, significantly increases the level of alkaline phosphatase activity and the overproduction of $\sigma^S$ inhibits it. Other PHO genes such as phoE and ugpB are likewise affected by $\sigma^S$. In contrast, pstS, which encodes a periplasmic phosphate-binding protein and is a negative regulator of PHO, is stimulated by $\sigma^S$. The effect of $\sigma^S$ on the PHO genes is at the transcriptional level. It is shown that a cytosine residue at position −13 is important for the positive effect of $\sigma^S$ on pst. The interpretation of these observations is based on the competition between $\sigma^D$ and $\sigma^S$ for the binding to the core RNA polymerase.

INTRODUCTION

The RNA polymerase holoenzyme of *Escherichia coli* is formed by the subunits $\alpha, \beta, \beta'$ and $\omega$ that compose the core of the enzyme and a $\sigma$ subunit. The $\sigma$ subunit, or $\sigma$ factor, is responsible for promoter recognition and transcription initiation, while the core enzyme executes transcription elongation. Seven different $\sigma$ factors have been identified in *E. coli*. The two most important ones are $\sigma^D$ (also known as $\sigma^{70}$) and $\sigma^S$ (or $\sigma^{38}$). $\sigma^D$, when associated with the core enzyme (Ec$^D$), initiates transcription of the majority of the *E. coli* genes while Ec$^S$ recognizes promoters and initiates transcription of genes associated with stationary phase survival and with the response to different stresses, such as osmolarity, pH and temperature shifts (Hengge-Aronis, 2000). More than 70 $\sigma^S$-dependent genes have so far been identified and more will probably be found in the future (Hengge-Aronis, 2002a).

Ec$^D$ and Ec$^S$ recognize similar promoter sequences (Wise et al., 1996; Espinosa-Urgel et al., 1996; Gaal et al., 2001; Lee & Gralla, 2001) and *in vitro* studies have shown that many genes are transcribed by both Ec$^D$ and Ec$^S$ (Nguyen et al., 1993; Tanaka et al., 1993; Kusano et al., 1996; Colland et al., 2000; Bordes et al., 2000), indicating that there is some overlap in promoter recognition by the two sigma factors. However, despite the similarities in promoter recognition *in vitro*, the two sigma factors are normally able to distinguish *in vivo* between $\sigma^D$- and $\sigma^S$-dependent promoters. No significant differences were found in the consensus sequence of the $\sigma^D$- and $\sigma^S$- dependent −35 elements (Becker & Hengge-Aronis, 2001; Lee & Gralla, 2001) except that the −35 region in $\sigma^S$ promoters can be more degenerate than in $\sigma^D$ promoters (Gaal et al., 2001), suggesting that Ec$^S$ interacts weakly or not at all with the −35 element. *In vitro* selection of an optimized $\sigma^S$ promoter ended with identical consensus elements that agree with those of $\sigma^D$-dependent promoters, both in the −10 and −35 positions (Gaal et al., 2001). However, a compilation of 41 $\sigma^S$-dependent promoters has led to the consensus CTACACT at positions −13 to −7 (Lee & Gralla, 2001) and another compilation of 56 promoters reached the consensus TG(n)0–2CYATACT (Lacour et al., 2003). These studies have revealed that over 80% of the natural $\sigma^S$- controlled promoters possess a cytosine at the −13 position (Espinosa-Urgel et al., 1996; Becker & Hengge-Aronis, 2001).

The PHO regulon of *E. coli* consists of more than 40 genes and operons whose transcription is induced under conditions of inorganic phosphate (Pi) starvation and that are related to the uptake and assimilation of Pi and phosphorylated compounds. The best characterized ones are phoA, *phoE*, the *pst* operon and the *ugp* operon, which encode, respectively, alkaline phosphatase (AP), the anion porin PhoE, the Pi transporter Pst and the glycerol-3-phosphate transporter Ugp. Apart from its role as a Pi-transporter, the Pst system also functions as a negative regulator of the PHO regulon, because most mutations in the *pst* operon lead to the constitutive synthesis of all PHO genes (Wanner, 1996). The promoters of the PHO genes display one or more

