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Constitutive Activation of the *Escherichia coli* Pho Regulon Upregulates *rpoS* Translation in an Hfq-Dependent Fashion

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Regulation of the σ factor RpoS occurs at the levels of transcription, translation, and protein stability activity, and it determines whether *Escherichia coli* turns on or off the stationary-phase response. To better understand the regulation of RpoS, we conducted genetic screens and found that mutations in the *pst* locus cause accumulation of RpoS during exponential growth. The *pst* locus encodes for the components of the high-affinity transport system for inorganic phosphate (P_i), which is involved in sensing P_i levels in the environment. When the Pst transporter is compromised (either by mutation or by P_i starvation), the two-component system PhoBR activates the transcription of the Pho regulon, a subset of genes that encode proteins for transporting and metabolizing alternative phosphate sources. Our data show that strains carrying mutations which constitutively activate the Pho regulon have increased *rpoS* translation during exponential growth. This upregulation of *rpoS* translation is Hfq dependent, suggesting the involvement of a small regulatory RNA (sRNA). The transcription of this yet-to-be-identified sRNA is regulated by the PhoBR two-component system.

The preferred source of phosphorus for *Escherichia coli* is inorganic phosphate (P_i), and *E. coli* uses several transporters to import P_i into the cytoplasm. These are the two low-affinity P_i transporters PitA and PitB (8, 10) and the high-affinity transporter Pst. The Pst transporter is a complex composed of the periplasmic P_i-binding protein PstS, the integral inner membrane proteins PstA and PstC, and the ATP-binding protein PstB (for a review see reference 32), and it serves as the cell's gauge for the extracellular availability of P_i as follows. The Pst transport system is associated with the PhoR histidine kinase/phosphatase, which controls the phosphorylation state of the response regulator PhoB. The functional status of the Pst transport system is sensed through an unknown mechanism by PhoR, so that if Pst can easily transport P_i into the cytoplasm (because of high extracellular P_i concentration), PhoR acts as a PhoB phosphatase. Consequently, unphosphorylated PhoB cannot activate the transcription of the Pho regulon, a group of genes that encode proteins involved in scavenging and metabolizing phosphorus sources other than P_i. On the contrary, low extracellular P_i concentrations reduce transport by Pst, which increases the kinase activity of PhoR, resulting in the accumulation of phosphorylated PhoB (PhoB-P). Likewise, loss-of-function mutations in the *pst* genes lead to the constitutive phosphorylation of PhoB, regardless of the external P_i concentration. Once phosphorylated, PhoB-P transcriptionally activates the Pho regulon, and an effort to scavenge phosphorus begins. However, because phosphorus is limiting in nature, even the induction of the Pho regulon might not be enough to ensure cell survival. Therefore, to protect itself during P_i starvation, *E. coli* triggers a developmental program known as stationary phase (5).

Stationary phase is a general stress response that leads to dramatic changes in the protein profile and cellular composition and metabolism, which increase the cell's resistance to many different harmful conditions (for a review see reference 9). The central regulator of stationary-phase transcription is the sigma factor RpoS (or σ^s), whose concentration in the cell is tightly regulated at the levels of transcription, translation, and protein stability (9). Although recent studies have identified new factors involved in the transcriptional control of *rpoS* (9), much more is known about the mechanisms controlling its translation and stability. During exponential growth, *rpoS* translation is maintained at very low levels by the inhibitory action of a *cis*-acting element present in the *rpoS* mRNA. The *rpoS* message has an unusually long 5′ untranslated region (UTR) that contains a region capable of binding to the ribosome-binding site (rbs) and the initiation region for *rpoS* translation (for a review see reference 7). As a result of this RNA-RNA interaction, access to the rbs by the ribosome is blocked, so *rpoS* translation is reduced. This inhibitory binding can be modulated by the action of several regulatory noncoding small RNAs (sRNAs) and the RNA chaperone Hfq. Specifically, the DsrA and RprA sRNAs positively regulate *rpoS* translation by pairing with the upstream element of the 5′ UTR that occludes the rbs, while the OxyS sRNA negatively regulates *rpoS* translation through an unknown mechanism (7, 9, 16, 18, 19, 35). All three sRNAs require the RNA chaperone, Hfq (19, 30, 35, 36), to regulate *rpoS* translation, but how Hfq promotes interactions between these different sRNAs and *rpoS* is not clear.

Another mechanism for the cell to increase RpoS levels is by preventing its degradation. Besides reduced *rpoS* synthesis, degradation of RpoS by the ClpXP proteolytic complex ensures that the RpoS content in the cell is very low during exponential growth (26). The regulatory mechanism controlling this proteolysis involves SprE (also named RssB), an orphan response regulator (20, 22). In vitro studies have shown

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TABLE 1. Strains used in this study

Strain	Genotype	Reference or source
MC4100	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flbB5301 deoC1 ptsF25 rbsR*	2
RO91	MC4100 λφ(*rpoS742′-′lacZ*)	14
LP810	RO91 *sprE19*::*cam*	22
AF633	MC4100 λφ(*uspB′-lacZ*$^+$)	4
TJP1	AF633 Δ(*pta ackA hisQ hisP*)	3
TJP2	TJP1 *pstS*::*cam*	
NR350	AF633 *pstS*::*cam*	
NR364	NR350 Δ(*brnQphoBR*)	Barry Wanner[a]
NR365	AF633 Δ(*brnQphoBR*)	
NR419	AF633 *rssA2*::*cam*	25
NR612	MC4100 λφ(*rpoS750′-lacZ*$^+$)	29
NR613	MC4100 λφ(*rpoS750′-′lacZ*)	29
NR614	NR612 *sprE*::*tet*	25
NR616	NR612 *hfq1*::*kan*	31
NR619	NR612 *pstS*::*cam*	
NR620	NR613 *sprE*::*tet*	
NR625	NR613 *pstS*::*cam*	
NR628	NR613 *pstS*::*cam sprE*::*tet*	
NR629	MC4100 λφ(*rpoS477′-′lacZ*)	Eric Masse and Susan Gottesman
NR630	NR629 *sprE*::*tet*	
NR632	NR629 *pstS*::*cam*	
NR633	NR629 *hfq1*::*kan*	
NR634	NR630 *hfq1*::*kan*	
NR635	NR632 *hfq1*::*kan*	
NR641	NR629 *zed-3069*::Tn*10*	29
NR642	NR629 *zed-3069*::Tn*10* ΔdsrA	29
NR643	NR632 *zed-3069*::Tn*10*	
NR644	NR632 *zed-3069*::Tn*10* ΔdsrA	

[a] Deletion Δ(*brnQphoBR*) was derived from strain BW 13746 as described in Materials and Methods.

that SprE catalytically delivers RpoS to the ClpXP protease (11, 37), but it still remains unknown how SprE's activity is regulated. Understanding the regulation of this proteolysis is further complicated by the existence of a negative feedback loop; namely, transcription of *sprE* is RpoS dependent (25).

