

A differential effect of σ^S on the expression of the PHO regulon genes of *Escherichia coli*

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The RNA polymerase core associated with σ^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 σ^D -associated RNA polymerase. A mutation in the gene that encodes σ^S , *rpoS*, significantly increases the level of alkaline phosphatase activity and the overproduction of σ^S inhibits it. Other PHO genes such as *phoE* and *ugpB* are likewise affected by σ^S . In contrast, *pstS*, which encodes a periplasmic phosphate-binding protein and is a negative regulator of PHO, is stimulated by σ^S . The effect of σ^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 σ^S on *pst*. The interpretation of these observations is based on the competition between σ^S and σ^D for the binding to the core RNA polymerase.

INTRODUCTION

The RNA polymerase holoenzyme of *Escherichia coli* is formed by the subunits α , β , β' and ω that compose the core of the enzyme and a σ subunit. The σ subunit, or σ factor, is responsible for promoter recognition and transcription initiation, while the core enzyme executes transcription elongation. Seven different σ factors have been identified in *E. coli*. The two most important ones are σ^D (also known as σ^{70}) and σ^S (or σ^{38}). σ^D , when associated with the core enzyme ($E\sigma^D$), initiates transcription of the majority of the *E. coli* genes while $E\sigma^S$ 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 σ^S -dependent genes have so far been identified and more will probably be found in the future (Hengge-Aronis, 2002a).

$E\sigma^D$ and $E\sigma^S$ 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 $E\sigma^D$ and $E\sigma^S$ (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 σ^D - and σ^S -dependent

promoters. No significant differences were found in the consensus sequence of the σ^D - and σ^S -dependent -35 elements (Becker & Hengge-Aronis, 2001; Lee & Gralla, 2001) except that the -35 region in σ^S promoters can be more degenerate than in σ^D promoters (Gaal *et al.*, 2001), suggesting that $E\sigma^S$ interacts weakly or not at all with the -35 element. *In vitro* selection of an optimized σ^S promoter ended with identical consensus elements that agree with those of σ^D -dependent promoters, both in the -10 and -35 positions (Gaal *et al.*, 2001). However, a compilation of 41 σ^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 σ^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 $E\sigma^D$ (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 σ^S is involved in the regulation of AP. Here we demonstrate that σ^S negatively affects the expression of *phoA*, *phoB*, *phoE* and *ugpB*, but not *pstS*. The competition between σ^S and σ^D for the core RNA polymerase is proposed to explain this differential effect of σ^S 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 KH_2PO_4 in the high-Pi minimal medium or 0.1 mM KH_2PO_4 in the low-Pi minimal medium. For the assay of the kinetics of AP induction, cells were grown in a high-Pi minimal medium until they reached an OD_{540} 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 KH_2PO_4 .

PCR amplifications. The *rpoS* fragment was amplified using genomic DNA extracted from strain MG1655 as template and the oligonucleotides *rpoS*⁺ (ATACTGCAGGCAGCAAAGGACAGG) and *rpoS*[−] (CGTCGCGGCTGAAGCTTACAACAC). 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*⁺ (CAGCATTCCTGCAGACGATAC) and *phoA*[−] (GATCAAGCTTAATGTATTTGTACATGGAGAA), *phoB*⁺ (TCAAAACCTCAAGCGCGAG) and *phoB*[−] (GCTCCAGTGCTT-TACGCA), *phoE*⁺ (ACCTGGGGGCGTTGTATGAC) and *phoE*[−] (TTGGTGCATCTGAGTTGGTAT), *pstS*⁺ (CTTCCTGCGCCG-TGTATGC) and *pstS*[−] (TCAGCGGAGATCAGTTTGGTGTT) and *ugpB*⁺ (GACGCGGTGCTGGAGTTCAATA) and *ugpB*[−] (CCGC-CCCTGGGTTTTTCTCATA), respectively.

Plasmid construction. Plasmids pNP1 and pNP5 were constructed by digesting the *rpoS* PCR fragment with *Pst*I and *Hind*III followed by ligation to the same sites of plasmids pKK223-3 and pACT3, respectively. Plasmid pBS11 was constructed by digesting a *pst* PCR fragment with *Dra*I and *Bst*YI followed by ligation to pKK232-8 digested with *Sma*I and *Bam*HI.

