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Genetic Evidence Suggests that the Intergenic Region between *pstA* and *pstB* Plays a Role in the Regulation of *rpoS* Translation during Phosphate Limitation[▽]

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In addition to the Pho regulon, phosphate starvation also stimulates the accumulation of RpoS. Several deletion mutations within the *pstSCAB-phoU* operon were tested for the accumulation of RpoS during exponential growth. Our data suggest that the processed 3′ end of the *pstA* message stimulates translation of *rpoS*.

Escherichia coli cells adapt to phosphate (P_i) limitation by increasing the synthesis of genes for the high-affinity acquisition of P_i and the utilization of alternate phosphorous sources (22). These genes are dispersed throughout the chromosome, are positively regulated, and are called the Pho regulon. Our current model for the regulation of this response is that the PstSCAB transporter senses P_i levels and communicates through the PhoU protein to the histidine kinase PhoR, which controls the phosphorylation of the response regulator PhoB. Phospho-PhoB binds to specific DNA sequences upstream of Pho regulon genes, interacts with σ^{70}, and stimulates transcription (11, 13). P_i sufficiency generates a signal that shuts off the Pho regulon by activating the phospho-PhoB phosphatase activity of PhoR. However, when P_i is limiting or when mutations eliminate any component of the PstSCAB transporter or PhoU, the Pho regulon is turned on. This low-P_i signal stimulates the kinase activity of PhoR, allowing it to serve as an efficient phospho donor to PhoB (12). These seven signaling proteins, encoded within the *phoBR* and *pstSCAB-phoU* operons, are themselves members of the Pho regulon.

Phosphate starvation also triggers the general stress response in which cells become increasingly resistant to many environmental stresses (4, 19). The master regulator of this response is σ^S (RpoS), an alternate sigma factor that competes with σ^{70} to direct the transcription of genes under its control (6). The regulation of σ^S is very complex and has numerous inputs (6, 17). Its cellular amounts are controlled at the levels of transcription, translation, and protein turnover. During exponential growth, the long 5′ untranslated region of the *rpoS* message forms a hairpin structure that occludes the ribosome binding site and prevents translational initiation. However, two small regulatory RNAs (DsrA and RprA) have been shown to regulate *rpoS* translation by base pairing it with an untranslated leader sequence of the *rpoS* mRNA that unmasks the ribosome binding site of the message and stimulates translation (5, 9, 10). This translational stimulation requires the RNA chaperone Hfq (10, 20). At the level of protein stability, the half-life of σ^S is very short during exponential growth but is extended during stationary phase (8). This protein turnover is mediated by the RssB (SprE) adapter protein and the ClpXP protease (7, 25). During exponential growth, RssB binds to σ^S and targets it to the protease for degradation (7, 15, 18, 25). In response to stationary growth phase or certain stress signals, the activity of RssB is inhibited, which leads to σ^S accumulation.

It has recently been demonstrated that in response to phosphate limitation, the cellular levels of σ^S are increased by inhibiting its turnover (2). Upon phosphate limitation, the IraP protein binds to the RssB adapter protein and blocks its activity, thereby preventing the degradation of σ^S and allowing its accumulation.

It has also recently been suggested that a small regulatory RNA mediates the induction of the general stress response due to phosphate limitation (17, 19). Ruiz and Silhavy showed that a Tn *cam* minitransposon mutation in the *pstS* gene, oriented so that transcription of its *cam* cassette is in the same direction as that of *pstS*, led to constitutive expression of the Pho regulon and resulted in increased levels of σ^S during logarithmic growth (19). They demonstrated that Hfq was required for this increase in σ^S and concluded that an uncharacterized small RNA controls translation of the *rpoS* message during phosphate limitation. They also showed that this effect was dependent upon a functional PhoB protein.