Here we show that mutations that disrupt genes encoding the Pst transporter cause accumulation of RpoS during exponential growth. As described above, mutations that disrupt this P_i transporter lead to the constitutive activation of the Pho regulon. Like the activation of the Pho regulon, the upregulation of RpoS levels also requires PhoBR. Using *lacZ* reporter fusions, we determined that the mutation in *pstS* increases *rpoS* translation. Because this regulation requires Hfq, we propose that there is involvement of a PhoBR-regulated sRNA.

MATERIALS AND METHODS

Bacterial strains, bacteriophages, and *rpoS-lacZ* reporter fusions. Standard microbial techniques were used for strain construction (27). The bacterial strains used are derivatives of MC4100 (2) and are listed in Table 1. The allele referred to in these studies as Δ*phoBR* is the Δ(*brnQ phoB phoR*) allele from strain BW13746 (from the B. L. Wanner laboratory collection) and was introduced by P1 transduction into various strains carrying a linked *proC*::Tn*10* allele by selecting for growth in glucose minimal medium lacking proline. The *hfq1*::*kan*, *rprA*::*kan*, and Δ*dsrA* alleles used were described previously (19, 29, 31). The λ vectors NK1323 and NK1324, which carry Tn*tet* and Tn*cam* minitransposons, respectively, were used for mutagenesis (12). The location of the minitransposon on the chromosome was determined by DNA sequencing by the Princeton University Department of Molecular Biology Synthesis and Sequencing Facility, as previously described (6).

We used four different *rpoS-lacZ* reporter fusions: *rpoS750′-lacZ*$^+$ to follow *rpoS* transcription (fusion joint at nucleotide +750 of *rpoS* [29]); *rpoS477′-′lacZ* to measure *rpoS* transcription and translation (fusion joint at nucleotide +477 of *rpoS* [personal communication, Eric Masse and Susan Gottesman]); and the *rpoS742′-′lacZ* and *rpoS750′-′lacZ* fusions, which serve as reporters of transcription, translation, and stability of RpoS (fusion joint at nucleotides +742 and +750 of *rpoS*, respectively [14, 29]).

Media and growth conditions. Luria-Bertani (LB) and lactose MacConkey agar media were prepared as described previously (27). Morpholinepropanesulfonic acid (MOPS) minimal medium was made as described by Bochner and Ames (1). For starvation experiments, MOPS medium was made without KH_2PO_4. All liquid cultures were grown under aeration at 37°C, and their growth was monitored by measuring the optical density at 600 nm (OD_{600}). Unless otherwise indicated, all experiments were done by growing cells in LB broth.

Starvation experiments. Cultures grown overnight in complete minimal MOPS medium were diluted 1:100 into fresh complete minimal MOPS medium. When the OD_{600} reached 0.3 to 0.4, samples were taken and processed for Western blot analysis or β-galactosidase assays as described below. The rest of the cells were pelleted for 5 min at 1,300 × *g* at room temperature, washed once in prewarmed (37°C) medium lacking KH_2PO_4, resuspended in prewarmed MOPS minimal medium lacking KH_2PO_4, and returned to the 37°C incubator. At the times indicated, samples were taken and processed for Western blot analysis or β-galactosidase assays.

Western blot analysis. Once cells reached the indicated OD_{600}, 1-ml samples were pelleted. To standardize samples, pellets were resuspended in a volume (in ml) of sodium dodecyl sulfate sample buffer equal to OD_{600}/6. Samples were boiled for 10 min, and equal volumes were subjected to electrophoresis in 12% polyacrylamide gels containing sodium dodecyl sulfate as described by Laemmli (13). Proteins were transferred to nitrocellulose membranes (Schleicher & Schuell), and Western blot analysis was performed as previously described (25). When appropriate, polyclonal sera against RpoS (from our laboratory collection) or alkaline phosphatase (5-3 Prime, Inc.) were used as primary antibodies at a dilution of 1:6,000 and 1:10,000, respectively. Donkey anti-rabbit immunoglobulin G horseradish peroxidase conjugate (Amersham Pharmacia Biotech) was used as secondary antibody at a 1:6,000 dilution. For visualization of bands, the ECL antibody detection kit (Amersham Pharmacia Biotech) and X-Omat film (Kodak) were used.

β-Galactosidase assays. Cultures grown overnight in LB broth were diluted 1:1,000 into fresh LB broth and were grown to an OD_{600} of ~0.3 to 0.4 for logarithmic phase samples or to an OD_{600} of ~3.0 for stationary-phase samples. Samples subject to starvation were treated as explained above. β-Galactosidase assays were performed with a microtiter plate assay as described previously (28). The β-galactosidase activities are expressed as $\Delta(OD_{420}/\text{time})/(OD_{600} \times \text{volume})$, where volume refers to the amount (in milliters) of cell suspension used. For each experiment every sample was assayed three times, and their average activity and standard deviation are shown. The data presented were derived from a single experiment representative of at least three independent experiments.

RESULTS

Genetic screens. Our laboratory has devised several genetic screens to identify new factors that regulate RpoS in *E. coli*. For one such screen we used a strain carrying the *rpoS742′-′lacZ* reporter hybrid fusion and the *sprE19*::*cam* allele (strain LP810; Table 1). The *rpoS742′-′lacZ* reporter fusion is subject to the same transcriptional, translational, and proteolytic degradation regulation as RpoS itself (14), while the *sprE19*::*cam* allele causes overexpression of *sprE*. Because of the increased levels of SprE, our starting strain has low Lac activity (22), which allowed us to screen for mutants with increased Lac activity on lactose MacConkey agar following a Tn*tet* minitransposon mutagenesis (12). Among the mutations found to increase Lac activity we found an insertion in *pstA*.

