HHS Public Access

Author manuscript

Mol Microbiol. Author manuscript; available in PMC 2015 August 01.

Published in final edited form as:

Mol Microbiol. 2014 August ; 93(3): 479–493. doi:10.1111/mmi.12674.

Co-ordinated regulation of the extracytoplasmic stress factor, sigmaE, with other *Escherichia coli* sigma factors by (p)ppGpp and DksA may be achieved by specific regulation of individual holoenzymes

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Summary

The *E. coli* alternative sigma factor, σ^E, transcribes genes required to maintain the cell envelope and is activated by conditions that destabilize the envelope. σ^E is also activated during entry into stationary phase in the absence of envelope stress by the alarmone (p)ppGpp. (p)ppGpp controls a large regulatory network, reducing expression of σ^{70}-dependent genes required for rapid growth and activating σ^{70}-dependent and alternative sigma factor-dependent genes required for stress survival. The DksA protein often potentiates the effects of (p)ppGpp. Here we examine regulation of σ^E by (p)ppGpp and DksA following starvation for nutrients. We find that (p)ppGpp is required for increased σ^E activity under all conditions tested, but the requirement for DksA varies. DksA is required during amino acid starvation, but is dispensable during phosphate starvation. In contrast, regulation of σ^S is (p)ppGpp- and DksA-dependent under all conditions tested, while negative regulation of σ^{70} is DksA- but not (p)ppGpp-dependent during phosphate starvation, yet requires both factors during amino acid starvation. These findings suggest that the mechanism of transcriptional regulation by (p)ppGpp and/or DksA cannot yet be explained by a unifying model and is specific to individual promoters, individual holoenzymes, and specific starvation conditions.

Introduction

Most bacteria have an intricate array of stress responses that allow them to sense changes in their surroundings and adapt their transcriptional profiles in order to survive. One of the major classes of stress responses relies on the modular nature of the multisubunit RNA polymerase (RNAP). The α, β, β′ and ω subunits of RNAP form the core enzyme (E), which is responsible for transcription elongation, but cannot specifically initiate transcription (Borukhov and Nudler, 2008). The sigma subunit binds to the core enzyme, forming the holoenzyme (Eσ), and confers specific promoter recognition (Burgess *et al*., 1969). The bulk

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Supporting information

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of bacterial transcription is directed by a housekeeping sigma factor, σ^{70} in *E. coli*. However, many bacteria possess an array of alternative sigma factors that are induced by particular stresses or environmental conditions (Gruber and Gross, 2003; Paget and Helmann, 2003). Once activated, they bind to core RNAP and redirect the enzyme to promoters for genes required for adaptation and survival.

The alternative sigma factor system is very efficient, but is essentially reactionary. A stress occurs, a sigma factor is activated, and a transcriptional response is generated. However, when nutrients are in short supply, the bacterium may not have the resources to rapidly mount a response that requires the energy-consuming processes of transcription and translation. In *E. coli*, a growing body of evidence suggests that as nutrients are depleted, the alarmones pppGpp and ppGpp (collectively referred to as (p)ppGpp herein) co-ordinately activate the stress-responsive alternative sigma factors, σ^{E}, σ^{H}, σ^{S} and σ^{N}, essentially preloading the cell with factors to combat stresses should they arise (Gentry *et al*., 1993; Hirsch and Elliott, 2002; Jishage *et al*., 2002; Laurie *et al*., 2003; Costanzo and Ades, 2006; Costanzo *et al*., 2008; Österberg *et al*., 2011). At the same time, (p)ppGpp inhibits transcription of genes required for rapid growth (Durfee *et al*., 2008; Potrykus and Cashel, 2008; Traxler *et al*., 2008). In effect, (p)ppGpp mediates a switch from a transcriptional program for rapid growth to one optimized for stress survival (Nyström, 2004; Magnusson *et al*., 2005;Traxler *et al*.,2008).

The alternative sigma factor, σ^{E}, plays an essential role ensuring the integrity of the bacterial cell envelope (De Las Peñas *et al*., 1997a; Hayden and Ades, 2008). The amount of σ^{E} available to form σ^{E} holoenzyme ($E\sigma^{E}$) is regulated by the antisigma factor, RseA. RseA is an inner membrane protein whose cytoplasmic domain binds tightly to σ^{E} and prevents it from interacting with core RNAP (De Las Pehas *et al*., 1997b; Missiakas *et al*., 1997; Campbell *et al*., 2003). RseA is proteolytically unstable, and its degradation increases in response to cell envelope stress (Ades *et al*., 1999; 2003). When RseA is degraded, σ^{E} is released, binds to core RNAP, and directs transcription of the genes in its regulon. In addition to the stress-signalling pathway acting through RseA, (p)ppGpp increases transcription by $E\sigma^{E}$ in response to starvation, independently of RseA and cell envelope stress (Costanzo and Ades, 2006; Costanzo *et al*., 2008). Therefore, the overall amount of σ^{E} activity in the cell is determined by a combination of the two regulatory pathways. The stress induction pathway mediated through RseA has been extensively studied (Barchinger and Ades, 2013); however regulation of σ^{E} by (p)ppGpp is not well understood.

(p)ppGpp is a general signal of starvation stress. Its levels increase upon nutrient downshifts and are inversely correlated with growth rate (Dennis *et al*., 2004; Potrykus and Cashel, 2008). In *E. coli*, (p)ppGpp is synthesized by two enzymes, RelA and SpoT (Xiao *et al*., 1991). RelA is a strong (p)ppGpp synthase, while SpoT has weak synthase activity (Cashel *et al*., 1996). SpoT is also a hydrolase and is responsible for hydrolysing (p)ppGpp (Cashel *et al*., 1996). *spoT* is essential for viability due to toxicity associated with the high levels of (p)ppGpp that accumulate in its absence (Xiao *et al*., 1991). Cells lacking both *relA* and *spoT* are devoid of (p)ppGpp and referred to as $(p)ppGpp^{0}$. Changes in the amount of (p)ppGpp in the cell in response to limitation for various nutrients are achieved via regulation of RelA and/or SpoT activity.

The best-studied cellular role of (p)ppGpp is its involvement in balancing the protein synthetic capacity of the cell with nutrient availability by acting on $E\sigma^{70}$ to negatively regulate transcription of stable RNA operons (rRNA and tRNA) and positively regulate promoters of several amino acid biosynthetic operons (Cashel *et al*., 1996; Dennis *et al*., 2004; Paul *et al*., 2004a). (p)ppGpp regulates transcription at these promoters in concert with the protein, DksA, which potentiates the effects of (p)ppGpp (Paul *et al*., 2004b; 2005). Because DksA levels are thought to be constant, while those of (p)ppGpp change, (p)ppGpp is regarded as the stress signal for the system (Brown *et al*., 2002; Paul *et al*., 2004b). Both (p)ppGpp and DksA bind to core RNAP, (p)ppGpp at the interface between the β′ and ω subunits and DksA in the secondary channel (Perederina *et al*., 2004; Lennon *et al*., 2012; Mechold *et al*., 2013; Ross *et al*., 2013; Zuo *et al*., 2013). By binding directly to core RNAP, they are able to regulate transcription in a large part by altering the kinetics of transcription initiation (Paul *et al*., 2004b; 2005). The outcome of this regulation, activation or inhibition, depends on the overall kinetic and thermodynamic properties of individual promoters and whether (p)ppGpp and/or DksA affect a step that is rate-limiting for transcription initiation at that promoter.

(p)ppGpp has been proposed to regulate σ^E activity both directly and indirectly (Costanzo *et al*., 2008). ppGpp alone has little effect *in vitro* on transcription by $E\sigma^E$. However, ppGpp and DksA together directly activate transcription by $E\sigma^E$ in multi-round *in vitro* transcription assays (Costanzo *et al*., 2008). Direct activation of transcription by $E\sigma^N$, $E\sigma^{32}$ and $E\sigma^S$ has not been observed *in vitro*, so this mode of regulation of alternative sigma factors may be specific to $E\sigma^E$ (Jishage *et al*., 2002; Laurie *et al*., 2003; Szalewska-Palasz *et al*., 2007). (p)ppGpp has also been proposed to act indirectly by altering the competition among sigma factors for limiting core RNAP in favour of alternative sigmas through its effects on transcription of stable RNA genes by $E\sigma^{70}$ (Zhou and Jin, 1998; Jishage *et al*., 2002; Szalewska-Palasz *et al*., 2007; Potrykus and Cashel, 2008). In rapidly growing cells, ~ 70% of the RNAs transcribed in the cell are stable RNAs encoded in long operons (Dennis *et al*., 2004). (p)ppGpp together with DksA decreases transcription of these operons, thereby reducing the amount of RNAP actively engaged in their transcription and increasing the size of the pool of free core RNAP available to bind all sigma factors.

To better understand regulation of σ^E by (p)ppGpp and DksA and the extent to which σ^E is integrated into the cellular response to starvation, we investigated regulation of σ^E during entry into stationary phase caused by starvation for specific nutrients. Here, we show that (p)ppGpp is required for activation of σ^E under all starvation conditions tested. However, the requirement for DksA varies. DksA is dispensable for the increase in σ^E activity following phosphate starvation, while it is required during entry into stationary phase in rich medium and following amino acid starvation. We further show that the contribution of (p)ppGpp and/or DksA to the regulation of other sigma factors during phosphate starvation is not the same for each sigma factor. DksA, but not (p)ppGpp, is required for decreased transcription of the *rrnB* P1 rRNA operon promoter by $E\sigma^{70}$, while DksA and (p)ppGpp are both required for increased σ^S activity. These data suggest that regulation of transcription in response to phosphate starvation is specific to individual sigma factors and promoters, and

cannot be explained by a concerted global model for (p)ppGpp-dependent transcriptional regulation that affects all sigma factors equivalently.