Enzyme assays. AP was assayed using *p*-nitrophenyl-phosphate (*p*-NPP) as substrate as described by Spira *et al.* (1995). AP-specific activity is represented by the increase in absorbance at 410 nm min^{-1} (cell density)^{−1}. Catalase activity was measured qualitatively by mixing 50 μl cells ($\text{OD}_{540} = 3.0$) with 50 μl 3% hydrogen peroxide and observing the appearance of bubbles caused by the release of O_2 . 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 nmoles min^{-1} (mg protein)^{−1}.

RNA extraction and Northern-blot analysis. RNA was extracted by the guanidine 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

Strain	Genotype	Source
BS7	MG1655 Δ (<i>pstSCAB-phoU</i>) 560::KmR	Spira <i>et al.</i> (1995)
BS16	MG1655 <i>rpoS</i> ::Tn10 (P1 transduction from RH90 into MG1655)	This work
BW17335	DE3(<i>lac</i>)X74 Δ (<i>pstCAB-phoU</i>) 560::kan	Steed & Wanner (1993)
MG1655	Wild-type <i>E. coli</i> K-12	Lab collection
NP34	BS16 Δ (<i>pstSCAB-phoU</i>) 560::KmR (P1 transduction from BW17335 into BS16)	This work
RH90	MC4100 <i>rpoS</i> ::Tn10	Lange & Hengge-Aronis (1991)
Plasmids		
pACT3	Cloning vector	Dykxhoorn <i>et al.</i> (1996)
pBS11	<i>pstS</i> – <i>cat</i> transcriptional fusion	This work
pKK223-3	Cloning vector	Amersham Pharmacia Biotech
pKK232-8	Cloning vector	Amersham Pharmacia Biotech
pMRG7	<i>rpoD</i> ⁺ cloned under <i>Ptac</i>	R. Burgess
pNP1	<i>rpoS</i> ⁺ cloned in pKK223-3 under <i>Ptac</i>	This work
pNP5	<i>rpoS</i> ⁺ cloned in pACT3 under <i>Ptac</i>	This work
pNP6	pBS11 that carries a -13 C→T transition in the <i>pstS</i> promoter	This work
pRPOD	<i>rpoD</i> ⁺ in pBR322	K. Makino

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 [α - 32 P]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 *Bam*HI and *Sac*I 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 *Dpn*I, as described by Sambrook & Russell (2001). Plasmid pBS11, carrying a *pstS*–*cat* fusion, was used as a template for the PCR reaction. The oligonucleotides *pstS*mut+ (CTGTACCTGTTGTCTTATTTGCTTCTCGTAGCCAACAAAC) and *pstS*mut– (GTTTGTGGCTAGGAGCAAATAAGACAAACAGGTGACAG) contain the desired mutation (underlined). The product of the amplification was treated with *Dpn*I 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 σ^S 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

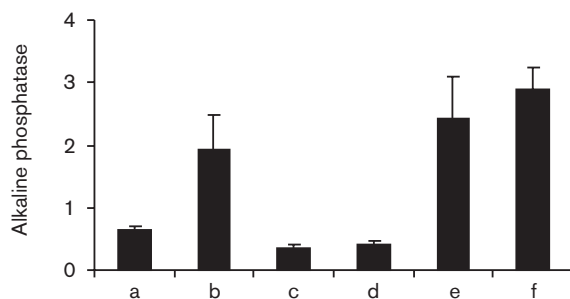


Fig. 1. Effect of *rpoS* on AP activity. Cells were grown overnight in medium A and assayed for AP activity. Bars: a, wild-type (strain MG1655); b, *rpoS*::Tn10 (strain BS16); c, wild-type transformed with pNP1; d, *rpoS*::Tn10 transformed with pNP1; e, wild-type transformed with pMRG7; f, *rpoS*::Tn10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

level of AP activity dropped to approximately half the level of the wild-type parent, suggesting that an excess of σ^S 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 3.7- and 1.5-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 $E\sigma^D$ (Makino *et al.*, 1993).