We conducted a second screen with a strain carrying the RpoS-regulated reporter fusion *uspB′-lacZ*$^+$ (4) that was incapable of producing acetyl phosphate due to the Δ(*ackA pta*) deletion (strain TJP1; Table 1). Because of the possible involvement of a two-component kinase in the regulation of RpoS, we chose to use a strain deficient in acetyl phosphate. This small phosphodonor has been reported to phosphorylate response regulators in the absence of their cognate histidine

kinases (3), so we reasoned that it could interfere with finding mutations in histidine kinases that affect RpoS.

A Tn*cam* minitransposon (12) was used to mutagenize TJP1, and chloramphenicol-resistant colonies were screened for increased Lac activity on lactose MacConkey agar. Because we were only interested in mutations that affected the fusion *uspB′-lacZ*$^+$ in an RpoS-dependent manner, we performed an epistasis test with an *rpoS*::*kan* null allele, and strains that retained increased Lac activity in the absence of RpoS were discarded. One candidate that answered our requirements carried an insertion in *pstS*. Thus, both screens described above yielded mutants carrying insertions in the *pst* locus (namely, in *pstA* and *pstS*). Since all subsequent characterizations revealed that both mutations behave identically, we will only focus on the *pstS*::*cam* mutation for simplicity.

Sequence analysis revealed that the Tn*cam* minitransposon had inserted between nucleotides 305 and 306 of the *pstS* open reading frame (the direction of transcription of the *cam* cassette is the same as that of *pstS*). The *pstS* gene is the first of the *pstSCAB-phoU* operon, which encodes the components of the Pst transport system, the high-affinity P_i transport system reviewed in reference 32. As noted above, the functional status of the Pst transporter regulates the activation of the Pho regulon via PhoBR, and mutations that disrupt the Pst transporter lead to the constitutive activation of the Pho regulon (32). To confirm that our *pstS*::*cam* allele causes the constitutive activation of the Pho regulon in a PhoBR-dependent manner, we monitored the levels of alkaline phosphatase (a member of the Pho regulon) by Western blot analysis. As Fig. 1 shows, strains carrying the *pstS*::*cam* allele produce constitutively high levels of alkaline phosphatase (compare wt and *pstS*::*cam* lanes in panel B) in a PhoBR-dependent manner (compare *pstS*::*cam* and *pstS*::*cam* Δ*phoBR* lanes in panel B).

Disruption of the *pst* locus increases RpoS levels during exponential growth in a PhoBR-dependent manner. As stated above, disruption of the Pst transporter increases the LacZ activity of both the *rpoS742′-′lacZ* and the *uspB′-lacZ*$^+$ fusions, suggesting that mutations in *pst* might increase the levels of RpoS. To test this hypothesis, we compared the amount of RpoS present in a wild-type and a *pstS*::*cam* mutant strain grown in LB broth by using Western blot analysis. As shown in Fig. 1, the *pstS*::*cam* allele caused a dramatic accumulation of RpoS in exponentially growing cells (compare wt and *pstS*::*cam* lanes in panel A). In contrast, no significant difference in RpoS levels was detectable between stationary-phase samples from wild-type and *pstS*::*cam* mutant strains (Fig. 1A). Similar results were obtained with cells grown in glucose MOPS minimal medium (data not shown). Together, these findings show that mutations in the genes encoding the Pst transport system increase RpoS levels during the exponential phase of growth.

Next we wanted to determine whether the increase in RpoS levels in the *pstS*::*cam* mutant is a result of the constitutive activation of the Pho regulon. If so, a deletion in *phoBR* would eliminate the increase in RpoS levels caused by the *pstS*::*cam* mutation. Figure 1 shows that, indeed, a Δ*phoBR* deletion suppressed the increase in RpoS caused by the *pstS* mutation (compare *pstS*::*cam* and *pstS*::*cam* Δ*phoBR* lanes in panel A, log samples). From these data we conclude that, during exponential growth, constitutive activation of the Pho regulon leads to high levels of RpoS.

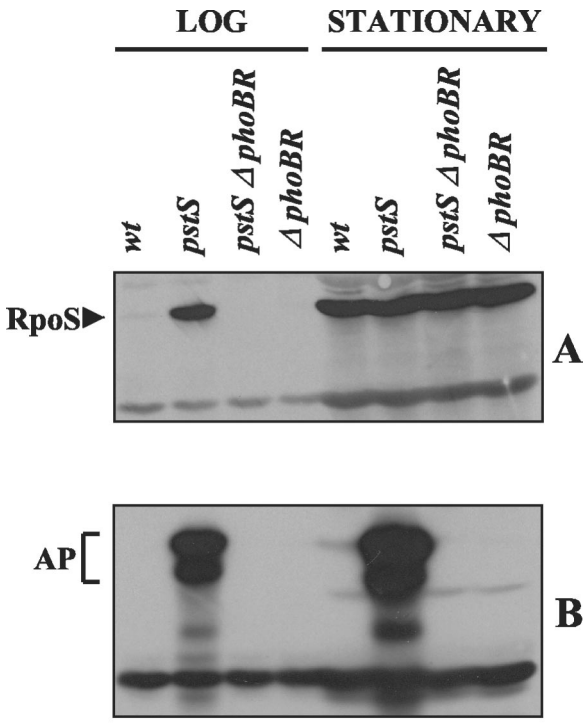

FIG. 1. Activation of the Pho regulon increases RpoS levels during logarithmic growth. (A) RpoS Western blot analysis demonstrates that disruption of *pstS* causes an increase in RpoS levels in a PhoBR-dependent manner. This increase is detectable in exponentially growing cells (LOG samples) but not in stationary-phase cells (STATIONARY samples). (B) Western blotting against alkaline phosphatase (AP) was used to determine the activation status of the Pho regulon. The constitutive activation of the Pho regulon caused by the *pstS* mutation requires PhoBR. The relevant genotype of the strains the samples were obtained from is indicated above the lanes (wt refers to the wild-type strain AF633, *pstS* refers to NR350, *pstS*Δ*phoBR* refers to NR364, and Δ*phoBR* refers to NR365 [Table 1]). Cells were grown in LB broth and were processed as described in Materials and Methods.