Results

Regulation of σ^E during nutrient limitation

Previous work on the regulation of σ^E by (p)ppGpp and DksA focused on entry into stationary phase in the rich growth medium, LB (Costanzo and Ades, 2006; Costanzo *et al*., 2008). To better understand the extent to which (p)ppGpp integrates σ^E into the cellular response to starvation, we investigated the regulation of σ^E by (p)ppGpp during entry into stationary phase under several different growth conditions known to be associated with increased (p)ppGpp production. *E. coil* MG1655 were grown in a MOPS-buffered, rich, defined medium (EZ Rich, Teknova), in EZ Rich with limiting phosphate to induce phosphate starvation or limiting isoleucine to induce amino acid starvation, and in LB. σ^E activity was measured throughout growth of the cultures using the σ^E-dependent *rpoHP30–lacZ* reporter, which has been used extensively as a measure of σ^E activity. For all culture conditions tested, σ^E activity increased when growth slowed (Fig. 1A – D). This increase was abrogated in all cases in a strain unable to make (p)ppGpp due to disruption of the *relA* and *spoT* genes (Fig. 1A – D), indicating that (p)ppGpp is involved in regulating σ^E. Because DksA is required for σ^E to respond to (p)ppGpp *in vivo* during entry into stationary phase in LB and *in vitro* in transcription reactions, we next examined σ^E activity in a strain lacking *dksA* under the same set of culture conditions (Costanzo *et al*., 2008). In contrast to (p)ppGpp, the requirement for DksA varied depending on the starvation condition. DksA was required for the increase in σ^E activity during amino acid starvation and entry into stationary phase in LB (Fig. 1C and D). However, deletion of *dksA* had little effect on σ^E activity in response to phosphate starvation (Fig. 1B). In EZ Rich itself, the increase in σ^E activity in the Δ*dksA* strain was complex and could be divided into two phases. When the culture first transitioned into stationary phase, σ^E activity was slightly lower than in the wild-type strain, indicating partial DksA-dependence (Fig. 1A, see Δ*dksAa*). However, σ^E activity was comparable to that of the wild-type strain at the end of the transition into stationary phase (Fig. 1A, see Δ*dksAb*), suggesting that after a brief adjustment period to stationary phase, *dksA* was no longer required. Similar results were obtained with the σ^E-dependent *PrybB-lacZ* reporter (data not shown).

σ^E activity is also known to increase when (p)ppGpp is made in the absence of starvation by overexpression of a fragment of *relA*, *relA*', which constitutively synthesizes (p)ppGpp independently of the ribosome (Svitil *et al*., 1993; Costanzo *et al*., 2008). The involvement of DksA in regulating σ^E activity under these conditions was not examined. Therefore, we measured σ^E activity following overexpression of *relA*' in wild-type and Δ*dksA* cells. Because high levels of (p)ppGpp are toxic to *E. coil*, we induced *relA* with a low concentration of IPTG that does not significantly alter the growth rate. When the *relA*'gene was overexpressed, σ^E activity increased in the wild-type strain, but not in the Δ*dksA* strain, indicating that DksA is required for σ^E to respond to (p)ppGpp in the absence of a starvation signal (Fig. 1E). These data indicate that σ^E activity increases under all conditions tested thus far that are known to lead to an increase in (p)ppGpp levels, and that disruption of

(p)ppGpp production disrupts the σ^E response. However, the mechanism of regulation differs with the culture conditions. In some cases DksA is required and in other cases it is not.

Regulation of σ^S during nutrient limitation

If the alternative sigma factors in *E. coil* are indeed regulated in concert by (p)ppGpp and DksA via their effects on σ^{70}, then other sigma factors should show the same pattern of regulation as σ^E. To test this idea, we examined the activity of the general stress factor, σ^S, in wild-type, (p)ppGpp^0 and Δ*dksA* strains under the same series of culture conditions used for σ^E. A σ^S-dependent *bolA–lacZ* fusion was used to monitor σ^S activity. Similar to σ^E, σ^S activity increased when cells entered stationary phase in the wild-type strain under each of the growth conditions (Fig. 2). However, σ^S did not exhibit the differential requirement for DksA. σ^S activity failed to increase in (p)ppGpp^0 or Δ*dksA* strains under all of the tested culture conditions, indicating that both factors are involved in regulating σ^S and that regulation of σ^S is therefore distinct from regulation of σ^E (Fig. 2). In addition, these data show that DksA is active during phosphate starvation.

Regulation of σ^{70} during phosphate starvation

Inhibition of transcription of rRNA promoters by (p)ppGpp and DksA has been proposed to indirectly activate σ^E and other alternative sigma factors by increasing the amount of free core RNAP (Zhou and Jin, 1998; Costanzo *et al*., 2008; Österberg *et al*., 2011). The results with the σ^S-dependent promoter fusion during phosphate starvation suggest that this indirect mechanism of regulation could still be applicable for alternative sigma factors other than σ^E, provided that (1) the rRNA operons are subject to regulation by (p)ppGpp and DksA, in which case σ^E is regulated in a unique manner that does not require DksA, or (2) the rRNA operons are regulated by (p)ppGpp only, in which case σ^S is regulated in a unique manner that requires DksA.

To the best of our knowledge, regulation of the rRNA promoters during phosphate starvation and the involvement of (p)ppGpp and DksA in any such regulation have not been reported, although (p)ppGpp levels have long been known to increase (Spira *et al*., 1995). To assess regulation of rRNA transcription following phosphate starvation, transcription of the stringently regulated *rrnB* P1–*lacZ* transcriptional reporter fusion was monitored. Decreased transcription is difficult to accurately measure using a β-galactosidase enzyme assay, in contrast to increased transcription, because β-galactosidase is a stable protein. A reduction in its production will be masked by the pre-existing enzyme. Therefore, we used primer extension to assay expression of the short-lived RNA transcript made from the *rrnB* P1–*lacZ* reporter (Schneider *et al*., 2003). *rrnB* P1 promoter activity decreased in the wild-type strain such that the primer extension product was undetectable after entry into phosphate starvation (Fig. 3). In contrast, promoter activity stayed relatively constant in the Δ*dksA* strain rather than decreasing, indicating that DksA is important for the observed regulation (Fig. 3A). Surprisingly, promoter activity also decreased in the (p)ppGpp^0 strain, suggesting that (p)ppGpp was not required for the response (Fig. 3A). Because strains lacking (p)ppGpp and DksA can accumulate suppressor mutations, the experiments were repeated in independently constructed (p)ppGpp^0 and Δ*dksA* strains and similar results were

obtained. To further ensure that the (p)ppGpp-independent, DksA-dependent regulation of *rrnB* P1 was not an artefact of our experimental system or due to a mutation in our strains, we analysed the response to amino acid starvation caused by the addition of serine hydroxamate. Both (p)ppGpp and DksA were required for negative regulation of *rrnB* P1 following amino acid starvation, consistent with previous reports (Fig. S1) (Paul *et al*., 2004b). In addition, we monitored transcription of the fusion in the absence of starvation and it was transcribed equivalently in the wild-type, (p)ppGpp^0 and Δ*dksA* strains (Fig. S1). These data suggest that DksA, but not (p)ppGpp, is necessary for inhibition of transcription of *rrnB* P1 by Eσ^{70} during phosphate starvation. The results do not support a model in which negative regulation of stable RNA operons is coupled with regulation of the alternative sigma factors during phosphate starvation, because regulation of transcription directed by σ^{70} is (p)ppGpp-independent and DksA-dependent, σ^S is (p)ppGpp- and DksA-dependent, and σ^E is (p)ppGpp-dependent and DksA-independent.

σ^E activity, not production, is regulated in response to phosphate limitation and the response is independent of envelope stress

The DksA-independence of the σ^E response to phosphate starvation indicates that the mechanism of regulation is not the same as the DksA-dependent mechanism that controls σ^E activity in response to amino acid starvation or entry into stationary phase in LB. To better understand the DksA-independent mode of regulation, we focused on phosphate starvation. Phosphate starvation could cause an envelope stress that is sensed by the RseA-dependent stress signalling pathway such that this pathway is involved in the regulation along with (p)ppGpp. If so, the response of σ^E to phosphate starvation should be disrupted in a strain lacking *rseA*. However, σ^E activity increased when culture growth slowed in the Δ*rseA* strain, indicating that the envelope stress pathway does not participate in regulation of σ^E in response to phosphate starvation (Fig. 4).

σ^E activity could increase due to changes in its expression, stability and/or activity. As such, we analysed the steady state level of σ^E, which reflects its expression and stability, in wild-type and (p)ppGpp^0 strains grown in EZ Rich with limiting phosphate. The total amount of σ^E increased as the cultures transitioned into stationary phase in both strains (Fig. 5A and B). This increase was not specific to phosphate starvation, because a similar accumulation of σ^E was observed during entry into stationary phase in LB (Costanzo *et al*., 2008). The regulator responsible for the increase in σ^E levels has not been identified. The finding that σ^E accumulates in the wild-type and (p)ppGpp^0 strains during phosphate starvation and entry into stationary phase in LB suggests that alterations in the amount of σ^E are not specific to the (p)ppGpp-dependent, DksA-independent regulation of σ^E. Therefore regulation is likely to be at the level of activity.

Low levels of phosphate are sensed in *E. coli* by a signalling complex that includes the PhoR/PhoB two-component system. Although σ^E has not been implicated as part of the PhoB regulon, transcriptomic and proteomic studies indicate that the expression of 287 genes and up to 400 proteins are affected by phosphate limitation (Hsieh and Wanner, 2010; Yang *et al*., 2012). A PhoB-regulated protein or sRNA could in turn regulate σ^E activity. To determine if PhoB contributes to activation of σ^E, σ^E activity was analysed in a Δ*phoB* strain

following phosphate starvation. Activity was unchanged compared to the wild-type strain indicating that PhoB does not play a role in the response of σ^E to phosphate limitation (Fig. S2).

(p)ppGpp synthesis during phosphate starvation

The σ^E response to starvation is dependent on (p)ppGpp under all conditions tested. It is possible that the lack of requirement for DksA following phosphate starvation could be caused by variations in the amount of (p)ppGpp produced among the strains. Therefore, growth, σ^E activity, and (p)ppGpp levels were measured in the wild-type, Δ*dksA* and (p)ppGpp^0 strains during phosphate starvation (Fig. 6). σ^E activity increased when growth slowed in the wild-type and Δ*dksA* strains, approximately one hour following the shift to medium with limiting phosphate (Fig. 6A). (p)ppGpp levels alone and as a fraction of guanosine nucleotide pools, (p)ppGpp/(GTP + (p)ppGpp), also peaked at the one hour time point and then declined for both wild-type and Δ*dksA* strains (Fig. 6B – D). The amount of (p)ppGpp was approximately twofold to fourfold higher in the Δ*dksA* strain than in the wild-type strain, whereas no (p)ppGpp was detected in the (p)ppGpp^0 strain (Fig. 6B – D). While measuring (p)ppGpp levels following phosphate starvation, we noticed that the ATP, GTP and CTP levels dropped substantially when growth slowed in all the strains tested, including the (p)ppGpp^0 strain (Fig. 6E). In contrast, the levels of these nucleotides stayed constant during amino acid starvation, while (p)ppGpp levels increased, similar to observations by others (Paul *et al*., 2004a) (Fig. S3).