The concentration of σ^S 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 σ^S 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*^q 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, \blacklozenge), while the activity of the *rpoS* mutant kept rising and reached a threefold increase over the wild-type at 210 min (Fig. 2, \blacksquare). The data suggest that at the early starvation phase (as of 90 min) the amount of σ^S 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

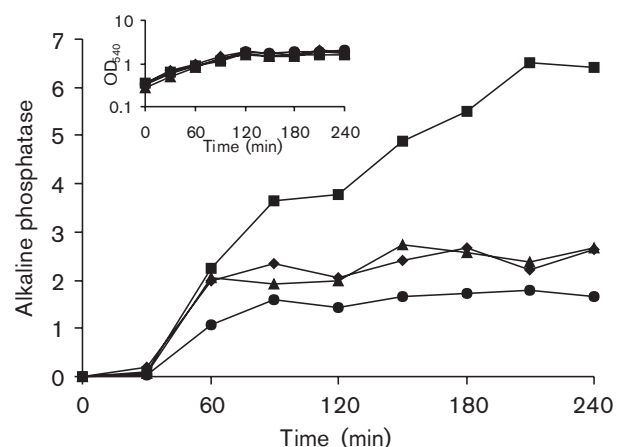


Fig. 2. Kinetics of the effect of *rpoS* on AP activity during Pi starvation. Exponential-phase cells were suspended in a low-Pi minimal medium and monitored for growth (insert) and AP activity. Wild-type strain (\blacklozenge); *rpoS*::Tn10 (\blacksquare); *rpoS*⁺ plasmid pNP5 in *rpoS*::Tn10 without IPTG (\blacktriangle) and with 100 μ M IPTG (\bullet).

of the *rpoS* mutation (Fig. 2, ▲). However, the presence of 100 μ M 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 σ^S on AP synthesis has already begun at the early Pi-starvation phase and that it is stronger when σ^S is overexpressed.

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

To test if σ^S 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 σ^S (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 E σ^S polymerase led to the expression of genes related to cell survival, such as catalase, and caused the arrest of AP synthesis. Due to its

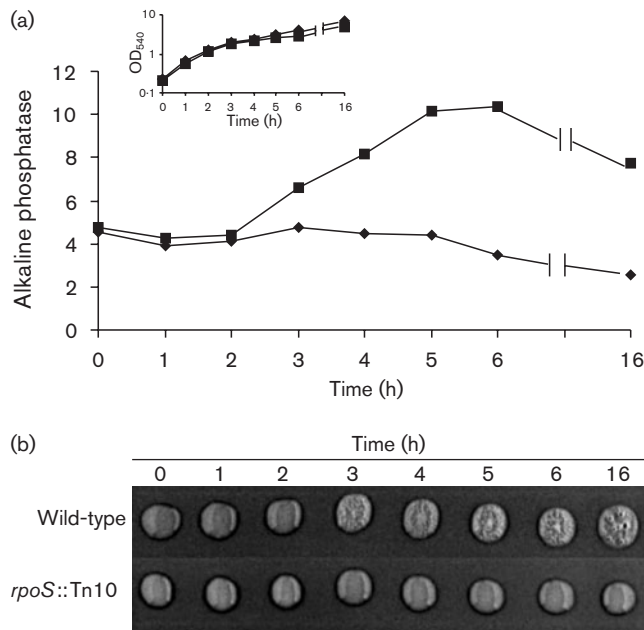


Fig. 3. Effect of *rpoS* on AP activity of PHO-constitutive non-starved cells. Exponential growing cells BS7 (Δpst ; ◆) and NP34 (Δpst *rpoS*::Tn10; ■) were suspended in LB medium and monitored for growth (insert), for AP activity (a) and for catalase activity, which is observed by the bubbled spots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