Disruption of *pstS* does not halt RpoS degradation by ClpXP. During exponential growth, reduced levels of RpoS are maintained because RpoS synthesis is kept at low levels, and most of the RpoS that is made is rapidly degraded by the ClpXP protease (14, 26). Although many factors regulate the synthesis of RpoS, only ClpXP and SprE have been shown to be required for the proteolytic regulation of RpoS (9). Because the high levels of RpoS found in the *pstS*::*cam* mutant during exponential growth resembled those of a *sprE* or *clp* null strain, we wanted to test whether mutations in the RpoS degradation pathway are additive or epistatic to *pstS*::*cam*.

As expected, strains carrying either a *sprE* or a *pstS* null allele contained high levels of RpoS with respect to wild-type cells during exponential growth (Fig. 2A). These levels increased even further in a *pstS sprE* double mutant (Fig. 2A). We also demonstrated this additivity by using the RpoS750-LacZ hybrid protein which, like RpoS itself, is regulated at the levels of transcription, translation, and protein stability. The levels of this RpoS750-LacZ hybrid protein in the *pstS sprE* double mutant strain were higher than in each of the single mutants (Fig. 2B). This additivity between the *pstS* and *sprE* mutations suggests that *pstS*::*cam* does not increase RpoS lev-

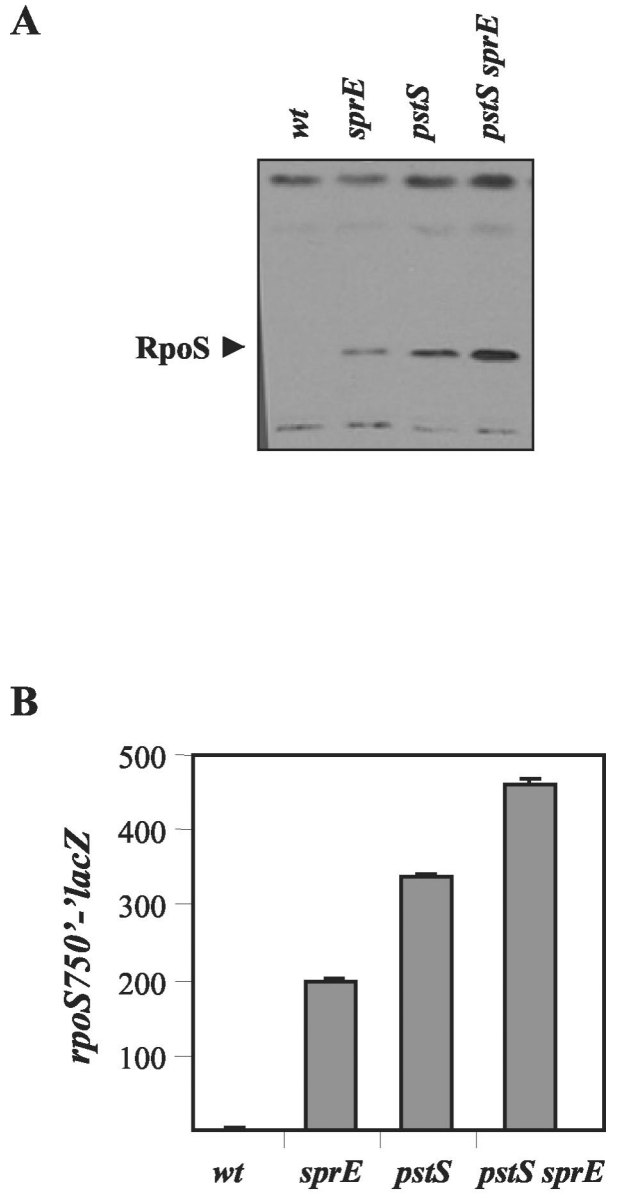

FIG. 2. The *sprE* and *pstS* alleles are additive. (A) Western blot analysis conducted with exponentially growing cells demonstrates additivity of *sprE* and *pstS* null alleles. The *sprE pstS* double mutant contains more RpoS than either single mutant. (B) The results presented in panel A were confirmed by using the *rpoS750′-'lacZ* reporter fusion. Epistasis analysis shows that the already high levels of LacZ present in the *sprE* and *pstS* single mutants increase even further in the *pstS sprE* double mutant. Bar values represent the relative LacZ activity present in each strain, and they are presented as the averages ± standard deviations (error bars) of each sample assayed in triplicate. The data are representative of at least three experiments. The relevant strain genotype is indicated above the lanes of the Western blot presented in panel A and below the bars in panel B (wt refers to the wild-type strain NR613, *sprE* refers to NR620, *pstS* refers to NR625, and *pstS sprE* refers to NR628 [Table 1]). Samples were processed as described in Materials and Methods.

els by inhibiting its ClpXP-dependent proteolysis. Rather, *pstS*::*cam* must increase RpoS synthesis.

We note that although RpoS is still degraded in a *pstS* mutant, it appears to be more stable than in the wild-type strain (data not shown). However, the effect of the *pstS* mutation on turnover cannot be direct. SprE and ClpXP are the only factors that have been identified to directly control RpoS degradation. When null mutations in either of these factors are introduced, RpoS is not subject to detectable degradation. If the *pstS* mutation was directly affecting RpoS degradation, we would not see an increase in RpoS levels in the *pst sprE* double mutant just as there is no increase in strains carrying both *clpP* and *sprE* null alleles. We therefore believe that the effect of the *pstS* mutation on *rpoS* synthesis indirectly affects its stability. This is in agreement with previous reports showing that increasing the synthesis of RpoS can result in an increase in its half-life, because the high levels of RpoS overwhelm the degradation machinery (23). In fact, alleles of *sprE* that produce higher levels of SprE than the wild-type strain (the *rssA2*::*cam* allele; see below) reduce but do not eliminate the accumulation of RpoS in a *pstS* mutant strain (data not shown). Taken together, all these data demonstrate that the *sprE* and *pstS* mutations directly act at different levels of RpoS regulation.

***rpoS* translation is increased in a *pstS*::*cam* mutant.** Since RpoS synthesis can be regulated both transcriptionally and translationally, we used two reporter fusions in parallel to distinguish at what level the *pstS* mutation might be increasing *rpoS* synthesis. The *rpoS750′-lacZ*$^+$ fusion is a reporter for transcription only, while the *rpoS477′-'lacZ* fusion is subject to both transcriptional and translational regulation. The hybrid protein made by the latter fusion does not contain the domain in RpoS that is required for the ClpXP degradation; therefore, unlike RpoS, this fusion protein is not regulated at the level of protein stability.