In *E. coli*, both ppGpp and pppGpp accumulate during starvation, although their relative amounts vary depending on the starvation condition. For example, both pppGpp and ppGpp accumulate during amino acid starvation, while ppGpp primarily accumulates during carbon starvation (Cashel, 1975; Gallant, 1979; Mechold *et al*., 2013). Our previous work demonstrated that DksA was required for Eσ^E to respond to ppGpp in *in vitro* transcription reactions. However, the effects of pppGpp on transcription by Eσ^E were not measured, leaving open the possibility that pppGpp may have different effects on Eσ^E than ppGpp and perhaps not require DksA. An accumulation of more pppGpp than ppGpp during phosphate starvation could then provide an explanation for the differential requirement of DksA. We examined the amount of pppGpp that accumulated during phosphate starvation using TLC conditions that separate pppGpp from ppGpp. The levels of pppGpp were very low compared to ppGpp (< 10% of the amount of ppGpp) (Fig. 7) indicating that ppGpp is the main regulator, and pppGpp is unlikely to contribute significantly to regulation of σ^E during phosphate starvation.

The source of (p)ppGpp does not affect the increase in σ^E activity in response to phosphate starvation

(p)ppGpp levels are thought to increase following amino acid starvation and phosphate starvation by different mechanisms. During amino acid starvation RelA is activated by the binding of uncharged tRNA in the A site of the ribosome (Haseltine and Block, 1973; Wendrich *et al*., 2002). During phosphate starvation, (p)ppGpp levels are thought to increase due to inhibition of the SpoT hydrolase, although the signalling mechanism is not known (Spira *et al*., 1995). To determine if the mechanism by which (p)ppGpp levels increase

influences regulation of σ^E, σ^E activity and (p)ppGpp production were measured in a series of strains that differ in their ability to synthesize and hydrolyse (p)ppGpp. It was not possible to use a strain in which (p)ppGpp levels were solely controlled by RelA, because the *spoT* gene is essential in the presence of a functional *relA* gene. Instead we used a strain carrying the *spoT*E319Q (*spoT*$^{syn-}$) mutation that inactivates the SpoT synthase (Harinarayanan *et al*., 2008). (p)ppGpp levels in this strain are controlled by RelA-dependent synthesis and SpoT-dependent hydrolysis. Growth, σ^E activity and (p)ppGpp accumulation in the *spoT*$^{syn-}$ mutant in response to phosphate starvation were comparable to that measured for the wild-type strain (Fig. 8A – D), indicating that control of (p)ppGpp synthesis by RelA and hydrolysis by SpoT are sufficient for regulation of σ^E.

σ^E activity and (p)ppGpp production were also measured in a Δ*relA* strain in which (p)ppGpp levels are controlled by SpoT-dependent synthesis and hydrolysis. σ^E activity increased in the Δ*relA* mutant, like it did in the wild-type strain (Fig. 8A). However, growth of the Δ*relA* cultures slowed more following phosphate starvation compared to the *relA*$^+$ strains, suggesting that the Δ*relA* mutant adapts less efficiently to lower phosphate levels (Fig. 8B). (p)ppGpp also accumulated somewhat slower than it did in the wild-type strain and the levels were still elevated at the 90 min. time point, whereas they decreased after a peak at 60 min. in the other strains (Fig. 8C–E). Finally, to isolate effects due to regulation of (p)ppGpp levels by control of the SpoT hydrolase, we examined a strain lacking *relA* and carrying the *spoT*R39A (*spoT*$^{hyd-}$) mutation that inactivates the SpoT hydrolase (Harinarayanan *et al*., 2008). In this strain, (p)ppGpp accumulates from the weak synthase activity of SpoT. Similar to the Δ*relA* strain, growth of this strain slowed more during entry into phosphate starvation than strains with *relA* (Fig. 8B). σ^E activity increased when culture growth slowed, but about twofold less than in the other strains (Fig. 8A). In the Δ*relA* *spoT*$^{hyd-}$ mutant, (p)ppGpp levels stayed relatively constant and were at least 10-fold lower than the peak amount of (p)ppGpp detected in the other strains (Fig. 8C–E). However, the (p)ppGpp/(GTP+(p)ppGpp) ratio increased due to the decrease in GTP levels.

σ^E activity increased in response to phosphate depletion in all of the strains that were able to synthesize (p)ppGpp. However, activity was not strictly correlated with the amount of (p)ppGpp (compare (p)ppGpp levels and σ^E activity in the Δ*relA* *spoT*$^{hyd-}$ strain to those in the other strains). The (p)ppGpp^0 strain was the only mutant examined in which the σ^E response to phosphate starvation was completely disrupted, and this strain is the only strain lacking *spoT*. These data suggest that the SpoT protein itself could modulate σ^E activity, independently of (p)ppGpp production. To test this idea, we combined the *spoT*$^{syn-}$ allele with a *relA* deletion. This strain does not make (p)ppGpp (data not shown), but does express a full-length SpoT protein (Harinarayanan *et al*., 2008). σ^E did not respond to phosphate starvation in this strain and (p)ppGpp was not detected, suggesting that synthesis of (p)ppGpp is required for the increase in σ^E activity (Fig. 8A). Taken together, these data show that σ^E responds to phosphate starvation independently of the source of (p)ppGpp.

Secondary channel binding proteins and σ^E activity

DksA exerts its effects on transcription by binding in the secondary channel of core RNAP. E *coll* possesses several other proteins, GreA, GreB and Rnk, which also bind in the

secondary channel (Rutherford *et al*., 2007; Lamour *et al*., 2008; Vinella *et al*., 2012). GreA and GreB are well-characterized for their role in transcriptional pausing (Nickels and Hochschild, 2004). Both factors can also substitute for DksA and regulate transcription initiation by $E\sigma^{70}$ under certain circumstances (Potrykus *et al*., 2006; Rutherford *et al*., 2007; Vinella *et al*., 2012). The Rnk protein is a structural homologue of DksA and the Gre factors. It can compete with DksA for binding to the secondary channel of RNAP, although its role in transcriptional regulation is not clear (Lamour *et al*., 2008). To determine if any of these factors could substitute for DksA in regulating σ^E during phosphate starvation, we measured σ^E activity in strains lacking *greA*, *greB*, or *rnk* alone or in combination with the *dksA* deletion. σ^E activity in response to phosphate starvation was similar to that of the wild-type strain for all mutants, except the Δ*greA*Δ*greB* double mutant in which the increase in σ^E activity was about twofold lower than that of wild type (Figs S4 and S5). These results indicate that no single one of these proteins takes the place of DksA in regulating σ^E. It is possible that either *greA* or *greB* may play a small role in regulating σ^E, however their effect is significantly less than that observed in the $ppGpp^0$ strain in which σ^E activity does not increase upon phosphate depletion.

In addition to the known secondary channel binding proteins, we examined two other genes for their effects on σ^E activity, *ybil* and *crl*. Overexpression of *ybil* alleviates several of the amino acid auxotrophies associated with the Δ*dksA* strain, suggesting that it is a functional homologue of DksA (Blankschien *et al*., 2009). Crl is a small protein that facilitates the association of σ^S and σ^H with core RNAP *In vitro* and has global regulatory effects via σ^S during stationary phase *In vivo* (Gaal *et al*., 2006; Typas *et al*., 2007). We deleted these genes alone and in combination with a deletion of *dksA*. The σ^E response to phosphate starvation was similar to that of the wild-type parent for each of these strains (data not shown), indicating that Ybil and Crl are also not necessary for activation of σ^E and do not substitute for DksA (Fig. S5).

Discussion

In this work we demonstrate that the *E. coli* extracytoplasmic stress sigma factor, σ^E, can be activated in the absence of cell envelope stress by the alarmone (p)ppGpp in response to different starvation conditions using at least two mechanisms, one dependent on DksA and the other DksA independent. Our previous model that (p)ppGpp and DksA regulate σ^E directly by acting on $E\sigma^E$ and indirectly via effects on transcription of stable RNA genes by $E\sigma^{70}$ (Costanzo *et al*., 2008) cannot explain the (p)ppGpp-dependent, DksA-independent regulation of σ^E during phosphate starvation described here. In addition, the observations that inhibition of σ^{70}-dependent transcription of stable RNA promoters requires only DksA and activation of σ^S-dependent transcription requires both ppGpp and DksA further invalidate the model that decreased transcription of the stable RNA genes contributes to activation of alternative sigma factors in concert.

Because DksA and (p)ppGpp often work together and DksA was thought to be required for σ^E to respond to (p)ppGpp, the differential requirement for DksA during phosphate starvation was surprising. However, a number of reports indicate that the effects of DksA and (p)ppGpp on both cellular physiology and transcription can differ. The phenotypes of

ppGpp^0 and Δ*dksA* mutants are similar, but not completely overlapping (Magnusson *et al*., 2007; Aberg *et al*., 2009). Similarly, the changes in gene expression in ppGpp^0 and Δ*dksA* mutants are also largely, but not completely overlapping, suggesting that (p)ppGpp and DksA can have independent and even opposing effects on transcription (Aberg *et al*., 2009). Opposing effects of ppGpp and DksA have also been demonstrated for the phage λ pR promoter (Magnusson *et al*., 2007). The extent of differential regulation by (p)ppGpp and DksA is not known and adds another level of complexity, as demonstrated here, to their ability to sculpt the transcriptome.