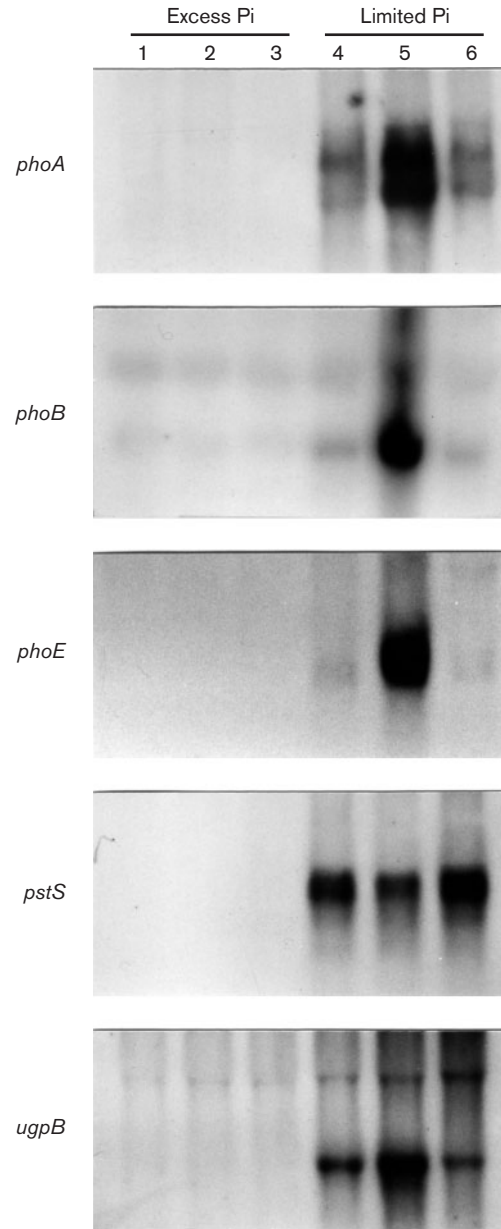


Fig. 4. Effect of *rpoS* on the mRNA level of *phoA*, *phoB*, *phoE*, *pstS* and *ugpB*. Exponential-phase wild-type cells (strain MG1655), *rpoS*::Tn10 mutant (strain BS16) and the *rpoS* mutant transformed with plasmid pNP1 were resuspended in medium A supplemented or not with Pi and grown for 2 h. 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.

strong stability (Torriani, 1960) the activity of AP remained constant. The slight and gradual decline of its specific activity [EU (cell density)⁻¹] is due to the gradual increase in the optical density of the stationary cells (see insert). In contrast, in the *rpoS*⁻ cells, no σ^S is formed to compete with σ^D , and many of the $E\sigma^D$ -dependent housekeeping genes cease to transcribe, providing excess $E\sigma^D$ 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 σ^S . These results demonstrate that σ^S 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 σ^S on AP is at the transcriptional level and if the expression of other genes that belong to the PHO regulon are also affected by σ^S , 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 Pi-starved and from non-starved wild-type cells, *rpoS*::Tn10 mutants and *rpoS*::Tn10 mutants transformed with a *prpoS*⁺ plasmid (pNP1). Fig. 4 shows that, as expected, all probes strongly hybridized with mRNA extracted from Pi-starved cells (lanes 4, 5 and 6), 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 σ^S 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 σ^S . These results suggest that σ^S 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 σ^S recognition of the *pst* promoter

The inconsistency between *pstS* and the other PHO genes with regard to their response to σ^S may reflect sequence 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 $E\sigma^S$ (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 $E\sigma^S$ 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 *pst* operon 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 *pstS* 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 *rpoS*::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 *pstS* promoter (-13C) was 25% lower in the *rpoS* mutant when compared to its *rpoS*⁺ parent (bar c), confirming the lower *pstS* mRNA level in the absence of σ^S (Fig. 4). In contrast, the modified promoter where the -13C residue

PHO-boxes	-10	
AGCTGTCATAAAGTTGTCAC	GGCCGAGACTT	TATAGTCGCT <i>phoA</i>
GCTTTTCATAAATCTGTCA	AAATCTGACG	CATAATGACG <i>phoB</i>
ATCTGTAATATATCTTTAA	AATCTCAGG	TAAAAACTTT <i>phoE</i>
CACTGTCATCACTCTGTCA	CTTCCAGT	AGAAACTAATG <i>phoH</i>
ATCTGTTAGTCACCTTTTAA	TAACCAAATCG	TACAATAATC <i>phnC</i>
AGSTTGAACAAAACATACAC	AAATATAGATC	TCCGTCACATTTTTCGCTTATACAGGAA <i>psiE</i>
CTCTGTCATAAACTGTCA	ATTCCTTACATATAACTGTCA	CTGTTTGTCCTATTTTGCTT <i>pstS</i>
TACTATCTTACAAATGTAAC	AAAAAGTTATTTTCTGTAA	TCGAGCATGTCATGTTACCC <i>ugpB</i>