To determine the effects of the *pstS* mutation on *rpoS* transcription, we measured the β-galactosidase activity during exponential growth of various strains carrying the *rpoS750′-lacZ*$^+$ fusion. As Fig. 3A shows, the *pstS* mutation did not significantly change the levels of *rpoS* transcription with respect to the wild-type parent strain. Likewise, mutations in genes encoding factors known to regulate RpoS stability (*sprE*) or translation (*hfq*) did not alter the activity of this *rpoS750′-lacZ*$^+$ transcriptional fusion (Fig. 3A).

We next measured the effect of the mutations above on the *rpoS477′-'lacZ* fusion. As expected, a mutation in *sprE* did not change the LacZ activity of cells carrying this translational fusion (Fig. 3B). In contrast, the *pstS* mutation increased the LacZ activity in cells carrying the *rpoS477′-'lacZ* fusion approximately three- to fourfold (Fig. 3B). When we compare data from both fusions, we can conclude that disruption of *pstS* results in an upregulation of *rpoS* translation but not transcription.

Hfq is necessary for the increase in RpoS caused by the disruption of Pst. The *rpoS* mRNA contains a 5′ UTR that interacts with a region just upstream of the start codon, occluding the rbs, and therefore inhibiting *rpoS* translation (9). Factors affecting this RNA-RNA interaction modulate *rpoS* translation, and they include sRNAs and Hfq. To this date, three sRNAs have been shown to either decrease (OxyS) or increase (DsrA and RprA) *rpoS* translation, and Hfq is required for their action (17, 21, 30, 35).

Since the *pstS* mutation increases *rpoS* translation, we thought it likely that Hfq and an sRNA would be involved. To test whether Hfq participates in the regulation of RpoS by *pstS*,

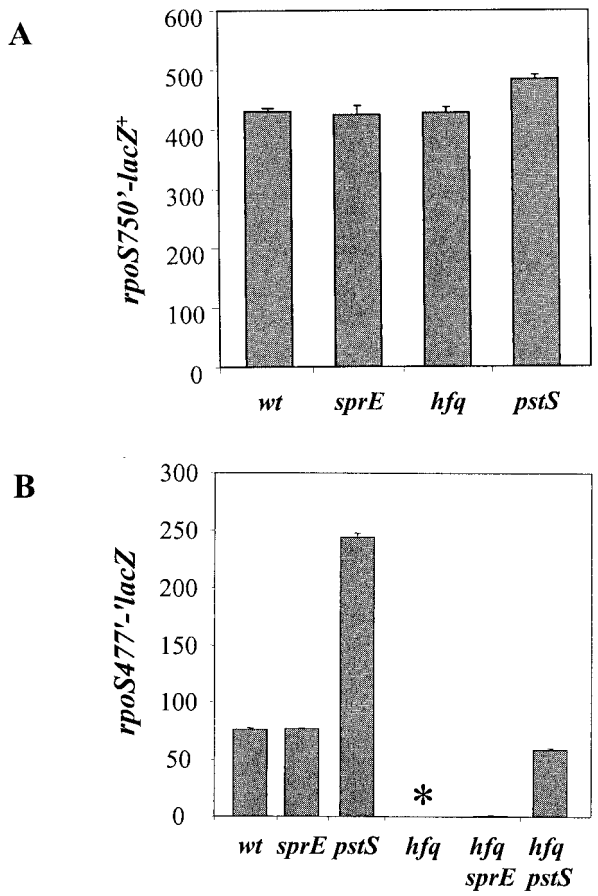

FIG. 3. Disruption of *pstS* increases translation but not transcription of *rpoS*. (A) The reporter fusion *rpoS750′-lacZ*$^+$, which reflects *rpoS* transcription, was used to determine at what levels of regulation the *pstS* mutation increases *rpoS* synthesis. Samples from exponentially growing cells were processed, and their LacZ activity was determined as indicated in Materials and Methods. The *pstS* mutation did not significantly increase the LacZ activity of this transcriptional reporter fusion. As controls, samples from strains carrying mutations (*sprE* and *hfq*) known not to affect *rpoS* transcription were also analyzed. (B) The LacZ activity of a strain carrying the *rpoS* translation reporter fusion *rpoS477′-′lacZ* was increased in the presence of a *pstS* mutation. As expected, the activity of this fusion was not affected by a mutation in *sprE*, but it was below the detection limits (indicated with an asterisk) in samples from a strain carrying a mutation in *hfq*. While a *sprE* mutation cannot suppress the reduction in *rpoS* translation of an *hfq* mutation, a *pstS* mutation partially can. The relevant genotype for each strain is given below each bar (in panel A, wt refers to the wild-type strain NR612, *sprE* refers to NR614, *hfq* refers to NR616, and *pstS* refers to NR619; in panel B, wt refers to the wild-type strain NR629, *sprE* refers to NR630, *pstS* refers to NR632, *hfq* refers to NR633, *hfq sprE* refers to NR634, and *hfq pstS* refers to NR635 [Table 1]). Bar values represent the relative LacZ activity present in each strain, and they are presented as the averages ± standard deviations (error bars) of each sample assayed in triplicate. The data are representative of at least three experiments.

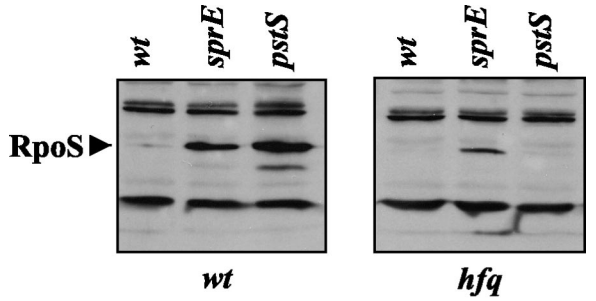

FIG. 4. Hfq is necessary for the increase in RpoS levels caused by a *pstS* mutation. The role of Hfq in the accumulation of RpoS caused by the *sprE* and *pstS* mutations was determined by Western blot analysis. Samples from strains carrying either the *sprE* or the *pstS* mutation (indicated above the panels) in wild-type or *hfq* mutant backgrounds (indicated below the panels) were obtained during exponential growth and were processed as indicated in Materials and Methods. Hfq is required for the increase in RpoS levels caused by the *pstS* mutation but not for that caused by the *sprE* mutation. For the wild-type background, wt refers to strain NR629, *sprE* refers to NR630, and *pstS* refers to NR632 (Table 1). For the *hfq*::Ω*kan* background, wt refers to strain NR633, *sprE* refers to NR634, and *pstS* refers to NR635 (Table 1).