Regulation of Eσ^E by (p)ppGpp and DksA during phosphate starvation

Our data with the Δ*rseA* strain indicate that phosphate starvation does not create a cell envelope stress to activate σ^E, nor does it increase the expression of *rpoE*. Therefore, the observed increase in σ^E activity is most likely due to (p)ppGpp-dependent increased transcription by Eσ^E. How does (p)ppGpp control σ^E activity without DksA in response to phosphate starvation? A potential explanation is that the twofold to fourfold increased amount of (p)ppGpp in the Δ*dksA* strain compensates for the absence of DksA, and DksA is still required in wild-type cells with lower amounts of (p)ppGpp. We think this model unlikely because it predicts that once a threshold amount of (p)ppGpp accumulates, DksA is no longer required for increased σ^E activity. However, DksA is required for the σ^E response to amino acid starvation, and our work, together with other studies, indicates that (p)ppGpp levels are actually somewhat higher during amino acid starvation than during phosphate starvation (unpublished observations and Rao *et al*., 1998; Spira *et al*., 1995). Additionally, *in vitro* multi-round transcription assays with high amounts of (p)ppGpp in the absence of DksA show no increase in Eσ^E-dependent transcription (Costanzo *et al*., 2008). Another explanation for DksA-independence of the response is that the decrease in NTP levels caused by phosphate starvation is important for regulation. Yet, it is difficult to envision a mechanism by which low NTP availability would increase transcription.

We propose that an as yet undiscovered factor participates in the control of σ^E during phosphate starvation along with (p)ppGpp. Results from experiments with the Δ*relA spoT*$^{hyd-}$ mutant indicate that the levels of (p)ppGpp in this mutant increased very little, if at all, suggesting that the signal for phosphate starvation is not simply increased levels of (p)ppGpp, but a function of this missing factor. Taking a candidate approach, we tested whether the missing factor was a transcriptional regulator known to respond to phosphate starvation (*phoB*), one of a series of structural and/or functional homologues of DksA (*greA, greB, ybil* and *rnk*), or an additional modulator of polymerase activity (*crl*). None of these regulators appear to be involved, so the identity of the factor remains a mystery.

Regulation of Eσ^{70} in response to phosphate starvation

Although transcription of the *rrnB* P1 promoter by (p)ppGpp and DksA has been extensively studied during amino acid starvation, at different growth rates, throughout the growth curve, and in response to nutrient upshifts and downshifts, phosphate starvation had not been investigated to our knowledge (Dennis *et al*., 2004; Paul *et al*., 2004a; Potrykus and Cashel, 2008). Because (p)ppGpp levels increase, we assumed that (p)ppGpp would be required for downregulation of transcription by E σ ^{70} at the *rrnB* P1 promoter. Instead, (p)ppGpp was

found to be dispensable, while DksA was required. The explanation for why (p)ppGpp is not needed may lie in the observation that nucleotide levels drop significantly when phosphate is depleted from the growth medium. At the *rrnB* P1 promoter and other stringently regulated rRNA promoters, $E\sigma^{70}$ forms unstable open complexes, which are stabilized by binding of initiating nucleotides (iNTPs) and destabilized by DksA and (p)ppGpp (Gaal *et al*., 1997; Barker *et al*., 2001; Murray *et al*., 2003; Murray and Gourse, 2004; Paul *et al*., 2004b; Kolmsee *et al*., 2011). A higher concentration of iNTP is required for transcription initiation at *rrnB* P1 in the presence of DksA than in its absence (Paul *et al*., 2004b). Because nucleotide levels drop during phosphate starvation, the decreased availability of nucleotides in combination with destabilization of open complexes by DksA may reduce transcription initiation, eliminating the requirement for (p)ppGpp. Because most other *rrn* promoters form similarly unstable open complexes limited by iNTP concentrations, this mechanism of regulation is likely to be shared and not particular to *rrnB* P1 (Gaal *et al*., 1997; Barker *et al*., 2001; Murray *et al*., 2003; Murray and Gourse, 2004; Paul *et al*., 2004a; Kolmsee *et al*., 2011).

Regulation of $E\sigma^S$ in response to phosphate starvation

σ^S is regulated by a complex network of signalling pathways that are activated under different conditions and affect σ^S at the transcriptional, translational and post-translational levels (Battesti *et al*., 2011). Translation of *rpoS* increases and proteolysis of σ^S decreases in response to phosphate limitation, presumably leading to increased levels of σ^S and increased expression of the σ^S regulon (Gentry *et al*., 1993; Bougdour and Gottesman, 2007). The stability of σ^S is modulated by the expression of the adaptor protein RssB, which delivers σ^S to the ClpXP protease for degradation (Muffler *et al*., 1996; Pratt and Silhavy, 1996; Zhou and Gottesman, 1998). A series of anti-adaptor proteins, expressed under different physiological conditions, prevent RssB from binding σ^S, thereby stabilizing σ^S (Bougdour *et al*., 2008). The IraP anti-adaptor is responsible for stabilization of σ^S during phosphate starvation, and its transcription is reduced in strains lacking (p)ppGpp and DksA (Bougdour *et al*., 2008). Therefore, the requirement for DksA and (p)ppGpp for activation of σ^S is likely to be due, at least in part, to positive regulation of $E\sigma^{70}$–dependent transcription of *iraP* by DksA and (p)ppGpp. DksA and (p)ppGpp may also contribute to translational regulation of *rpoS* (Battesti *et al*., 2011). Both factors have been shown to be important for upregulation of *rpoS* translation during entry into stationary phase (Brown *et al*., 2002). However, their role in translational regulation of *rpoS* during phosphate starvation has not been examined.

Production of (p)ppGpp in response to phosphate starvation

The prevailing model for the increased amounts of (p)ppGpp during phosphate starvation is that phosphate levels are sensed by the SpoT hydrolase through an unknown mechanism that results in decreased hydrolysis of (p)ppGpp. This model is based in a large part on observations that during phosphate starvation (p)ppGpp levels increased in a Δ*relA* strain and (p)ppGpp-dependent regulation of several factors including σ^S activity and *iraP* expression was not disrupted (Spira *et al*., 1995; Rao *et al*., 1998; Bougdour and Gottesman, 2007). However, the observation that strains lacking *relA* stop growing abruptly at the onset of phosphate starvation and that the rise in (p)ppGpp follows different kinetics in the Δ*relA*

strain than in the wild-type strain (also seen by Spira *et al*., 1995) suggest a role for RelA in phosphate starvation. Because RelA shares a similar domain structure with SpoT, although the hydrolase of RelA is inactive, it is possible that the production of (p)ppGpp by RelA is subject to regulation during phosphate starvation along with SpoT and regulation of both factors yields the optimal response (Gentry and Cashel, 1996; Gropp *et al*., 2001; Mechold *et al*., 2002).

Global regulation of alternative sigma factors by (p)ppGpp and DksA

In *E. coli*, the activity of the alternative sigma factors, σ^E, σ^H, σ^N and σ^S, have all been shown to increase under a variety of nutrient limitation conditions or entry into stationary phase in a (p)ppGpp-dependent manner (Gentry *et al*., 1993; Jishage *et al*., 2002; Laurie *et al*., 2003; Costanzo and Ades, 2006; Costanzo *et al*., 2008; Österberg *et al*., 2011). The current model explaining how this happens is that (p)ppGpp activates alternative sigma factor-dependent transcription by altering the competition among sigma factors for limiting core RNAP in favour of the alternative sigma factors. As described above, this model does not explain the results presented in this work. A model for the mechanism of action of (p)ppGpp that explains its ability to regulate all alternative sigma factors in concert is certainly attractive. Nevertheless, our results suggest that there may not be a global mechanism by which (p)ppGpp orchestrates changes in the transcriptome that encompasses all sigma factors. Instead, (p)ppGpp may directly regulate transcription of individual promoters by different holoenzymes, with or without DksA and other transcription factors depending on the physiological conditions. (p)ppGpp and/or DksA may also control the expression of specific regulators of a particular sigma factor associated with a particular starvation condition (i.e. regulation of RpoS via IraP). As such, (p)ppGpp may achieve global changes in transcription by acting locally on individual promoters, sigma factors and holoenzymes.

Experimental procedures

Media, strains and plasmids

Strains used in this paper are derivatives of the MG1655 *E. coli* K12 strain and are listed in Table 1. Cells were grown at 30°C or 37°C, as indicated, with aeration in LB (0.5% yeast extract, 1% tryptone, 0.5% NaCI, pH 7.4) or EZ Rich [a MOPS-based medium (Neidhardt *et al*., 1974) purchased from Teknova]. Antibiotics were used at the following concentrations: ampicillin 100 μg ml^{-1}, kanamycin 15 μg ml^{-1}, tetracycline 10 μg mL^{-1} and chloramphenicol 20 μg ml^{-1}. For phosphate limitation experiments, EZ Rich was prepared with 0.132 mM K_2HPO_4, instead of the standard 1.32 mM K_2HPO_4. For amino acid limitation experiments, isoleucine was included at 0.06 mM, instead of the standard 0.4 mM. The limiting isoleucine medium was prepared using Teknova components with the exception of the 5× supplement, which was prepared according to the recipe from the *E. coli* Genome Project (http://www.genome.wisc.edu/resources/protocols/ezmedium.htm). The amino acid starvation system utilizing limiting isoleucine is based on that described in Traxler *et al*. (2008). For *E. coli* K12, excess valine in the presence of low concentrations of isoleucine leads to feedback inhibition of enzymes responsible for synthesizing isoleucine. As a result, *E. coli* K12 strains grown in low concentrations of isoleucine will slowly enter

amino acid starvation when isoleucine is depleted and de novo synthesis is inhibited. An advantage to this system is that protein synthesis can continue, enabling use of β-galactosidase reporters, and the multi-auxotrophic ppGpp^0 strain can grow because all amino acids are present.

Mutant alleles were moved into appropriate strains by transduction with P1$_{vir}$ according to standard techniques (Miller, 1992). Transductants were isolated by selection on medium containing the appropriate antibiotic. Experiments with ppGpp^0 and *spoT*$^{hyd-}$ strains were performed with at least three independent transductants to ensure that the results were not affected by spontaneous suppressor mutations. In addition, ppGpp^0 strains were tested for their inability to grow on minimal media lacking amino acids, because many spontaneous suppressor mutations restore amino acid prototrophy. SEA6462 (Δ*rseA yfic::kan*) was made by transduction of the *yfiC::kan* allele from the Keio collection (Baba *et al*., 2006) into SEA2000 (SEA001 *nadB*::Tn*10* Δ*rseA*). Transductants were screened for loss of tetracycline sensitivity and increased σ^E activity due to loss of *rseA*. SEA7062 was made by targeted disruption of the *ybil* gene in SEA001 according to the procedure of Datsenko and Wanner (2000).