Fig. 5. DNA sequences of PHO promoters. The PHO-boxes are framed and the -10 sequences are underlined. The -13 position is in bold.

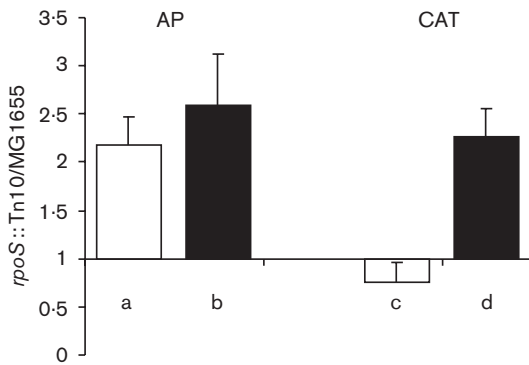


Fig. 6. Effect of *rpoS* on AP and CAT activities in strains carrying a plasmid with a $-13T$ mutation in the promoter region of *pstS*. Cells were grown overnight in medium A and assayed for AP and CAT. Bars represent the enzyme levels of the *rpoS*::Tn10 mutant (strain BS16) divided by those of the wild-type strain (strain MG1655). Bars a and c, strains carrying the wild-type *pst* promoter (plasmid pBS11, $-13C$); b and d, strains carrying the mutated ($-13T$) *pst* promoter (pNP6). Each bar represents the mean \pm SE of at least five independent experiments.

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 \rightarrow T transition at the -13 position of the *pst* promoter caused it to act like the promoters of *phoA*, *phoE* and *ugp*. 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; Sundareshwar *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^S 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 compatible, 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^S 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^S . 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 $E\sigma^D$ and $E\sigma^S$, and that the other PHO genes are transcribed only by $E\sigma^D$. 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^S -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^S competition against σ^D 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 σ^S -dependent catalase activity was induced only upon entry into the stationary phase, suggest that even if σ^S is expressed at high levels in the exponential phase in PHO-constitutive mutants, it is not able to induce the synthesis of σ^S -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 σ^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 σ^S is present in the exponential phase, it is probably inactive.

In conclusion, we have shown that σ^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 σ^S and σ^D for the core RNA polymerase. Since the PHO genes are transcribed by $E\sigma^D$, accumulation of σ^S in the cytosol during the starvation phase reduces their transcription. In contrast, *pst*, which is also a negative regulator of PHO, may be transcribed by both σ^S and σ^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