we introduced an *hfq* null allele into strains carrying either a wild-type or mutant *pstS* allele and monitored the activity of the *rpoS477′-′lacZ* reporter fusion (Fig. 3B) as well as the levels of RpoS itself by Western blot analysis (Fig. 4). As controls, we also monitored the effects of the *sprE* null allele in the wild-type and the *hfq* mutant strains. As expected, the *hfq* null allele reduced the RpoS content (Fig. 4, compare wt lanes in both panels), because without Hfq, *rpoS* translation occurs at low levels (Fig. 3B) (21). Also as expected, this reduction of *rpoS* translation in the absence of Hfq was not suppressed by a *sprE* null allele (Fig. 3B), since this latter mutation affects RpoS stability and not translation. However, despite its decreased synthesis, the levels of RpoS itself are higher in the *hfq sprE* double mutant than in the wild-type and the *hfq* mutant strains (Fig. 4), since the small amount of RpoS that is made is not subject to proteolysis by ClpXP. Thus, the results from the Western blot analysis show additivity of the effects caused by the *hfq* and *sprE* null alleles (compare wt and *sprE* lanes in both panels of Fig. 4) as expected, since Hfq and SprE act at two different levels of RpoS regulation (20–22).

Before we discuss epistasis tests between the *pstS* and *hfq* alleles, we must clarify that in contrast to those just described, epistasis tests between *hfq* and sRNAs are complicated. Since Hfq acts as an accessory protein, a null allele in *hfq* reduces *rpoS* translation and mitigates the effect of sRNAs but does not totally eliminate their regulation of *rpoS* translation. Indeed, because sRNAs can still regulate *rpoS* in the absence of Hfq, albeit with reduced efficiency, their overexpression can partially complement an *hfq* null allele (30). Thus, they can appear to be additive with *hfq* mutations in epistasis tests.

The *rpoS477′-′lacZ* fusion shows that an *hfq* null allele decreases *rpoS* translation while the *pstS* increases it (Fig. 3B). As expected, the activity from this fusion is lower in a *pstS hfq* double mutant than in a *pstS* single mutant. However, the *pstS hfq* double mutant has increased LacZ activity with respect to the *hfq* single mutant (Fig. 3B). As noted above, we think this reflects the accessory role of Hfq.

We next examined by Western blotting the dependency on Hfq of the *pstS* effect on RpoS levels. Because of the additivity of the *sprE* and *pstS* alleles, we expected that the effects of the *hfq* mutation in a strain carrying a *pstS* mutation would be

different from those reported above for the *sprE* null strain. Indeed, while the *pstS* single mutant contained high levels of RpoS similar to those found in a *sprE* single mutant, the *pstS hfq* double mutant contained significantly less RpoS than the *sprE hfq* double mutant (Fig. 4). Thus, the increase in RpoS levels induced by the *pstS* mutation was dramatically reduced by the *hfq* null allele. These results suggest that Hfq is required for the *pstS* null mutation to induce *rpoS* translation to high levels. Since Hfq is believed to regulate *rpoS* translation by increasing the interaction between regulatory sRNAs and the *rpoS* 5′ UTR, we propose that the mechanism by which the *pstS* mutation increases *rpoS* translation involves a regulatory sRNA. Specifically, we propose here that disruption of the Pst transport system leads to either the production of a regulatory sRNA that activates *rpoS* translation or the repression of an sRNA that inhibits *rpoS* translation. The expression of this sRNA is directly or indirectly controlled by PhoBR, and for its regulatory action on *rpoS* translation this sRNA requires Hfq.

We favor the idea that this sRNA is a positive (i.e., its expression is activated in a PhoBR-dependent manner) and not a negative (i.e., its expression is repressed in a PhoBR-dependent manner) regulator of *rpoS* translation, because our results resemble those obtained in studies with DsrA. Here as well, overexpression of the positively acting DsrA had only minor effects on RpoS levels (determined by Western blot analysis) in an *hfq* mutant strain, while reporter fusions showed an eightfold increase in translation (30).

Neither RprA nor DsrA mediate the effect of the *pstS* null mutation on *rpoS* translation. As described above, our data suggest that disruption of the Pst transport system leads to increased production of a regulatory sRNA that upregulates *rpoS* translation. Since a malfunctioning Pst transporter constitutively activates the Pho regulon and the effects of a *pstS* null allele on RpoS are PhoBR dependent, the synthesis of this sRNA must also be PhoBR dependent. We are not aware of the existence of any untranslated sRNA whose expression is PhoB-dependent, but there are two sRNAs that have been characterized as positive regulators of *rpoS* translation, RprA and DsrA (17, 29). We thus tested the effects of deleting either RprA or DsrA on the *pst*-mediated upregulation of *rpoS* translation.

Introducing an *rprA*::*kan* null allele into our strains did not alter the high levels of *rpoS* translation caused by the *pstS* null allele (data not shown). Indeed, under the conditions tested, the *rprA* null allele had only a mild effect even in the wild-type background (data not shown). This was expected, since Majdalani et al. have reported that the effect of RprA on *rpoS* translation is clearly seen only when the expression of *rprA* is upregulated (17). We therefore rule out RprA as the sRNA involved in the regulation of *rpoS* translation by the *pstS* mutation.

Epistasis tests with a deletion allele of *dsrA* (Δ*dsrA*) also showed that DsrA is not involved in the activation pathway of *rpoS* translation by *pstS*. As expected from the studies of Sledjeski et al., Fig. 5 shows that deletion of *dsrA* alone greatly decreases *rpoS* translation (29). However, introduction of the *pstS* null allele into the Δ*dsrA* strain increased *rpoS* translation (Fig. 5). Thus, both alleles behave in additive fashion. It is worth noting that unlike the epistasis tests between *pstS* and *hfq* null alleles, epistasis tests between *pstS* and *dsrA* null alleles are simple to interpret. This difference stems from the fact that while an *hfq* null allele does not totally eliminate sRNA regulation of *rpoS* translation because of the accessory role of Hfq discussed above, a *dsrA* null allele completely abolishes any DsrA-mediated regulation.