β-Galactosidase assays

β-galactosidase assays were performed as described (Costanzo and Ades, 2006). Data are presented as differential rate plots in which β-galactosidase activity in a fixed volume of culture is plotted versus the optical density (OD$_{600}$) of the sample. The slope of the linear portions of the curve represents the accumulation of σ-dependent β-galactosidase activity as a function of increased cell density. To calculate how σ^E or σ^S activity changes during entry into stationary phase in rich media or in response to nutrient limitation, the slope of the linear portion of the differential rate plot for the relevant points corresponding to entry into stationary phase was determined. All experiments were performed at least three times with independent cultures and activity varied by < 10%.

Western blotting

Western blotting was performed as described (Costanzo *et al*., 2008). Briefly, whole cell extracts were precipitated with acetone and resuspended in 2% SDS. Protein concentrations were determined using the BCA protein assay (Pierce) and 10 μg of total protein from each sample were loaded. σ^E-containing bands were detected using a polyclonal antibody (gift from CA Gross) and alkaline phosphatase conjugated secondary antibody in conjunction with the ECF reagent (GE Healthcare) followed by scanning with the Typhoon 8600 Imager in fluorescence mode.

Primer extension

Primer extension was performed as described (Ross and Gourse, 2009). SEA6583 was grown with shaking to an OD$_{600}$ of 0.25–0.3 in EZ Rich. For phosphate limitation experiments, cells were then pelleted by centrifugation, resuspended to the same OD$_{600}$ in EZ Rich with limiting phosphate, and growth resumed. For amino acid starvation, serine hydroxamate was added to 1 mg ml^{-1} final concentration. Samples were taken at the indicated times after the change in growth medium or addition of serine hydroxamate, and

total RNA prepared by the boiling lysis method. To control for sample degradation and variations in gel loading, 5 μg of recovery marker RNA was added to each sample during the phenol-chloroform extraction step of the boiling lysis RNA preparation protocol. Recovery marker RNA was prepared from strain SEA7070, which carries a *lacUV5–lacZ* promoter fusion. The 5414 primer used for primer extension anneals to this fusion as well as the rm6P1–*lacZ* fusion and produces primer extension products of different sizes from each reporter. For the primer extension reactions, 15 μg of total RNA was annealed to [32]P end-labelled primer 5414 (5′-TGGTGTTCGTCCCGGCTGTAATGTTCTGGC-3′) and the extension reaction was carried out with Moloney Murine Leukemia Virus reverse transcriptase (M-MLV RT from Promega) for 30 min. at 42°C. Reactions were stopped with formamide loading buffer. Extension products were separated by electrophoresis on denaturing 6% polyacrylamide gels and visualized by phosphorimaging.

Measurement of (p)ppGpp and NTP levels

To measure NTP and (p)ppGpp levels following starvation for phosphate, cultures were grown in EZ Rich at 37°C with shaking to an OD_{600} of approximately 0.2. Cells were collected by centrifugation, and pellets resuspended in EZ Rich with limiting phosphate to the same optical density and divided between two flasks. [32]P-orthophosphoric acid (specific activity 8500 Ci $mmol^{-1}$, Perkin-Elmer) was added to one culture to a concentration of 20 μCi ml^{-1}. The other culture was used to monitor OD_{600} and activity of the *rpoHP3–lacZ* reporter. At the indicated times, samples were taken from the [32]P labelled culture and nucleotides were isolated by formic acid extraction (Schneider *et al*., 2003). Nucleotides were separated by PEI cellulose thin-layer chromatography using 0.85 M KH_2PO_4 as the running buffer. Because pppGpp and ppGpp are not resolved using 0.85 M KH_2PO_4, a running buffer with 1.5 M KH_2PO_4 was used to separate pppGpp from ppGpp for the TLC presented in Fig. 7. Nucleotides were detected by phospho-imaging and quantified using Image-Quant 5.2 (Molecular Dynamics). Because equal volumes of samples were spotted onto the TLC plates, the intensities of the nucleotide spots were normalized to the OD of the cultures at the time of sampling during the data analysis. These values are presented in Figs 6A and 8B. To compare samples from different cultures, the amounts of (p)ppGpp as a fraction of (p)ppGpp and GTP were calculated (Figs 6D and 8E).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank Richard Gourse for providing the *rrnB P1–lacZ* fusion strain RLG6583 and *lacUV5–lacZ* fusion strain RLG4993, Michael Cashel for the $spoT^{syn-}$ and $spoT^{hyd-}$ mutant strains CF11605 and CF11608, and Steve Finkel for the *bolA–lacZ* reporter. We would also like to thank Richard Gourse, Tomas Gaal, Karen Wassarman and Jade Wang for advice on nucleotide extraction experiments, and the Gourse lab for advice on the primer extension experiments. This project was supported by Award number NSF0347302 from the National Science Foundation and Award number GM097365 from the National Institute of General Medicine.

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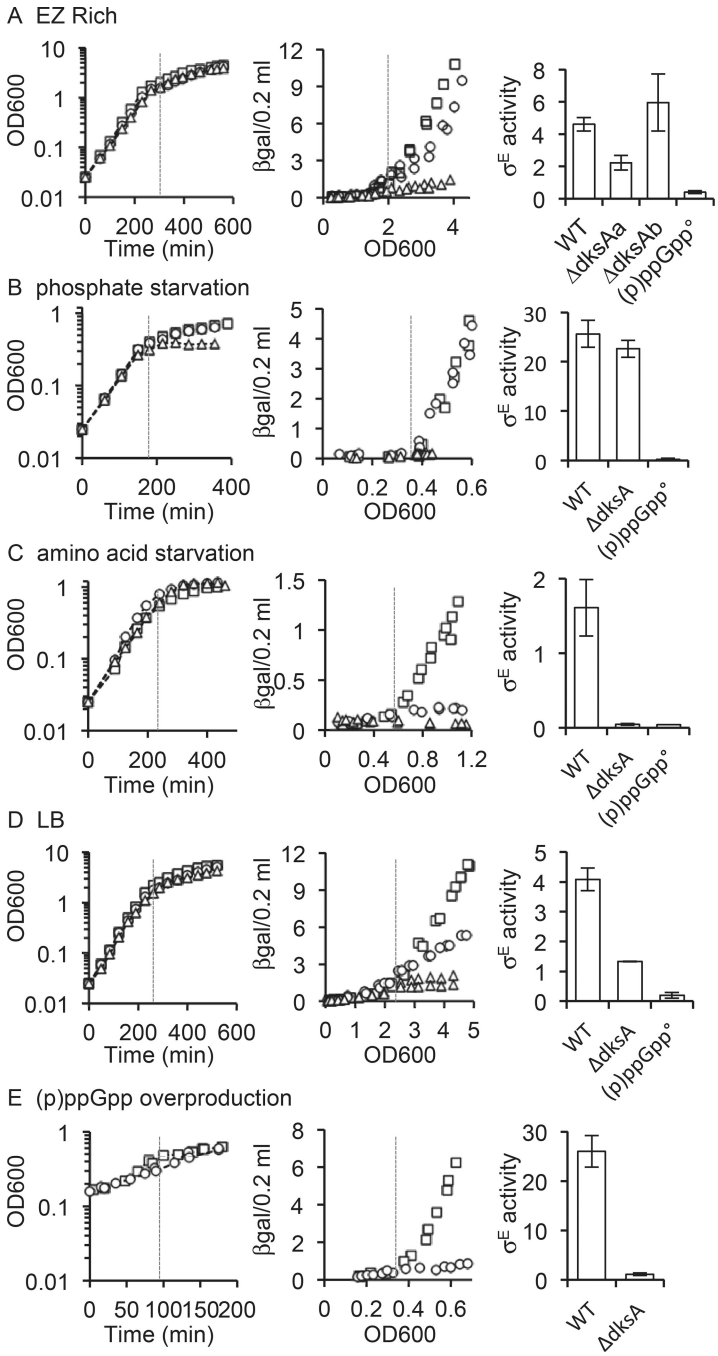

Fig. 1.

The increase in σ^E activity during entry into stationary phase is dependent on (p)ppGpp under all conditions, but dependent on *dksA* only under certain conditions. σ^E activity was measured throughout the growth curve in wild-type (squares), Δ*dksA* (circles) and (p)ppGpp^0 (triangles) cultures grown in (A) EZ Rich, (B) EZ Rich with limiting phosphate (phosphate starvation), (C) EZ Rich with limiting isoleucine (amino acid starvation), (D) LB, and (E) following gratuitous production of (p)ppGpp in the absence of starvation. In E IPTG was added to cultures grown in LB at time 0 (OD_{600} = 0.15) to induce expression of

relA′ and overproduction of (p)ppGpp. Growth curves are shown on the left. σ^E activity is shown in the right two graphs. The graphs in the middle are differential rate plots in which β-galactosidase activity from the σ^E-dependent reporter in fixed volume of culture is displayed as a function of culture growth throughout the growth curve. The dashed vertical lines on the left and middle graphs correspond to the OD_{600} after which σ^E activity increases. On the right, the increase in σ^E activity is quantified for each strain, as calculated from the slope on the differential rate plot for the points to the right of the dashed line. In EZ Rich, the increase in σ^E activity in the Δ*dksA* strain is biphasic, low during entry into stationary phase (OD_{600} = 2.0–3.5) then increasing at the end of the transition into stationary phase (OD_{600} = 3.5–4.5), and both slopes are quantified (Δ*dksAa* and Δ*dksAb* respectively). Data from at least two representative experiments are shown in each graph.