- Agüena, M., Yagil, E. & Spira, B. (2002). Transcriptional analysis of the *pst* operon of *Escherichia coli*. *Mol Genet Genomics* **268**, 518–524.
- Barik, S. K., Prurshothaman, C. S. & Mohanty, A. N. (2001). Phosphatase activity with reference to bacteria and phosphorus in tropical freshwater aquaculture pond systems. *Aquac Res* **32**, 819–832.
- Becker, G. & Hengge-Aronis, R. (2001). What makes an *Escherichia coli* promoter σ^S dependent? Role of the –13/–14 nucleotide promoter positions and region 2·5 of σ^S . *Mol Microbiol* **39**, 1153–1165.
- Bordes, P., Repoila, F., Kolb, A. & Gutierrez, C. (2000). Involvement of differential efficiency of transcription by $E\sigma^S$ and $E\sigma^{70}$ RNA polymerase holoenzymes in growth phase regulation of the *Escherichia coli* *osmE* promoter. *Mol Microbiol* **35**, 845–853.
- Bradford, M. M. (1976). A rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* **72**, 248–254.
- Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate phenol chloroform extraction. *Anal Biochem* **162**, 156–159.
- Colland, F., Barth, M., Hengge-Aronis, R. & Kolb, A. (2000). σ factor selectivity of *Escherichia coli* RNA polymerase: role for CRP, IHF and Irp transcription factors. *EMBO J* **19**, 3028–3037.
- Dove, S. L., Smith, S. G. & Dorman, C. J. (1997). Control of *Escherichia coli* type 1 fimbrial gene expression in stationary phase: a negative role for RpoS. *Mol Gen Genet* **254**, 13–20.
- Dykxhoorn, D. M., St Pierre, R. & Linn, T. (1996). A set of compatible *tac* promoter expression vectors. *Gene* **177**, 133–136.
- Echols, H., Garen, A., Garen, S. & Torriani, A. (1961). Genetic control of repression of alkaline phosphatase in *E. coli*. *J Mol Biol* **3**, 425–438.
- Espinosa-Urgel, M., Chamizo, C. & Tormo, A. (1996). A consensus structure for σ^S -dependent promoters. *Mol Microbiol* **21**, 657–659.
- Farewell, A., Kvint, K. & Nyström, T. (1998). Negative regulation by RpoS: a case of sigma factor competition. *Mol Microbiol* **29**, 1039–1051.
- Gaal, T., Ross, W., Estrem, S. T., Nguyen, L. H., Burgess, R. R. & Gourse, R. L. (2001). Promoter recognition and discrimination by $E\sigma^S$ RNA polymerase. *Mol Microbiol* **42**, 939–954.
- Gentry, D. R., Hernandez, V. J., Nguyen, L. H., Jensen, D. B. & Cashel, M. (1993). Synthesis of the stationary-phase sigma factor σ^S is positively regulated by ppGpp. *J Bacteriol* **175**, 7982–7989.
- Hengge-Aronis, R. (1993). Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. *Cell* **72**, 165–168.
- Hengge-Aronis, R. (2000). The general stress response in *Escherichia coli*. In *Bacterial Stress Responses*, pp. 161–178. Edited by G. Storz & R. Hengge-Aronis. Washington, DC: American Society for Microbiology.
- Hengge-Aronis, R. (2002a). Signal transduction and regulatory mechanisms involved in control of the σ^S (RpoS) subunit of RNA polymerase. *Microbiol Mol Biol Rev* **66**, 373–395.
- Hengge-Aronis, R. (2002b). Stationary phase gene regulation: what makes an *Escherichia coli* promoter σ^S -selective? *Curr Opin Microbiol* **5**, 591–595.
- Kusano, S., Ding, Q., Fujita, N. & Ishihama, A. (1996). Promoter selectivity of *Escherichia coli* RNA polymerase $E\sigma^{70}$ and $E\sigma^{38}$ holoenzymes. Effect of DNA supercoiling. *J Biol Chem* **271**, 1998–2004.
- Kvint, K., Farewell, A. & Nyström, T. (2000). RpoS-dependent promoters require guanosine tetraphosphate for induction even in the presence of high levels of σ^S . *J Biol Chem* **275**, 14795–14798.
- Lacour, S., Kolb, A. & Landini, P. (2003). Nucleotides from –16 to –12 determine specific promoter recognition by bacterial σ^S -RNA polymerase. *J Biol Chem* **278**, 37160–37168.
- Lange, R. & Hengge-Aronis, R. (1991). Identification of a central regulator of stationary-phase gene expression in *Escherichia coli*. *Mol Microbiol* **5**, 49–59.
- Lee, S. J. & Gralla, J. D. (2001). Sigma38 (*rpoS*) RNA polymerase promoter engagement via –10 region nucleotides. *J Biol Chem* **276**, 30064–30071.
- Levinthal, C., Signer, E. R. & Fetherolf, K. (1962). Reactivation and hybridization of reduced alkaline phosphatase. *Proc Natl Acad Sci U S A* **48**, 1230–1237.
- Makino, K., Amemura, M., Kim, S. K., Nakata, A. & Shinagawa, H. (1993). Role of the sigma 70 subunit of RNA polymerase in transcriptional activation by activator protein PhoB in *Escherichia coli*. *Genes Dev* **7**, 149–160.
- Makino, K., Amemura, M., Kawamoto, T., Kimura, S., Shinagawa, H., Nakata, A. & Suzuki, M. (1996). DNA binding of PhoB and its interaction with RNA polymerase. *J Mol Biol* **259**, 15–26.
- Miller, J. H. (1992). *A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