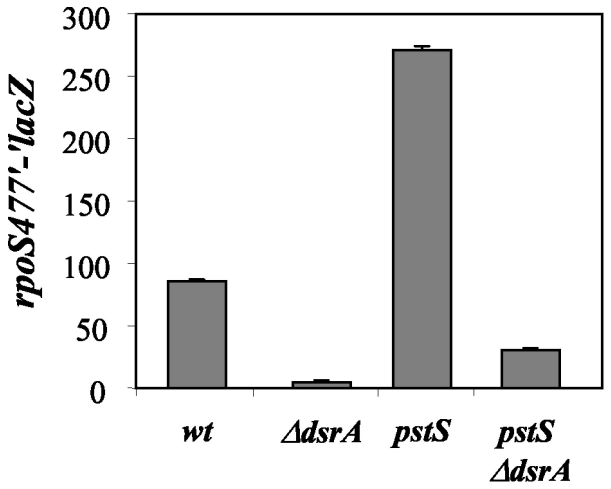

FIG. 5. DsrA is not involved in the increase in *rpoS* translation caused by a *pstS* mutation. Epistasis analysis between mutations in *dsrA* and *pstS* were conducted with strains carrying the *rpoS* translation reporter fusion *rpoS477′-′lacZ*. Although deletion of *dsrA* dramatically decreases the fusion activity, it does not abolish the threefold induction caused by the *pstS* mutation. The relevant genotype for each strain is given below each bar (wt strain refers to the wild-type strain NR641, Δ*dsrA* refers to NR642, *pstS* refers to NR643, and *pstS* Δ*dsrA* refers to NR644). Bar values represent the relative LacZ activity present in each strain, and they are presented as the averages ± standard deviations (error bars) of each sample assayed in triplicate. The data are representative of at least three experiments.

Furthermore, the *pstS* null allele did not significantly increase the transcription of *dsrA* (data not shown) as determined by the use of *dsrA′-lacZ*$^+$ reporter fusions (24). Because these data show that neither RprA nor DsrA are involved in the upregulation of *rpoS* translation triggered in a *pstS* mutant, we believe that there is yet another sRNA that positively regulates *rpoS* translation.

Regulation of RpoS during P_i starvation. The mechanism(s) of induction of RpoS in response to P_i starvation remains unknown. Since disrupting Pst mimics P_i starvation and increases RpoS levels in a PhoBR-dependent manner, and because RpoS has been shown to be induced upon P_i starvation (5), we wanted to investigate if the Pho regulon plays a role in the induction of RpoS in response to P_i starvation. There are conflicting reports implicating a role of transcriptional regulation of *rpoS* in response to P_i starvation (14, 15, 33). Moreover, although it had been reported that the accumulation of RpoS upon P_i starvation requires ppGpp (5), we have found that the induction of *rpoS* synthesis that results from the presence of mutations in *pst*, as reported here, does not require ppGpp (data not shown). We therefore wanted to combine the use of reporter LacZ fusions, Western blot analysis, and available mutations to probe at the signaling mechanism(s) that leads to RpoS induction upon P_i starvation.

As previously reported (5), Western blot analysis shows that immediate P_i starvation leads to the accumulation of RpoS

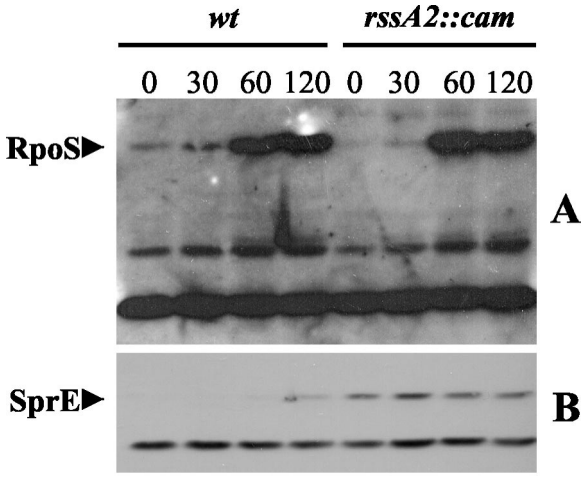

FIG. 6. RpoS accumulation upon P_i starvation is largely caused by increased stability. The induction kinetics of RpoS in response to P_i starvation does not depend on the cellular levels of SprE. Either a wild-type (strain AF633; Table 1) or a strain carrying an allele that causes constitutively elevated expression of *sprE* (*rssA2*::*cam*; strain NR419; Table 1) were grown exponentially in MOPS minimal medium. After cells were subjected to sudden P_i starvation (see Materials and Methods), samples were collected at various times (indicated above the lanes in minutes) and were processed for Western blot analysis against RpoS (A) and SprE (B). Although before P_i starvation (time zero) the strain carrying the *rssA2*::*cam* allele contained less RpoS (because of increased SprE levels), it accumulated RpoS following the same kinetics and to the same levels as the wild-type (wt) strain.

(Fig. 6). To our surprise, this induction was not affected by a deletion of *phoBR* (data not shown), suggesting that either the signaling pathway that leads to the upregulation of *rpoS* translation via PhoBR caused by the *pstS* mutation is not induced during P_i starvation or that it can be induced in a PhoBR-independent manner. In addition, multiple redundant mechanisms that increase RpoS levels could be operating during P_i starvation.

To better understand at what level of regulation RpoS is induced in response to immediate P_i starvation, we used the LacZ reporter fusions described above. During the first 90 min after P_i depletion in the culture medium, we observed that the LacZ activity in a strain carrying the *rpoS750′-lacZ*$^+$ fusion (reporter for *rpoS* transcription) did not increase. In contrast, during that same period there was an approximately two- and ninefold induction of LacZ activity in strains carrying the *rpoS477′-′lacZ* and *rpoS750′-′lacZ* hybrid fusions, respectively. Recall that the *rpoS477′-′lacZ* fusion reflects the regulation of *rpoS* transcription and translation, while the fusion protein encoded by *rpoS750′-′lacZ* is also subject to the stability regulation by SprE and ClpXP.

It has been shown that upregulation of *rpoS* synthesis can increase RpoS stability, since SprE is limiting (23). Although we cannot determine its exact contribution directly, we believe that the elevated *rpoS* translation that we report to occur during P_i starvation is not the sole factor increasing RpoS stability. We propose that the SprE-ClpXP-mediated proteolysis of RpoS is either abolished or greatly reduced in response to the absence of P_i in the culture medium. The following findings support this idea.