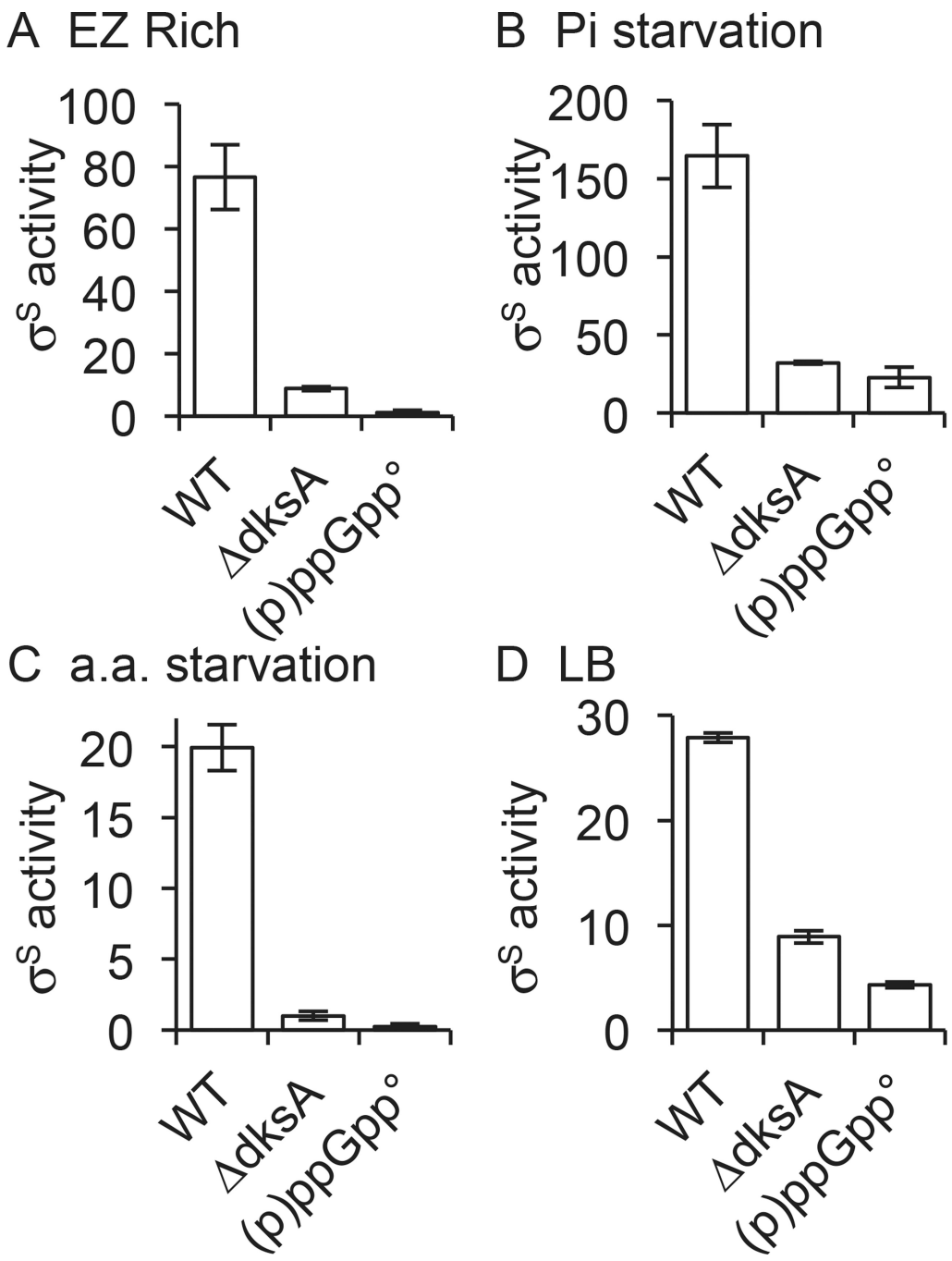

Fig. 2.

The increase in σ^S activity during entry into stationary phase is dependent on (p)ppGpp and *dksA* under all conditions tested. σ^S activity was measured from the *bolA–lacZ* fusion throughout the growth curve in wild-type, Δ*dksA* and (p)ppGpp^0 cultures grown in (A) EZ Rich, (B) EZ Rich with limiting phosphate (phosphate starvation), (C) EZ Rich with limiting isoleucine (amino acid starvation), and (D) LB. The increase in σ^S activity when growth slows is quantified for each strain as described in Fig. 1.

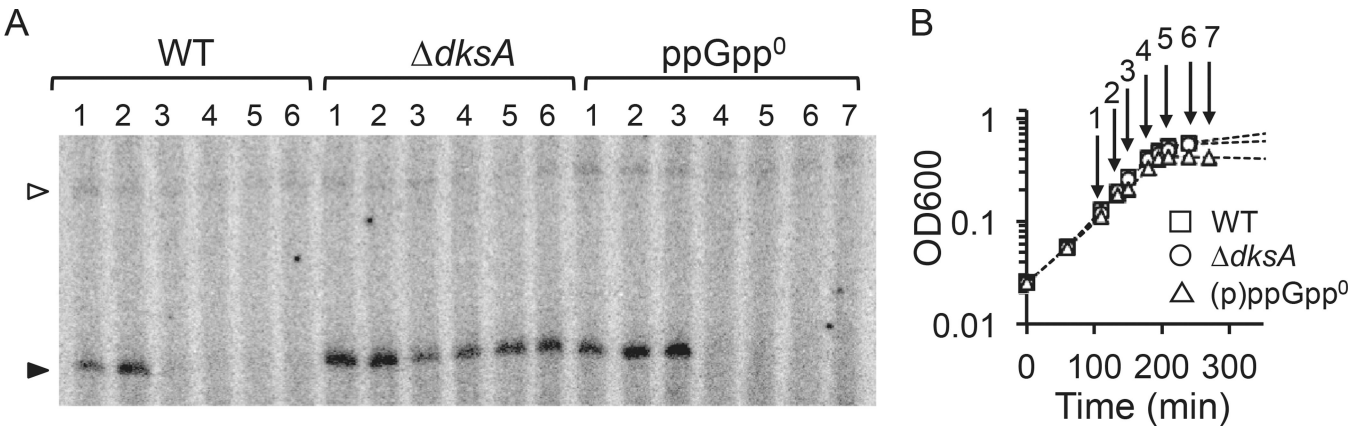

Fig. 3.

DksA, but not ppGpp, is required for full inhibition of transcription from *rrnB* P1 by $E\sigma^{70}$ following phosphate depletion.

A. Primer extension was used to measure mRNA production from the *rrnB* P1–*lacZ* fusion. The primer extension product is indicated by the closed arrowhead and a recovery marker by the open arrowhead.

B. Growth curves of the reporter strains are shown on the right and the times at which samples were taken for primer extension are indicated.

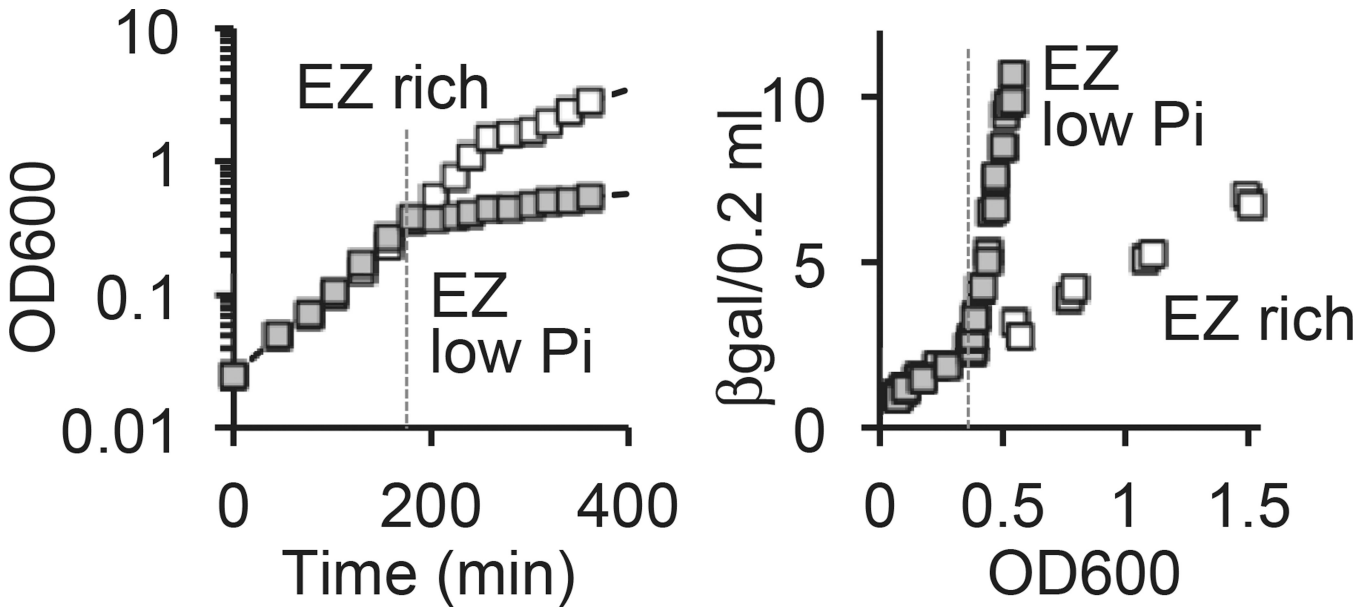

Fig. 4.

σ^E activity increases following phosphate depletion independently of *rseA*. The Δ*rseA* strain was grown in EZ Rich (open squares) and EZ Rich with limiting phosphate (shaded squares). Growth curves are shown on the left and σ^E activity is shown on the right in a differential rate plot in which β-galactosidase from the σ^E-dependent reporter in 0.2 ml culture is displayed as a function of culture growth. The dashed vertical lines correspond to the OD_{600} after which σ^E activity increases in EZ Rich with limiting phosphate.