Abbreviations: AP, alkaline phosphatase; CAT, chloramphenicol acetyl transferase.
consensus regulatory sequences known as PHO-boxes that replace the −35 element. Transcription is regulated by a two-component system that is composed of the proteins PhoB and PhoR. When the concentration of Pi in the medium decreases below a certain level, the sensor protein PhoR auto-phosphorylates and transfers the Pi group to the regulatory protein PhoB, which in turn binds to the PHO-boxes and allows transcription of the PHO genes by interacting with EσD (Wanner, 1996; Makino et al., 1996).

In preliminary experiments we have noticed that in rpoS mutants the expression of AP was considerably stronger than in the wild-type strain, implying that σD is involved in the regulation of AP. Here we demonstrate that σD negatively affects the expression of phoA, phoB, phoE and ugpB, but not pstS. The competition between σD and σD for the core RNA polymerase is proposed to explain this differential effect of σD on the expression of the PHO genes.

### METHODS

#### Strains and plasmids

These are listed in Table 1.

#### Growth media and growth conditions

The rich medium was LB (Miller, 1992). Medium A is a semi-rich medium that is low in Pi (Levinthal et al., 1962). T-salts medium is a Tris-buffered minimal medium supplemented with 0.4 % glucose (Echols et al., 1961) that contains either 1 mM KH2PO4 in the high-Pi minimal medium or 0.1 mM KH2PO4 in the low-Pi minimal medium. For the assay of the kinetics of AP induction, cells were grown in a high-Pi minimal medium supplemented with 0.1 mM KH2PO4 in the low-Pi minimal medium. For the assay of AP activity, cells were grown in minimal high-Pi medium until they reached an OD540 of 0.2–0.3. They were then washed and resuspended in a minimal low-Pi medium. Samples were taken at 30 min intervals for AP assays. For RNA extraction, a 20 ml sample was taken from bacteria growing in minimal high-Pi medium and a second sample was taken from a culture grown for 2 h in minimal low-Pi medium. For the assays of AP and chloramphenicol acetyl transferase (CAT), cells were grown overnight in medium A, and in medium A supplemented with 1 mM KH2PO4.

#### PCR amplifications

The rpoS fragment was amplified using genomic DNA extracted from strain MG1655 as template and the oligonucleotides rpoS+ (TAATCGAGCCAGCAAGGACAGG) and rpoS− (GGCGCGCGGTGAAGCTTACACAAC). Bold letters indicate restriction sites. The DNA fragments used as probes for phoA, phoB, phoE, pstS and ugpB were amplified as above using the oligonucleotides phoA+ (CAGGATCTCAGGAGGATAC) and phoA− (GATCAAGCTTATATGGTAGATTTGAA) and phoB+ (TGCTCATGCTCTTTTGGGAAG) and phoB− (TTGGTTGCATCTGGAATTGATGAT). AP was assayed using pGEM-PLP-NTP (p-NPP) as substrate as described by Spira (1995). AP activity is represented by the increase in absorbance at 410 nm (cell density). Catalase activity was measured qualitatively by mixing 0.1 ml cells (OD540 = 3.0) with 50 ml 3 % hydrogen peroxide and observing the appearance of bubbles caused by the release of O2. CAT assays were performed essentially as described by Shaw (1975). Cells were disrupted by sonication and protein concentration was determined by the method of Bradford (1976). The substrate was 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) together with acetyl-CoA and chloramphenicol in a total volume of 500 μl. The reaction was started by adding chloramphenicol at a final concentration of 0.1 mM to a cuvette containing 0.4 mg DTNB, 0.5 mM acetyl-CoA and cell extract. The absorbance increase rate at 412 nm was recorded. CAT activity was calculated as nmols min−1 (mg protein)−1.