The development of our hypothesis. An analysis of the transcription of the *pstSCAB-phoU* operon by Aguena et al. showed that there is a single promoter upstream of the *pstS* gene and that the full-length message is processed at several sites; the first is downstream of the *pstS* gene, and another site lies in a 184-bp intergenic region between the *pstA* and *pstB* genes (Fig. 1A) (1). Examination of the DNA sequence in this intergenic region showed that it displayed some complementarity to the untranslated leader region of the *rpoS* gene (Fig. 1B). We hypothesized that the processed 3′ end of the *pstCA* message (containing the small intergenic region) interacts with the untranslated leader portion of the *rpoS* mRNA in conjunction with Hfq and enhances the translation of *rpoS*. Since this message is normally made only in response to phosphate starvation, it would provide a regulated input into the complex network that controls RpoS levels in the cell.

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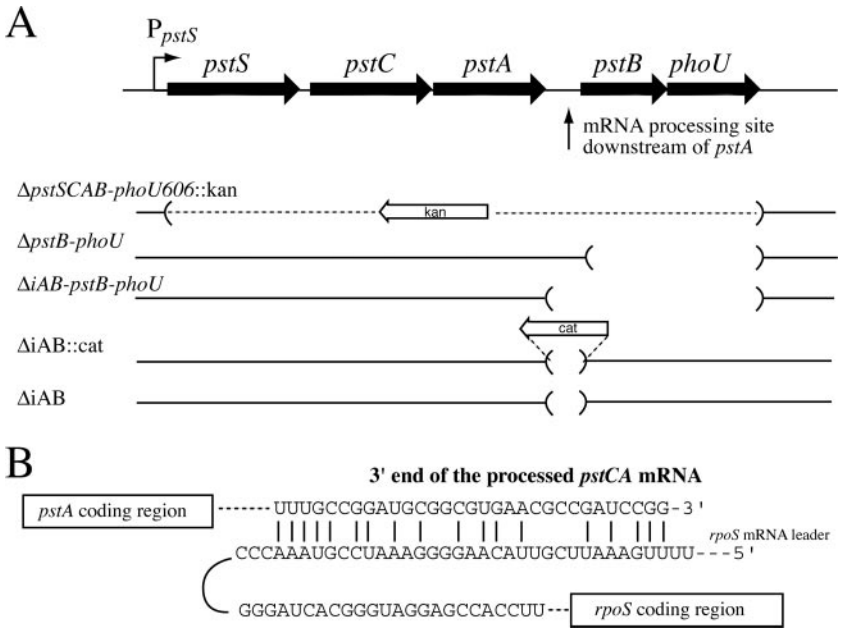

FIG. 1. The *pstSCAB-phoU* operon of *E coli*. (A) The operon is transcribed from a single promoter and processed as several sites, one of which lies in an intergenic region between *pstA* and *pstB*. The structures of deletion mutations used in this study are shown below the genetic map. Parentheses denote the deletion borders, and dotted lines indicate the presence of internal antibiotic resistance cassettes that are flanked by Flip recombinase recognition sites. (B) The proposed interaction between the 3′ end of the processed *pstA* mRNA and the 5′ untranslated leader of the *rpoS* mRNA.

Construction and characteristics of deletion strains. To test this hypothesis, we initially examined three deletion mutations within the *pstSCAB-phoU* operon that constitutively expressed the Pho regulon for the ability to turn on the general stress response during exponential growth. The first mutation consisted of a precise deletion of the entire operon (Δ*pstSCAB-phoU606*::*kan*) (Table 1 and Fig. 1A). This allele was kindly provided by Barry Wanner for strain BW26336 (3). The other two mutations were similar *pstB-phoU* deletions that differed only in the presence or absence of the short intergenic region upstream of *pstB*. For ease of discussion, we subsequently refer to this intergenic region as iAB (for the intergenic region between *pstA* and *pstB*). The mutations were introduced into *E. coli* strain BW25113 using the procedure described by Datsenko and Wanner (3). The Δ*pstB-phoU* mutation was created using the pstBfor and phoUrev primers, and the ΔiAB-*pstB-phoU* mutation used the iABfor and phoUrev primers (Table 1). The mutations were confirmed by PCR and by plasmid complementation (not shown). An important feature of these mutations is that they, like other deletion mutations in this operon, highly activate the Pho regulon even under conditions of phosphate sufficiency by dis-