As previously reported (25), a strain carrying the *rssA2*::*cam* allele contains high levels of SprE compared to that of its wild-type parent strain (Fig. 6B, compare time 0 lanes for the wild type and *rssA2*::*cam*) because *sprE* transcription is constitutively driven from the *cam* promoter of the mini-Tn*cam* transposon. This higher content of SprE results in decreased RpoS levels (Fig. 6A, compare time 0 lanes for the wild type and *rssA2*::*cam*). Despite this, when we subjected these strains to sudden P_i starvation, both the wild type and the strain carrying the *rssA2*::*cam* allele induced RpoS to the same level and with equivalent kinetics (Fig. 6A). Because RpoS degradation by ClpXP is regulated by SprE activity, we believe that our results show that SprE is inactivated upon P_i starvation, contributing to RpoS accumulation under these conditions.

The twofold increase in *rpoS* translation that occurs upon P_i starvation is lower but is comparable to the one caused by the *pstS* mutation during exponential growth (three- to fourfold; see Fig. 3B). Mutations in *pst* are additive with the *rssA2*::*cam* allele (i.e., the double mutants have less RpoS than the *pst* single mutant but have more than the *rssA2*::*cam* single mutant [data not shown]). Therefore, since RpoS levels increase to the same levels in the *rssA2*::*cam* mutant as in the wild type, we can conclude that the increase in RpoS levels upon P_i starvation in the *rssA2*::*cam* mutant results from inhibition of RpoS degradation (most likely by inactivating SprE), not just from overwhelming SprE by the increase in *rpoS* translation. This is further supported by the fact that RpoS accumulates significantly upon P_i starvation even in an *hfq* mutant strain, despite its decreased translation (data not shown). This is also in agreement with our own findings that in strains deficient in SprE or ClpXP there is only an approximately twofold increase in RpoS activity (measured using the *uspB′-lacZ*$^+$ fusion) during the first hours after immediate P_i starvation, while in the wild-type strain there is a greater than 10-fold increase. The increase seen in the strains deficient in RpoS proteolysis is likely due to increased *rpoS* translation.

As stated above, since the constitutive activation of the Pho regulon affects *rpoS* translation and does not inactivate SprE-ClpXP-mediated proteolysis, it is not surprising that the Δ*phoBR* mutant can still induce RpoS upon P_i starvation. Even if P_i starvation triggered a PhoBR-dependent increase in *rpoS* translation by the same sRNA that the *pstS* mutation induces, RpoS would still accumulate in a strain deficient in PhoBR because of SprE inactivation. In fact, a strain carrying a deletion in *phoBR* is still capable of accumulating RpoS in response to P_i starvation (see above).

We also tested whether the twofold increase (see above) in translation that occurs when P_i is absent from the medium requires PhoBR. Although the absolute LacZ levels in a Δ*phoBR* strain carrying the *rpoS477′-′lacZ* fusion are reproducibly lower than those of a wild-type strain, translation of *rpoS* increases almost twofold in the mutant strain in response to P_i starvation. This suggests that either the sRNA upregulating *rpoS* translation in the *pstS* mutant is not being produced in response to P_i starvation or that it can also be induced in a PhoBR-independent manner. Until we identify this sRNA, we cannot distinguish between these possibilities.

DISCUSSION

Here we report two independent genetic screens that indicate the involvement of the Pho regulon in the regulation of RpoS. We found that mutations that disrupt the P_i transporter Pst, which are known to cause the constitutive activation of the Pho regulon, cause an accumulation of RpoS during exponential growth. This increase in RpoS levels is due to elevated *rpoS* translation, and it likely requires an unknown sRNA together with Hfq. Although we have presented evidence showing that the upregulation in *rpoS* translation caused by the *pstS* mutation requires PhoBR, we do not know yet how PhoB controls the synthesis of the sRNA. That is, PhoB could either directly or indirectly control the expression of this sRNA. In addition, our data suggest that PhoB might not be the sole regulator of this unknown sRNA, but until we identify the sRNA involved we cannot address these issues.

It is clear that multiple sRNAs control *rpoS* translation. Because each of these sRNAs is expressed in response to particular environmental signals, this regulatory mechanism efficiently deals with the problem of signal integration. When conditions in the environment are favorable, *rpoS* translation is reduced because of the antisense pairing that occurs within the 5′ UTR that inhibits the access of the ribosome to the rbs. However, once an environmental parameter becomes harmful and induction of stationary phase is desired to ensure survival, *E. coli* can deploy a specific signaling cascade in response to that particular stress. The response would include the increase in the levels of an sRNA that can interfere by an anti-antisense mechanism with the intramolecular inhibition of *rpoS* translation by its own mRNA. Because the lack of external stress would result in the absence of any of these sRNAs, there is no conflict in integrating the signals from all the environment sampling systems; inhibition of *rpoS* translation still occurs in the absence of stress because it is intrinsic to its own mRNA.

There are additional advantages to regulating RpoS via sRNAs. Their accumulation in response to the inducing signal can occur quickly because of their small size, rapid synthesis, and great stability. This, in turn, means a rapid induction of RpoS under hazardous conditions, which could be essential for cell survival. Furthermore, because a sufficiently large increase in *rpoS* synthesis via an sRNA can overcome ClpXP-mediated degradation of RpoS, their action is independent of SprE's activity and, therefore, of other regulatory mechanisms acting on RpoS.

We have found that a major regulatory mechanism responsible for the accumulation of RpoS in response to P_i starvation is an increase in RpoS stability. In fact, this SprE-dependent increase in RpoS stability could explain why a strain deficient in PhoBR still upregulates RpoS in response to P_i starvation. Interestingly, RpoS stability also increases dramatically upon carbon starvation (22, 34). Thus, the effect of both carbon and P_i starvation on SprE activity and ClpXP-mediated proteolysis of RpoS seems to be the same. However, because the signal inactivating SprE under either condition remains to be identified, we do not know whether both types of starvation converge on a common switch that controls SprE activity.

Despite the fact that both carbon and P_i starvation increase RpoS stability in SprE-dependent fashion, they appear to affect *rpoS* synthesis differently. Zgurskaya et al. (34) concluded that the accumulation of RpoS upon carbon starvation is solely due to its increased stability. We found that P_i starvation leads to an increase in *rpoS* translation and stability. However, there appears to be no significant difference in RpoS levels following carbon or P_i starvation (data not shown and reference 5). Additional studies are needed to understand this paradox, and the *pstS* mutation provides a useful tool.

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