#### Enzyme assays

AP was assayed using p-nitrophenyl-phosphate (p-NPP) as substrate described by Spira et al. (1995). AP-specific activity was represented by the increase in absorbance at 410 nm (cell density)−1. Catalase activity was measured qualitatively by mixing 50 μl cells (OD540 = 3.0) with 50 μl 3 % hydrogen peroxide and observing the appearance of bubbles caused by the release of O2. CAT assays were performed essentially as described by Shaw (1975). Cells were disrupted by sonication and protein concentration was determined by the method of Bradford (1976). The substrate was 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB) together with acetyl-CoA and chloramphenicol in a total volume of 500 μl. The reaction was started by adding chloramphenicol at a final concentration of 0.1 mM to a cuvette containing 0.4 mg DTNB, 0.5 mM acetyl-CoA and cell extract. The absorbance increase rate at 412 nm was recorded. CAT activity was calculated as nmols min−1 (mg protein)−1.

#### RNA extraction and Northern-blot analysis

RNA was extracted by the guandine thiocyanate method, as described by Chomczynski & Sacchi (1987). RNA (20 μg) was electrophoresed in a 1 % agarose

### Table 1. Strains and plasmids used in this study

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<th>Strain</th>
<th>Genotype</th>
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<tr>
<td>BW17335</td>
<td>Δ(ΔactAB) Δ(ϕoU) 560::kan</td>
<td>Steed &amp; Wanner (1993)</td>
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<tr>
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<td>RH90</td>
<td>MC1400 rpoS::Tn10</td>
<td>Lange &amp; Hengge-Aronis (1991)</td>
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### Plasmids

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<td>This work</td>
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<td>Cloning vector</td>
<td>Amersham Pharmacia Biotech</td>
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<tr>
<td>pKK232-8</td>
<td>Cloning vector</td>
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<td>rpoD+ cloned under PtaC</td>
<td>R. Burgess</td>
</tr>
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<td>pNP1</td>
<td>rpoS+ cloned in pKK223-3 under PtaC</td>
<td>This work</td>
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<td>pNP5</td>
<td>rpoS+ cloned in pACT3 under PtaC</td>
<td>This work</td>
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<tr>
<td>pNP6</td>
<td>pBS11 that carries a −13 C→T transition in the pstS promoter</td>
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<td>pRPOD</td>
<td>rpoD+ in pBR322</td>
<td>K. Makino</td>
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gel containing 7% formaldehyde for 3 h. The RNA was transferred to a nylon membrane by capillary action. Probes for phoA, phoB, phoE, pstS and ugpB were synthesized with [γ-32P]dCTP by random primer labelling using the DNA fragments obtained by PCR, as described above. For synthesis of the rpoD probe, a 1–5 kb fragment digested from plasmid pRPOD with BamHI and SacI was used. The labelled probes were hybridized with the membranes at 42°C for 16–20 h and the membranes were exposed to X-ray films.

Site-directed mutagenesis and DNA sequencing. Site-directed mutagenesis was performed by the circular mutagenesis method, using double stranded DNA templates and selection with DpnI, as described by Sambrook & Russell (2001). Plasmid pBS11, carrying a pstS–cat fusion, was used as a template for the PCR reaction. The oligonucleotides pstSmut+ (CTGTCCACCTGTTGCTATTTTG-CTTCTGGTACCACCAACCA) and pstSmut− (GTGTGTTGGCTAGGAGACAAATAAGACAAACGGTGACAG) contain the desired mutation (underlined). The product of the amplification was treated with DpnI and transformed into strains MG1655 and BS16. Both wild-type and mutated plasmids were sequenced in an automatic sequencer type ABI Prism 3100 Genetic Analyser (Applied Biosystems/Hitachi) to confirm the presence of the point mutation.