TABLE 1. Strains and primers used in this study

Strain or primer	Genotype or sequence	Source or reference
E. coli strains		
BW25113	Wild type	3
BW26336	BW25113 Δ*pstSCAB-phoU*::*kan*	3
BM121	BW25113 Δ*pstB-phoU*::*frt*	This study
BM122	BW25113 ΔiAB-*pstB-phoU*::*frt*	This study
BM123	BW25113 ΔiAB::*cam*	This study
BM124	BW25113 ΔiAB::*frt*	This study
NR629	MC4100 λϕ(*rpoS477′-′lacZ*)	19
BM125	NR629 Δ*pstSCAB-phoU*::*kan*	This study
BM126	NR629 Δ*pstB-phoU*::*frt*	This study
BM127	NR629 ΔiAB-*pstB-phoU*::*frt*	This study
BM128	NR629 ΔiAB::*cam*	This study
BM129	NR629 ΔiAB::*frt*	This study
BM130	NR629 Δ*pstB-phoU*::*frt* Δ*phoBR*::*kan*	24, this study
Primers		
iABfor	5′-TCTGGCGCGCGTTGTTTTTGCGAAGAATAAACACG GTTAGTGTAGGCTGGAGCTGCTTC	
iABrev	5′-CGAACCTGAATTTTACTCGGGGCAGTTTCAACCAT ACTCATATGAATATCCTCCTTAG	
pstBfor	5′-TTAGCGAAGTGCATCAGGCACGATGAGGAAAAGA TTGCGTGTAGGCTG GAGCTGCTTC	
phoUrev	5′-CAAATCCCAATAATTAAGTTATTGGGATTTGTCTG GTGAACATATGAAT ATCCTCCTTAG	

abling the negative regulatory features of the PstSCAB transporter and PhoU. The wild-type strain (BW25113) expressed ~1 arbitrary alkaline phosphatase unit when grown in LB medium to an optical density at 600 nm (OD_{600}) of 0.4, whereas the Δ*pstSCAB-phoU*, Δ*pstB-phoU*, and ΔiAB-*pstB-phoU* mutants expressed ~2,000 arbitrary units (assayed as described in reference 26). This feature allowed us to examine RpoS induction during exponential growth when other RpoS-activating inputs are shut off.

These two new deletion strains also displayed noticeable growth defects when incubated in LB medium. They produced smaller colonies when plated on LB agar and had doubling times about 60% longer than wild-type cells. We frequently observed these strains to accumulate mutations following overnight growth that disabled the Pho system and restored normal growth rates. Similar growth phenotypes have previously been observed for other mutations affecting the PstSCAB transporter, and the slow-growth phenotype could be attributed to phosphate poisoning (23). This growth defect could be alleviated by growing cells in minimal media containing 1.32 mM K_2HPO_4, which we routinely did.

Tests of RpoS induction. Since the expression of the *katE* gene, encoding HPII catalase, is under the control of σ^S, we first measured the catalase activities of these strains as reporters of RpoS levels (21). Strains were grown overnight in 5 ml of MOPS (morpholinepropanesulfonic acid) medium containing 1.32 mM P_i (16) and then diluted 1:200 into prewarmed LB medium. Cultures were grown until the OD_{600} reached 0.35 to 0.4 and were placed on ice until all cultures reached the same cell density. HPII activities were determined spectrophotometrically according to the method of Visick and Clarke (21). As shown in Fig. 2A, the wild type strain, the Δ*pstSCAB-phoU* mutant, and the ΔiAB-*pstB-phoU* mutant all showed low catalase levels during exponential growth. However, the Δ*pstB-phoU* mutant showed about a twofold increase in catalase activity. Since the only difference between the Δ*pstB-phoU* and ΔiAB-*pstB-phoU* strains is the intergenic region, these results are consistent with our hypothesis.