- Nguyen, L. H., Jensen, D. B., Thompson, N. E., Gentry, D. R. & Burgess, R. R. (1993).** *In vitro* functional characterization of overproduced *Escherichia coli* *katF/rpoS* gene product. *Biochemistry* **32**, 11112–11117.
- Notley-McRobb, L., King, T. & Ferenci, T. (2002).** *rpoS* mutations and loss of general stress resistance in *Escherichia coli* populations as a consequence of conflict between competing stress responses. *J Bacteriol* **184**, 806–811.
- Nyström, T. (2003).** Conditional senescence in bacteria: death of the immortals. *Mol Microbiol* **48**, 17–23.
- Pratt, L. A., Hsing, W., Gibson, K. E. & Silhavy, T. J. (1996).** From acids to *osmZ*: multiple factors influence synthesis of the OmpF and OmpC porins in *Escherichia coli*. *Mol Microbiol* **20**, 911–917.
- Ruiz, N. & Silhavy, T. J. (2003).** Constitutive activation of the *Escherichia coli* Pho regulon upregulates *rpoS* translation in an Hfq-dependent fashion. *J Bacteriol* **185**, 5984–5992.
- Sambrook, J. & Russell, D. W. (2001).** *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schellhorn, H. E., Audia, J. P., Wei, L. I. & Chang, L. (1998).** Identification of conserved, RpoS-dependent stationary-phase genes of *Escherichia coli*. *J Bacteriol* **180**, 6283–6291.
- Shaw, W. V. (1975).** Chloramphenicol acetyltransferase from chloramphenicol-resistant bacteria. *Methods Enzymol* **43**, 737–755.
- Spira, B. & Yagil, E. (1999).** The integration host factor (IHF) affects the expression of the phosphate-binding protein and of alkaline phosphatase in *Escherichia coli*. *Curr Microbiol* **38**, 80–85.
- Spira, B., Silberstein, N. & Yagil, E. (1995).** Guanosine 3',5'-bispyrophosphate (ppGpp) synthesis in cells of *Escherichia coli* starved for Pi. *J Bacteriol* **177**, 4053–4058.
- Steed, P. M. & Wanner, B. L. (1993).** Use of the *rep* technique for allele replacement to construct mutants with deletions of the *pstSCAB-phoU* operon: evidence of a new role for the PhoU protein in the phosphate regulon. *J Bacteriol* **175**, 6797–6809.
- Sundareshwar, P. V., Morris, J. T., Koepfler, E. K. & Fornwalt, B. (2003).** Phosphorus limitation of coastal ecosystem processes. *Science* **299**, 563–565.
- Tanaka, K., Takayanagi, Y., Fujita, N., Ishihama, A. & Takahashi, H. (1993).** Heterogeneity of the principal σ factor in *Escherichia coli*: the *rpoS* gene product, σ^{38} , is a second principal σ factor of RNA polymerase in stationary-phase *Escherichia coli*. *Proc Natl Acad Sci U S A* **90**, 3511–3515.
- Torriani, A. (1960).** Influence of inorganic phosphates in the formation of phosphatases of *Escherichia coli*. *Biochim Biophys Acta* **38**, 460–469.
- Wanner, B. (1996).** Phosphorus assimilation and control of the phosphate regulon. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, pp. 1357–1381. Edited by F. C. Neidhardt & others. Washington, DC: American Society for Microbiology.
- Wise, A., Brems, R., Ramakrishnan, V. & Villarejo, M. (1996).** Sequences in the –35 region of *Escherichia coli* *rpoS*-dependent genes promote transcription by $E\sigma^S$. *J Bacteriol* **178**, 2785–2793.
- Xu, J. & Johnson, R. C. (1995).** Identification of genes negatively regulated by Fis: Fis and RpoS comodulate growth-phase-dependent gene expression in *Escherichia coli*. *J Bacteriol* **177**, 938–947.