RESULTS

Effect of rpoS inactivation on AP synthesis

Overnight-grown cultures of the wild-type strain (MG1655) and its isogenic rpoS::Tn10 mutant (BS16) grown in excess or limited Pi media were assayed for AP activity. As expected, cells grown in limited Pi expressed high levels of AP (Fig. 1) while cells grown in excess Pi media showed only a basal level of AP activity that did not exceed a specific activity of 0–015 (not shown). The rpoS mutation caused a threefold increase in AP activity (bar b) when compared to the wild-type strain (bar a), indicating that σS negatively affected the expression of AP. When a multicopy plasmid that carries the wild-type rpoS+ gene under the control of the tac promoter (plasmid pNP1) was introduced into the wild-type strain (bar c) and into the rpoS mutant (bar d), the level of AP activity dropped to approximately half the level of the wild-type parent, suggesting that an excess of σS inhibited AP expression. Introducing plasmid pMRG7, which overexpresses rpoD+, into the wild-type (bar e) and the rpoS mutant (bar f) increased AP activity by 37- and 15-fold above the level of their untransformed parents, respectively. The elevated expression of AP in the presence of the multicopy rpoD+ plasmid supports a previous observation that phoA transcription is driven by EoD (Makino et al., 1993).

The concentration of σS is known to increase progressively in cells that enter the stationary growth phase and in cells that undergo carbon or Pi-starvation (Hengge-Aronis, 1993; Gentry et al., 1993; Ruiz & Silhavy, 2003). To test at what stage of the Pi-starvation process σS affects the expression of AP, exponentially growing cultures of the wild-type strain, rpoS::Tn10 mutant and its transformant carrying plasmid pNP5 (rpoS+ under the control of Ptac and the lacI allele that overproduces the Ptac repressor LacI) were suspended in a low-Pi minimal medium and monitored for AP activity for several hours (Fig. 2). All of them entered the Pi-starvation phase between 30 and 60 min as seen by the induction of AP and the subsequent deceleration of the growth rate (insert). The induced enzyme activity of the wild-type strain reached its maximal level at 90 min (Fig. 2, ), while the activity of the rpoS mutant kept rising and reached a threefold increase over the wild-type at 210 min (Fig. 2, ). The data suggest that at the early starvation phase (as of 90 min) the amount of σS in the cell was already sufficient to prevent further induction of AP. The pNP5 transformant showed a similar pattern of AP induction even in the absence of the inducer (IPTG), suggesting that the tac promoter was sufficiently leaky to suppress the effect

![Fig. 1.](image1) Effect of rpoS on AP activity. Cells were grown overnight in medium A and assayed for AP activity. Bars: a, wild-type (strain MG1655); b, rpoS::Tn10 (strain BS16); c, wild-type transformed with rpoS+ (plasmid pNP1); d, rpoS::Tn10 transformed with pNP1; e, wild-type transformed with rpoD+ (plasmid pMRG7); f, rpoS::Tn10 transformed with pMRG7. Bars represent the means±SE of at least four independent experiments.

![Fig. 2.](image2) Kinetics of the effect of rpoS on AP activity during Pi starvation. Exponential-phase cells were suspended in a low-Pi minimal medium and monitored for growth (insert) and AP activity. Wild-type strain ( ); rpoS::Tn10 ( ); rpoS+ plasmid pNP5 in rpoS::Tn10 without IPTG ( ) and with 100 μM IPTG ( ).
of the rpoS mutation (Fig. 2, ▲). However, the presence of 100 μM IPTG caused a further inhibition of AP activity from the very beginning of the starvation phase (Fig. 2, ●). These results indicate that the negative effect of σS on AP synthesis has already begun at the early Pi-starvation phase and that it is stronger when σS is overexpressed.

**rpoS inhibits AP expression in non-starved cells**

To test if σS is able to inhibit AP expression of non-starved cells, the rpoS::Tn10 mutation was introduced into a strain that carries a deletion of the entire pst operon (strain NP34). This is a constitutive mutant that produces AP independently of the external Pi concentration. Samples were withdrawn every hour from cultures of this mutant and of its rpoS+ parent grown in LB medium (a medium that contains excess Pi). The samples were assayed for growth rate, for AP activity and for catalase activity. Synthesis of catalase (encoded by katE) is strongly dependent on the presence of σS (Schellhorn et al., 1998). The insert in Fig. 3(a) shows that both strains grew exponentially for the first 120 min and entered the stationary phase thereafter. Fig. 3(a) shows that during the first 120 min of exponential growth, both strains presented a similar level of constitutive AP activity. Upon entry into the stationary phase, cells growth was drastically reduced and in the rpoS+ strain the enhanced formation of EσS polymerase led to the expression of genes related to cell survival, such as catalase, and caused the arrest of AP synthesis. Due to its