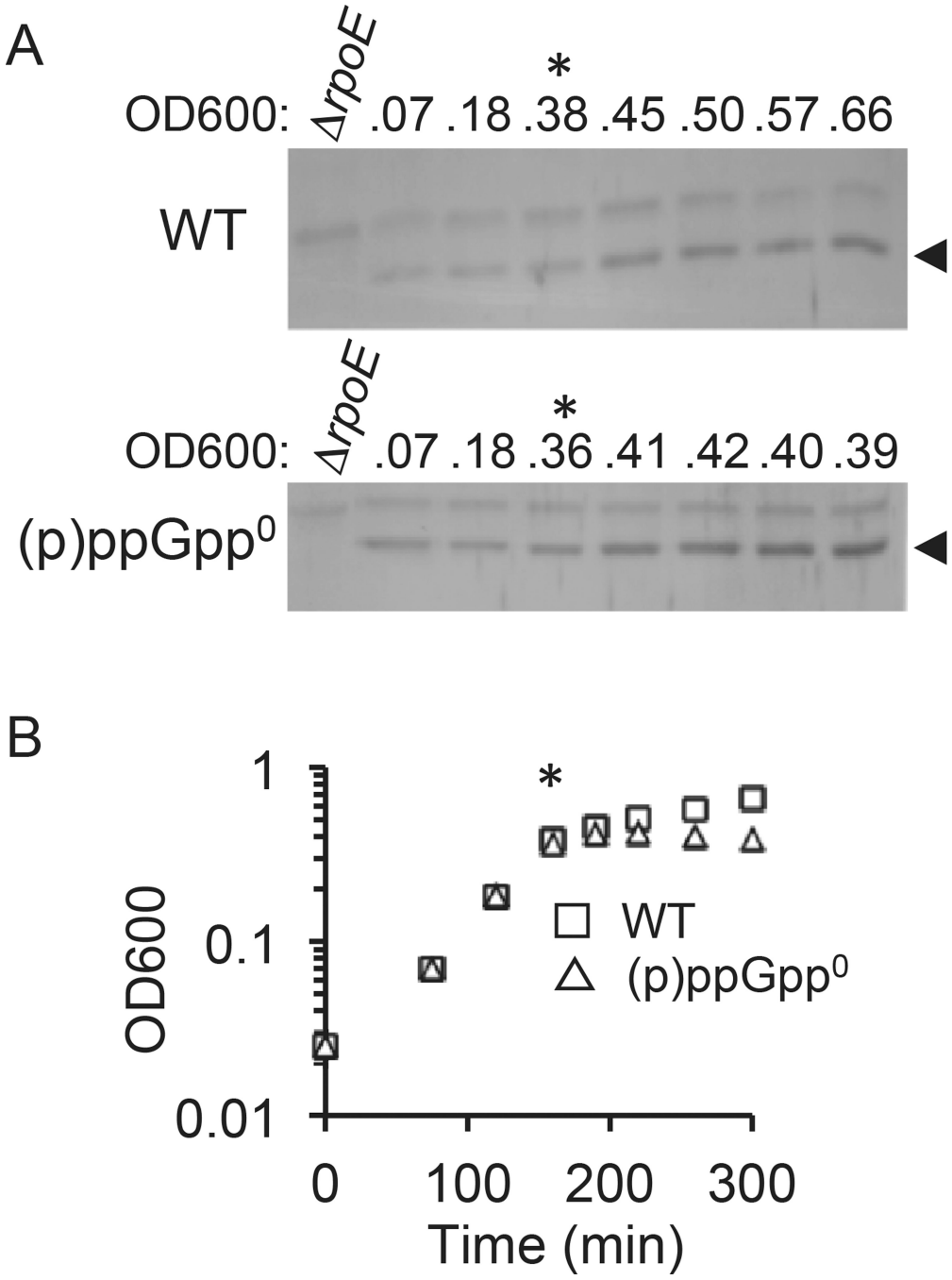

Fig. 5.

σ^E levels are similar in wild-type and (p)ppGpp^0 strains following phosphate depletion.

Wild-type and (p)ppGpp^0 strains were grown in EZ Rich with limiting phosphate.

A. Samples were taken at the indicated OD_{600} and cell extracts used for western blotting with an anti-σ^E polyclonal antibody. The band marked with an arrowhead corresponds to σ^E, while the top band is a cross-reacting band of unknown identity.

B. Growth curves for the cultures from which samples were taken are shown. Comparable amounts of protein were loaded in each lane. The asterisk marks the point at which growth slows and cultures start to enter stationary phase.

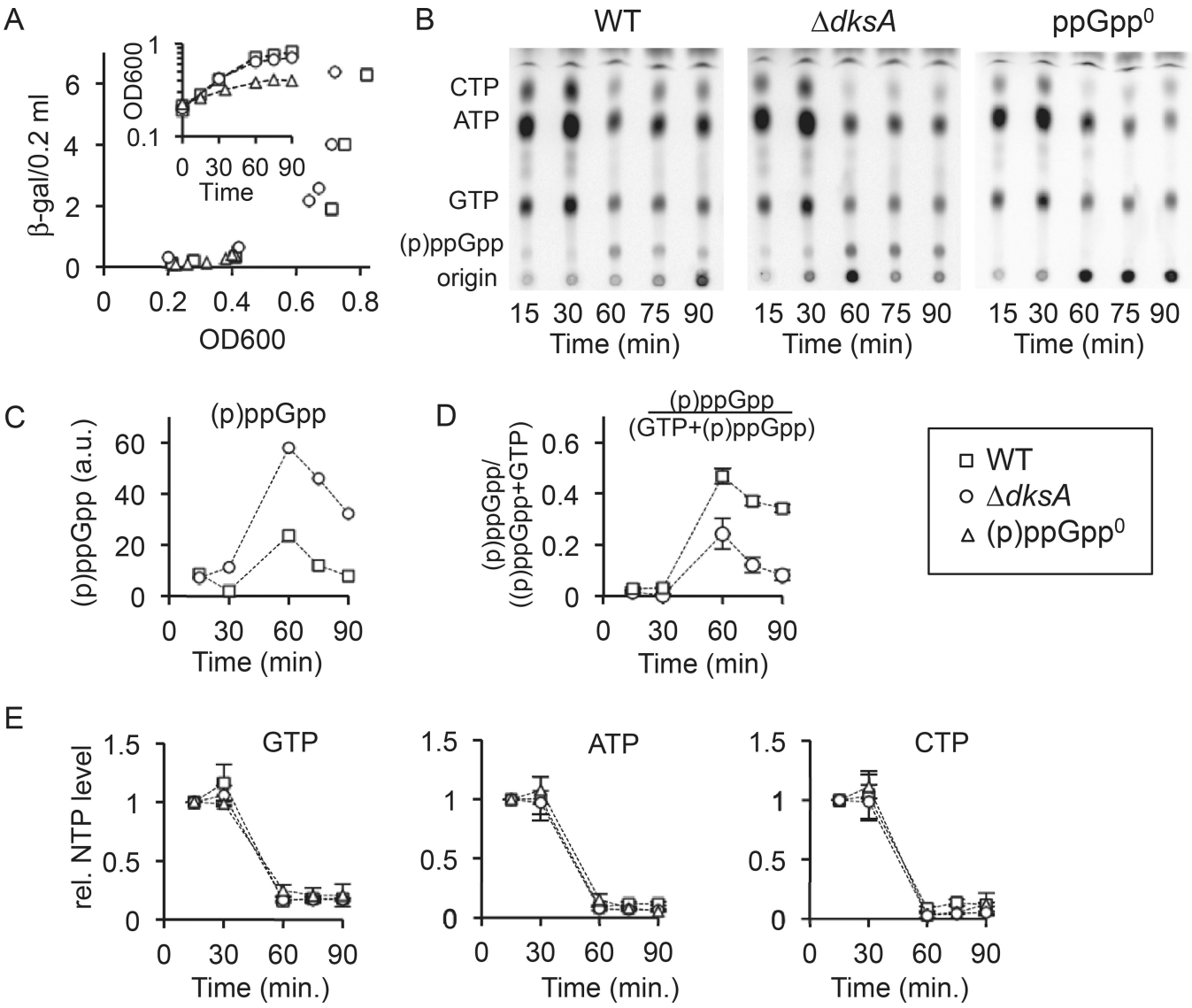

Fig. 6.

(p)ppGpp levels increase and GTP, ATP and CTP levels decrease following phosphate starvation.

A. Growth (inset graph) and σ^E activity (differential rate plot) from cultures used to isolate nucleotides are shown. Both graphs start with points after the shift to limiting phosphate (t = 0, OD_{600}~0.15).

B. TLC separation of nucleotides extracted with formic acid is shown for samples taken from cultures of WT, Δ*dksA* and (p)ppGpp^0 strains at the indicated times after a shift to EZ Rich with limiting phosphate.

C and D. (C) (p)ppGpp levels (arbitrary units, a.u.) were quantified from the TLC in part A and normalized to OD_{600} at the time of sampling or to (D) total (p)ppGpp and GTP pools.

E. GTP, ATP and CTP levels normalized to the level at 15 min. in each of the three strains after a shift to low phosphate. Symbols are the same in all parts of the figure as indicated in the legend.