Ruiz and Silhavy had previously used *rpoS-lacZ* fusions to demonstrate that high-level activation of the Pho regulon stimulates the translation of σ^S (19). The *rpoS477′-′lacZ* fusion is a reporter of *rpoS* transcription and translation but not post-translational processing. The deletion mutations described above were moved into the reporter strain NR629 by generalized transduction using phage P1 (14). Strains were grown to exponential phase as described above, and β-galactosidase assays were performed as described previously (19). As shown in Fig. 2B, elevated levels of β-galactosidase were observed during exponential growth in the strain that retained the intergenic region (BM126) but not in deletion strains that eliminated the intergenic region (NR629, BM125, and BM127). To test whether this increased production of β-galactosidase was dependent upon the PhoBR two-component signaling pathway, we introduced into the BM126 strain a *phoBR* deletion by P1 transduction from *E. coli* strain ANCH1 (24). This new strain, designated BM130, expressed low levels of β-galactosidase activity. This observation is consistent with the proposal that increased transcription of the *pstSCAB-phoU* operon, which is dependent on PhoB and PhoR, leads to elevated levels of RpoS. We then constructed another deletion strain, following the same procedure described above, using the iABfor and iABrev primers (Table 1), which eliminated only the iAB region. As expected, this strain, containing the ΔiAB::*frt* mutation, did not activate the Pho regulon during exponential growth because it expressed a functional PstSCAB transporter and PhoU. However, a strain that was constructed as an intermediate to the ΔiAB strain, which lacked the iAB region but in addition contained the *cat* gene expressed in the direction opposite that of the *pstSCAB-phoU* operon, did show full induction of the Pho regulon (ΔiAB::*cam*). Neither of these strains, one of which induced the Pho regulon and the other