![Fig. 3. Effect of rpoS on AP activity of PHO-constitutive non-starved cells. Exponential growing cells BS7 (Apst; ◆) and NP34 (Apst rpoS::Tn10; ■) were suspended in LB medium and monitored for growth (insert), for AP activity (a) and for catalase activity, which is observed by the bubbled spots (b).](image)

![Fig. 4. Effect of rpoS on the mRNA level of phoA, phoB, phoE, pstS and ugpB. Exponential-phase wild-type cells (strain MG1655), rpoS::Tn10 mutant (strain BS16) and the rpoS mutant transformed with plasmid pNP1 were resuspended in medium A supplemented or not with Pi and grown for 2 h. RNA was extracted and probed with labelled phoA, phoB, phoE, pstS and ugpB DNA fragments as described in Methods. To confirm that equal amounts of RNA were applied to the gels, the membranes were stripped and rehybridized with a labelled rpoD probe (not shown). Lanes 1 and 4, wild-type; 2 and 5, rpoS::Tn10; 3 and 6, plasmid pNP1 (rpoS+) in rpoS::Tn10. Lanes 1–3, cells grown in excess Pi; 4–6, cells grown in limited Pi.](image)
strong stability (Torriani, 1960) the activity of AP remained constant. The slight and gradual decline of its specific activity [EU (cell density)] is due to the gradual increase in the optical density of the stationary cells (see insert). In contrast, in the rpoSΔ cells, no σ² is formed to compete with σ³, and many of the Eσ-dependant housekeeping genes cease to transcribe, providing extra Eσ available for the increased expression of AP that is evident in these cells. The appearance of catalase activity at the onset of the stationary phase (Fig. 3b) testifies to the induction of σ³. These results demonstrate that σ³ down-regulates AP expression also in the presence of excess Pi and therefore this inhibition is not related to the mechanism of PHO induction by Pi-starvation.

rpoS inhibits the transcription of phoA, phoB, phoE and ugp but not that of pst mRNA

To test whether the effect of σ³ on AP is at the transcriptional level and if the expression of other genes that belong to the PHO regulon are also affected by σ³, Northern blot analyses were conducted. DNA probes that are specific for the genes phoA, phoB, phoE, pstS and ugpB were hybridized with RNA extracted from Eσ-starved and from non-starved wild-type cells, rpoSΔ::Tn10 mutants and rpoSΔ::Tn10 mutants transformed with a rpoSΔ plasmid (pNP1). Fig. 4 shows that, as expected, all probes strongly hybridized with mRNA extracted from Pi-starved cells (lanes 1, 2 and 3), while no signal was detected from hybridization of the probes with mRNA extracted from cells grown in excess Pi (lanes 1, 2 and 3). The signals corresponding to phoA, phoB, phoE and ugpB were significantly stronger in the rpoS mutant (lane 5 as compared to the wild-type lane 4) and were reduced by the overexpression of rpoS (lane 6), indicating that the negative effect on σ³ is at the transcriptional level. In contrast, the signal corresponding to pstS was moderately weaker in the rpoS mutant than in the wild-type and slightly stronger in the presence of the multicopy plasmid that expresses σ³. These results suggest that σ³ inhibits the transcription of phoA, phoB, phoE and ugp and moderately stimulates the transcription of pstS.