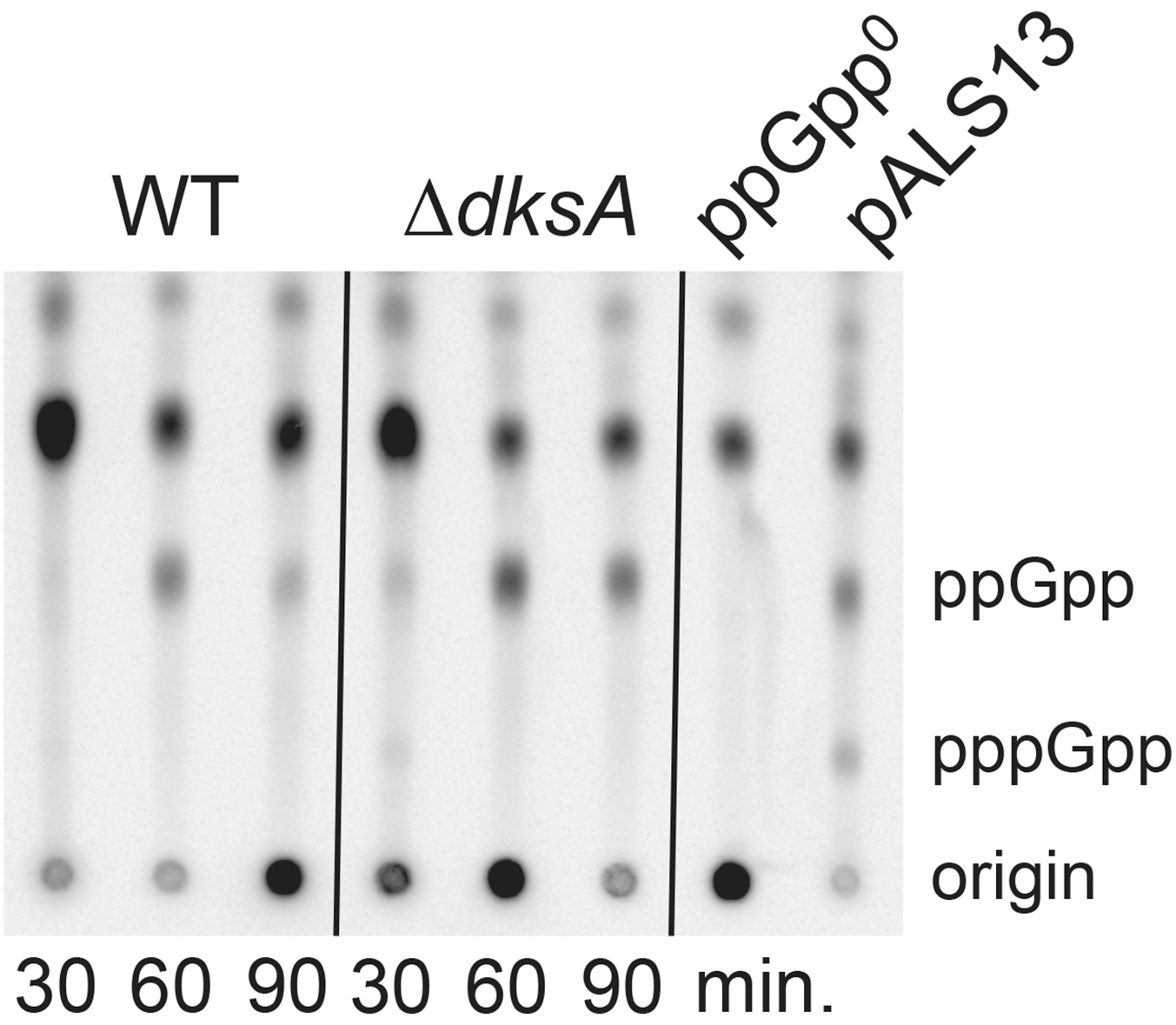

Fig. 7.

ppGpp accumulates to a greater extent than pppGpp following phosphate starvation in wild-type and Δ*dksA* strains. 1.5 M KH_2PO_4 was used as the running buffer in the TLC to resolve ppGpp from pppGpp. Extracts from a $ppGpp^0$ strain and a strain overexpressing the *relA′* fragment (pALS13) that accumulates both ppGpp and pppGpp are shown for reference.

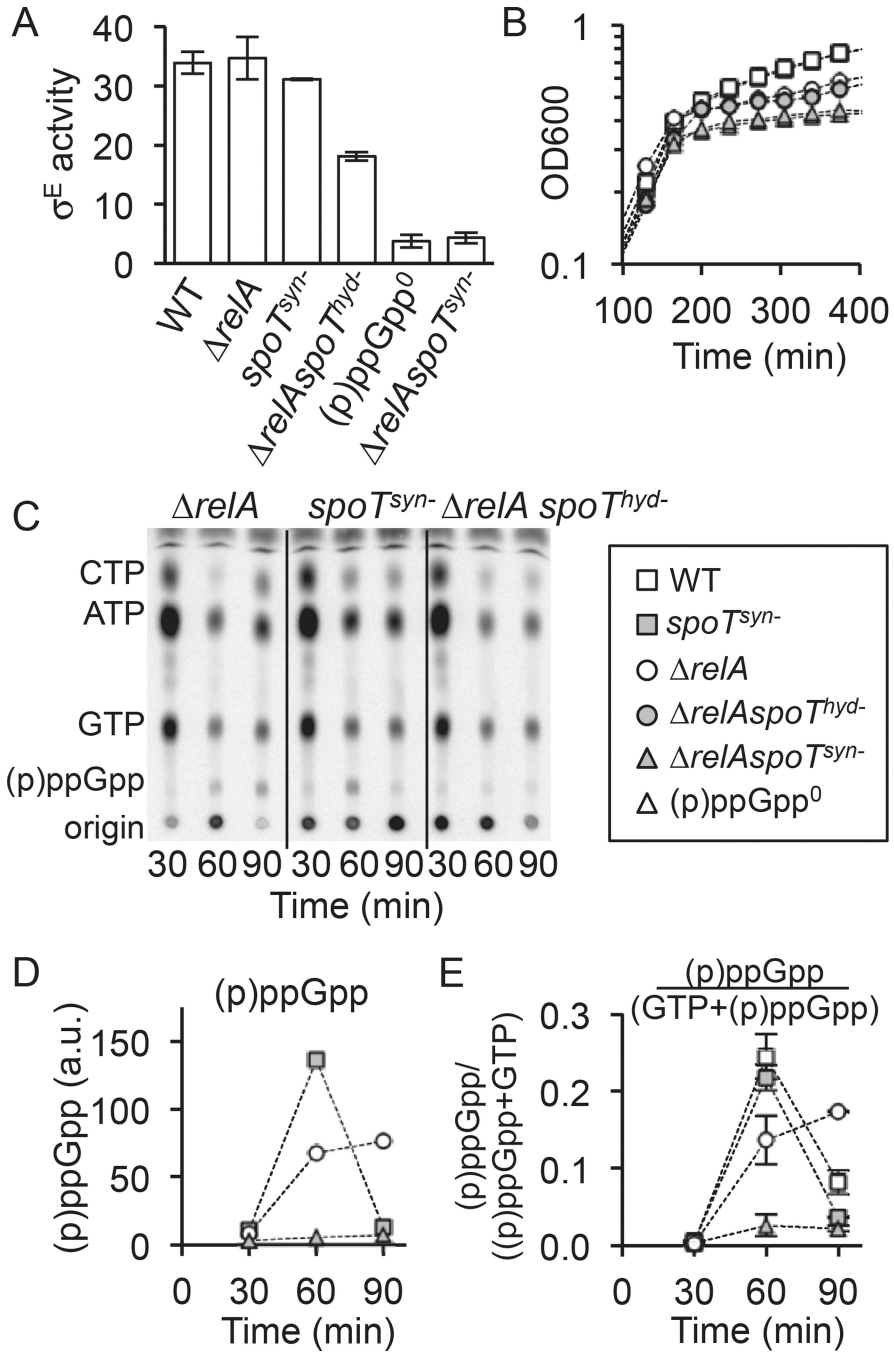

Fig. 8.

σ^E activity increases independently of the source of (p)ppGpp in all strains that make (p)ppGpp following phosphate starvation. The indicated strains were grown in EZ Rich and shifted to EZ Rich with limiting phosphate at time 0.

A and B. (A) σ^E activity measured when growth slows due to phosphate starvation is shown for each strain and (B) the corresponding growth curves are shown.

C and D. (C) TLC separation of nucleotides and (D) quantification of (p)ppGpp levels (arbitrary units) normalized to OD_{600} at time of sampling after the shift to low phosphate are shown.

E. (p)ppGpp levels normalized to total (p)ppGpp and GTP pools for wild-type, *spoT*$^{syn-}$, Δ*relA*, Δ*relAspoT*$^{hyd-}$ strains. Symbols are the same in all parts of the figure as indicated in the legend.

Table 1

Strains and plasmids used in this study.

Strain/plasmid	Genotype	Source, Reference, P1 donor strain
Strains		
SEA001	MG1655 Δ*lacX74* λ[*rpoH* P3::*lacZ*]	Costanzo and Ades (2006)
SEA2010	SEA001 Δ*relA*251::*kan* Δ*spoT*207::*chlor*	Costanzo and Ades (2006)
SEA4041	Δ*rpoE*::*kan* Suppressor+	This work, P1 donor CAG43113
SEA6020	SEA001 Δ*dksA*::tet	Costanzo and Ades (2006)
SEA6328	MG1655 Δ*lacX74* λ[*bolA*::*lacZ*]	provided by S. Finkel
SEA6329	SEA6328 Δ*dksA*::tet	This work, P1 donor SEA6020
SEA6401	SEA6328 Δ*relA*251::*kan*, Δ*spoT*207::*chlor*	This work, P1 donor SEA2010
SEA6462	SEA001 Δ*rseA yfic*::*kan*	This work
SEA6483	MG1655 λ[*rybB*::*lacZ*] pALS13	This work
SEA6487	SEA6483 Δ*dksA*::*tet*	This work
SEA6513	SEA001 Δ*relA*251::*kan*	Costanzo and Ades (2006)
SEA6565	SEA6513 *spoT*E319 *zib563*::Tn*10*	This work, P1 donor CF11608 provided by M. Cashel
SEA6575	SEA001 *spot*E319 *zib563*::Tn*10*	This work, P1 donor CF11608 provided by M. Cashel
SEA6583	VH1000 λ[*rrnB* P1–*lacZ*]	Provided by R. Gourse, RLG6583
SEA7019	SEA6513 *spoT*R39A *zib563*::Tn*10*	This work, P1 donor CF11605 provided by M. Cashel
SEA7025	SEA6583 Δ*dksA*::*tet*	This work, P1 donor SEA6020
SEA7027	SEA6583 Δ*relA*251::*kan* Δ*spoT*207::*chlor*	This work, P1 donor SEA2010
SEA7029	SEA001 Δ*greA*::*chlor*	This work, P1 donor RLG7239
SEA7030	SEA001 Δ*greB*::*kan*	This work, P1 donor RLG7240
SEA7031	SEA7029 Δ*greB*::*kan*	This work, P1 donor RLG7240
SEA7032	SEA7029 Δ*dksA*::*tet*	This work, P1 donor SEA6020
SEA7033	SEA7030 Δ*dksA*::*tet*	This work, P1 donor SEA6020
SEA7040	SEA7031 Δ*dksA*::*tet*	This work, P1 donor SEA6020
SEA7060	SEA001 Δ*rnk*::*kan*	This work, P1 donor JW0602
SEA7061	SEA7060 Δ*dksA*::*tet*	This work, P1 donor SEA6020
SEA7062	SEA001 Δ*ybil*::*chlor*	This work
SEA7063	SEA7062 Δ *dksA*::*tet*	This work, P1 donor SEA6020
SEA7070	VH1000 λ[*lacUV5–lacZ*]	Provided by R. Gourse RLG4993
SEA7149	SEA001 Δ*crl*::*kan*	This work, P1 donor JW0230
SEA7152	SEA7149 Δ*dksA*::*tet*	This work, P1 donor SEA6020
SEA7166	SEA001 Δ*phoB*::*kan*	This work, P1 donor JW0389, Baba *et al*. (2006)
Plasmids		
pALS13	p*tac* truncated *relA*, active protein, Ap^R	Svitil *et al*. (1993)