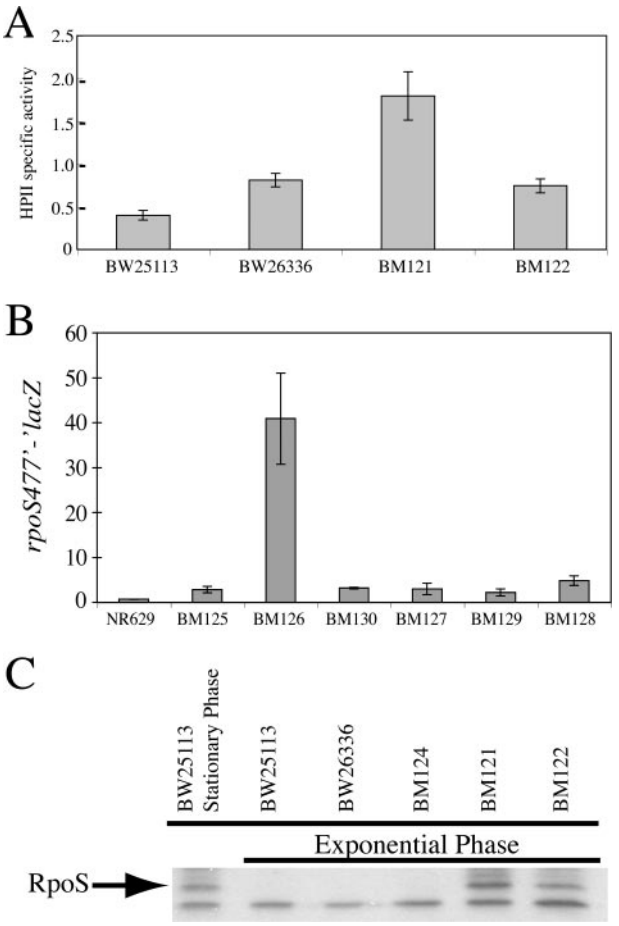

FIG. 2. The Δ*pstB-phoU* strain shows increased induction of the general stress response. Within each experiment, all strains are isogenic. (A) Catalase assays. Cells were grown in LB medium to mid-exponential phase and were prepared for HPII assays as described in the text. The values represent averages for duplicate trials, and the error bars represent standard deviations. BW25113, wild type; BW26336, Δ*pstSCAB-phoU*; BM121, Δ*pstB-phoU*; BM122, ΔiAB -*pstB-phoU*. (B) RpoS-LacZ fusion assays. The *rpoS477′-′lacZ* reporter is sensitive to a combination of transcriptional and translational activities but not protein turnover. The values represent averages for duplicate trials, and the error bars represent standard deviations. NR629, wild type; BM125, Δ*pstSCAB-phoU*; BM126, Δ*pstB-phoU*; BM130, Δ*pstSCAB-phoU* Δ*phoBR*; BM127, ΔiAB-*pstB-phoU*; BM129, ΔiAB::*frt*; BM128, ΔiAB::*cam*. (C) Western blot analysis on exponentially grown cells shows that the strain retaining the iAB region produces more RpoS than strains that lack it. BW25113, wild type; BW26336, Δ*pstSCAB-phoU*; BW124, ΔiAB::*frt*; BW121, Δ*pstB-phoU*; BW122, ΔiAB-*pstB-phoU*.

which did not, showed elevated levels of β-galactosidase activity. These results are also consistent with our proposal.

To confirm our findings, we performed a Western blot analysis on logarithmically grown cells containing various deletion mutations. Cells were grown in LB to an OD_{600} of ~0.4 and were placed on ice. When all samples reached the indicated cell density, a 1-ml sample of each strain was pelleted in a microcentrifuge and resuspended in a 1× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer as described by Ruiz and Silhavy (19). The samples were boiled for 10 min, and equal volumes were separated on a 12% sodium dodecyl sulfate-polyacrylamide gel, transferred to a nitrocellulose membrane, and subjected to immunological detection using an Invitrogen Western-Breeze chemiluminescent kit (Carlsbad, CA) and a commercially available mouse monoclonal antibody to RpoS (Neoclone, Madison, WI). As can be observed in Fig. 2C, no σ^S was observed in the wild-type strain or the Δ*pstSCAB-phoU* or ΔiAB mutant. However, σ^S was detected in both the Δ*pstB-phoU* and the ΔiAB-*pstB-phoU* strains. When the relative amounts of σ^S were compared using the faster-migrating, nonspecific, cross-reacting band shown in Fig. 2C as an internal loading standard, then the strain containing the iAB region showed three to four times the amount of σ^S as did the strain without, even though both strains showed identical growth rates and alkaline phosphatase levels. That we observed an RpoS band in the ΔiAB-*pstB-phoU* strain was unexpected, but since both strains showed decreased growth rates compared to the wild-type strain, this level of *rpoS* expression may be due to a lower growth rate and the elevated levels in the Δ*pstB-phoU* strain may reflect enhanced translation due to the presence of the iAB region.

Taken together, our results are consistent with a model in which phosphate limitation induces the Pho regulon, including the *pstSCAB-phoU* operon, by activating the PhoBR two-component signaling pathway. Following transcription of the entire operon, the message is cut at several sites, producing one product whose 3′ terminus is able to interact with the 5′ untranslated leader region of the *rpoS* message to increase the production of σ^S. We did not observe increased RpoS levels in cells that contained a plasmid expressing the full iAB region (data not shown). This may be because this genetic element may not contain the sequences for proper processing of the RNA. Our conclusions extend the findings of Ruiz and Silhavy (19) by showing that not all mutations that lead to constitutive activation of the Pho regulon lead to elevated levels of σ^S; only constitutive mutations that retain the iAB region stimulate σ^S accumulation. To the best of our knowledge, this is the first suggestion that an intergenic region from a processed message may serve as a regulatory RNA and may suggest a role for similar sites in other prokaryotic genomes. Given the importance of phosphate for cellular function, it is not surprising that multiple signaling mechanisms exist to communicate its limitation to the general stress response.

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