A cytosine residue at position –13 is important for σ² recognition of the pst promoter

The inconsistency between pstS and the other PHO genes with regard to their response to σ² may reflect differences in their promoters. PHO promoters are devoid of a –35 sequence, carrying one or more PHO-boxes instead. The –10 regions of all known PHO promoters are depicted in Fig. 5. phoA, phoE, phnC, psiE and ugpB all carry a thymine residue at position –13, phoH has an adenine and phoB a guanine residue, while pstS carries a cytosine at that position. Previous reports have suggested that promoters carrying a C residue at position –13 are preferred by Eσ (Espinosa-Urgel et al., 1996; Bordes et al., 2000; Gaal et al., 2001; Becker & Hengge-Aronis, 2001; Lee & Gralla, 2001). Therefore, the presence of the –13C residue might confer on the pst operon the ability to be transcribed also by Eσ explaining why, in contrast to the other PHO genes, the expression of pstS is reduced rather than induced by the rpoS mutation.

To test this hypothesis, the promoter of pstS, the first gene of the ps promoter and which governs the transcription of the entire operon (Aguena et al., 2002), was cloned in plasmid pKK232-8, creating an operon fusion between the ps promoter and the reporter gene that encodes CAT (cat, plasmid pBS11). The –13 cytosine residue of the pst promoter in this fusion was replaced by a thymine by site-directed mutagenesis (plasmid pNP6). Both plasmids were transformed into the wild-type strain (MG1655) and into its isogenic rpoSΔ::Tn10 mutant. The transformants were grown overnight under limited Pi concentrations and in excess Pi and were assayed for AP and CAT activity. Fig. 6 depicts the ratios of enzyme activities between the rpoS mutant and the wild-type strain transformants, grown under conditions of Pi-starvation. As before, AP activity was twofold higher in the rpoS mutant than in the wild-type (bar a). CAT activity that represents the wild-type ps promoter (–13C) was 25% lower in the rpoS mutant when compared to its rpoSΔ parent (bar c), confirming the lower pstS mRNA level in the absence of σ² (Fig. 4). In contrast, the modified promoter where the –13C residue

<table>
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<tr>
<td>AGTCTGCATAAAAATGTCACGCGGAGAATTATAGTCCGT</td>
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<td>phoB</td>
</tr>
<tr>
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<tr>
<td>TATCTGATAAAAATGTCACGCGGAGAATTATAGTCCGT</td>
<td>ugpB</td>
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Fig. 5. DNA sequences of PHO promoters. The PHO-boxes are framed and the –10 sequences are underlined. The –13 position is in bold.
was replaced by a T (−13T) showed more than a twofold increase in CAT activity in the rpoS mutant (bar d) that was similar to the stimulation of AP activity (bar b). Thus, the C→T transition at the −13 position of the pst promoter caused it to act like the promoters of phoA, phoE and nsp. Under excess Pi the cells showed only low basal levels of both enzymes, indicating that transcription of the mutant pstS is also PhoB-dependent (not shown). These results suggest that the presence of a −13C residue in the pst promoter is important for σ^S recognition of this promoter and below we present an evolutionary rationale to this observation.

**DISCUSSION**

Phosphorus limitation is very common in natural environments (Barik et al., 2001; Sundareswar et al., 2003); hence the PHO regulon of bacteria growing in their natural habitats should be constantly expressed. The activation of the general stress response controlled by σ^D is also frequent in nature (Hengge-Aronis, 2002b). At first sight, the molecular mechanisms used by bacteria to protect themselves against both types of stress should be competitive, if not synergistic. However, during its evolution, *E. coli* developed two separate and apparently antagonistic mechanisms to cope with each type of stress, the σ^S regulon and the PHO regulon. When cells enter the Pi-starvation phase, the PHO regulon is activated and σ^S starts to accumulate in the cytosol (Gentry et al., 1993; Wanner, 1996; Ruiz & Silhavy, 2003). The simultaneity of these events creates a situation where, on one hand, the PHO genes, whose expression is σ^D-dependent, demand their own transcription and, on the other hand, σ^S accumulates and competes with σ^D for the core RNA polymerase. Thus, in this case there is no apparent benefit from the competition between the sigma factors and therefore it is paradoxical that σ^S, which controls the general cell response to stress, inhibits the expression of genes related to phosphate starvation.

In *E. coli*, σ^S and the alarmone guanosine tetraphosphate (ppGpp) are the key factors that promote the transition from growth proliferation to stasis, where proteins related to protection against the deleterious effects of oxidation are expressed. This led to the suggestion that there is a trade-off between bacterial survival and proliferation, such that the expression of genes encoding proteins involved in cell growth is inhibited by factors that promote survival and vice-versa (Nyström, 2003). The PHO regulon genes, whose function is to acquire and assimilate Pi in order to restore cell growth, are inhibited by σ^D whose main concern is with the expression of genes related to cell survival. The competition between σ^S and σ^D for the core RNA polymerase inhibits the σ^D-transcribed PHO genes either directly or indirectly via the σ^S-promoted inhibition of the positive regulator PhoB.

Unlike all other PHO genes tested, *pstS* was somewhat stimulated by the induction of σ^D. In addition to its role in Pi transport the *pst* operon also serves as a negative regulator of the PHO genes (Wanner, 1996). Moreover, its promoter carries a feature shared by many σ^S-promoters (Hengge-Aronis, 2000), namely, the presence of a functional IHF binding site that helps elevate its expression and thereby reduce the expression of AP (Spira & Yagil, 1999). The *pst* promoter is the only one of eight known PHO promoters that possesses a cytosine residue at the −13 position (Fig. 5). Our results suggest that *pst* may be transcribed in vivo by both Eσ^D and Eσ^S, and that the other PHO genes are transcribed only by Eσ^D. Is there a teleological reason for the differential behaviour of *pstS* in relation to the other PHO genes? Being a negative regulator of PHO, an increase in Pst expression would reduce the transcription of the other PHO genes that are driven by σ^D, thereby providing more RNA polymerase core enzyme to interact with σ^S. As a result, σ^D-dependent genes that are important to bacterial survival during stress could be more readily transcribed. In such a trade-off way, the controlled repression of the PHO genes by Pst might be beneficial for cell survival during prolonged Pi starvation periods. The negative effect of *rpoS* on gene expression as a result of σ^D competition against σ^S was already reported for other σ^D-transcribed systems. These include the glucose transport-related genes mal and mgl (Notley-McRobb et al., 2002), the type 1 fimbrial genes fimA and fimB (Dove et al., 1997), ompF (Pratt et al., 1996), the stress-induced gene uspA (Farewell et al., 1998) and several other genes that were found to be hyperexpressed in the absence of a functional σ^S (Xu & Johnson, 1995; Farewell et al., 1998).

Ruiz & Silhavy (2003) have recently shown that in a *pstS* mutant that causes PHO constitutivity σ^S is already strongly
expressed in the exponential growth phase. The results shown in Fig. 3(b), where \( \sigma^S \)-dependent catalase activity was induced only upon entry into the stationary phase, suggest that even if \( \sigma^S \) is expressed at high levels in the exponential phase in PHO-constitutive mutants, it is not able to induce the synthesis of \( \sigma^D \)-dependent promoters. Also, there was no significant difference in the level of AP between the wild-type and the rpoS mutant during the exponential phase (Fig. 3a). Kvint et al. (2000) have demonstrated that \( \sigma^S \)-dependent promoters require ppGpp for induction in the stationary phase, but PHO-constitutive mutants present a low level of ppGpp in the exponential phase of growth (Spira et al., 1995). Thus, if \( \sigma^S \) is present in the exponential phase, it is probably inactive.

In conclusion, we have shown that \( \sigma^S \) negatively affects the expression of several PHO genes, but not that of the pst operon. We suggest that this effect is due to a competition between \( \sigma^S \) and \( \sigma^D \) for the core RNA polymerase. Since the PHO genes are transcribed by \( \sigma^D \), accumulation of \( \sigma^D \) in the cytosol during the starvation phase reduces their transcription. In contrast, \( \sigma^S \), which is also a negative regulator of PHO, may be transcribed by both \( \sigma^S \) and \( \sigma^D \). Through this mechanism the PHO regulon has evolved to maintain a trade-off balance between cell nutrition and cell survival during severe Pi-starvation stress.

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REFERENCES


