Growth Phase-Dependent Regulation of the Extracytoplasmic Stress Factor, σ^E , by Guanosine 3',5'-Bispyrophosphate (ppGpp)

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The sigma subunit of procaryotic RNA polymerases is responsible for specific promoter recognition and transcription initiation. In addition to the major sigma factor, σ^{70} , in *Escherichia coli*, which directs most of the transcription in the cell, bacteria possess multiple, alternative sigma factors that direct RNA polymerase to distinct sets of promoters in response to environmental signals. By activating an alternative sigma factor, gene expression can be rapidly reprogrammed to meet the needs of the cell as the environment changes. Sigma factors are subject to multiple levels of regulation that control their levels and activities. The alternative sigma factor σ^E in *Escherichia coli* is induced in response to extracytoplasmic stress. Here we demonstrate that σ^E can also respond to signals other than extracytoplasmic stress. σ^E activity increases in a growth phase-dependent manner as a culture enters stationary phase. The signaling pathway that activates σ^E during entry into stationary phase is dependent upon the alarmone guanosine 3',5'-bispyrophosphate (ppGpp) and is distinct from the pathway that signals extracytoplasmic stress. ppGpp is the first cytoplasmic factor shown to control σ^E activity, demonstrating that σ^E can respond to internal signals as well as signals originating in the cell envelope. ppGpp is a general signal of starvation stress and is also required for activation of the σ^S and σ^{54} alternative sigma factors upon entry into stationary phase, suggesting that this is a key mechanism by which alternative sigma factors can be activated in concert to provide a coordinated response to nutritional stress.

Stress responses allow organisms to survive and adapt to changing environments. In bacteria, many stress responses are mediated by alternative sigma factors that can rapidly reprogram gene expression in response to signals by recruiting RNA polymerase to specific subsets of promoters in the cell (16). Pathways that control the activity of these alternative sigma factors are central to the success of this regulatory strategy and ensure that the sigma factors are turned on and off at the appropriate times and with the appropriate kinetics. In general, regulation of alternative sigma factor activity is complex, with several layers of control over the expression of the sigma factor itself, as well as its activity. The alternative sigma factors can be activated both individually by dedicated signaling pathways that respond to specific signals and jointly by global regulatory pathways that activate multiple stress responses at once. These stress responses can also serve multiple functions in the cell, and some alternative sigma factors are activated by several different stresses.

In *Escherichia coli* there are six sigma factors in addition to the major sigma factor, σ^{70} , each of which has a unique role in stress survival and adaptation to environmental conditions. The alternative sigma factor σ^E plays a key role in the response to stress in the cell envelope (1, 4). Cell envelope stresses that activate σ^E are generally thought to disrupt the proper folding of outer membrane porins. Such stresses include chromosomal mutations in genes encoding periplasmic folding catalysts needed for outer membrane porin folding, heat shock, and

overproduction of porin genes (29, 31, 43). During the stress response, porin misfolding is communicated from the cell envelope to σ^E in the cytoplasm via a signal transduction pathway centered on the inner membrane protein RseA. RseA is a single-pass, membrane protein whose cytoplasmic domain acts as an anti-sigma factor preventing σ^E from binding to RNA polymerase, thereby inhibiting σ^E -dependent transcription (8, 12, 32). By spanning both compartments, RseA is poised to relay a signal from the cell envelope to σ^E in the cytoplasm.

In order for σ^E to initiate transcription, the interaction between RseA and σ^E must be disrupted. This is achieved primarily through the proteolysis of RseA by the sequential action of two inner membrane proteases, DegS and YaeL, and the cytoplasmic protease ClpXP (2, 5, 14, 22, 23, 26). The proteolysis of RseA is regulated such that in unstressed cells RseA is degraded at a moderate rate; when cells are stressed, the degradation rate increases significantly; and when the stress is removed, RseA is stabilized significantly (3). The factors that determine the rate of degradation of RseA are best understood for cell envelope stresses that disrupt outer membrane porin folding. The key step in this regulatory pathway is the activation of DegS through its interaction with a conserved peptide found at the C terminus of outer membrane porins. When porin folding is disrupted, the peptide is exposed and binds to DegS, converting DegS into an active conformation (43). This initiates the proteolytic cascade that results in the complete degradation of RseA. Once RseA is degraded, σ^E is released and is free to bind to RNA polymerase and initiate transcription. It is currently unclear whether the degradation rate of RseA is set entirely through the amount of unfolded porins in the cell or if other regulatory mechanisms are involved.

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In addition to its role during stress, σ^E is essential for viability under all conditions examined (11). Presumably, σ^E is essential because the proper expression of a gene or set of genes that it transcribes is required for viability, although these critical σ^E -dependent genes have yet to be identified. A variety of genetic and genomic methods have been used to define members of the σ^E regulon, and a large number of these genes encode proteins that affect the synthesis of the cell envelope or reside in the cell envelope (10, 39, 40). As expected from its role in porin folding, σ^E transcribes genes encoding several periplasmic proteases and chaperones whose products are important in refolding or degrading misfolded proteins during the stress response. The essential role of σ^E could, therefore, be related to its role in the stress response, combating cell envelope stresses that would otherwise be lethal. However, σ^E also transcribes a number of genes that are involved in other processes related to the cell envelope, including the biosynthesis of lipopolysaccharide, fatty acids, and membrane-derived oligosaccharides, as well as several lipoproteins (10, 35, 39, 40). This suggests that the role of σ^E in maintaining cell envelope integrity extends beyond ensuring that outer membrane porins are properly folded. Additional evidence that σ^E may have other roles comes from studies of σ^E in the closely related bacterium *Salmonella enterica* serovar Typhimurium that indicate a role for σ^E during stationary phase and in response to oxidative stress (7, 41).

In contrast to the alternative sigma factors σ^{32} and σ^S , which have been shown to be regulated at many levels, the only regulatory pathway characterized thus far that controls σ^E activity is the RseA-dependent stress signaling pathway (6, 17, 19). The likelihood that σ^E has additional roles in the cell beyond monitoring porin folding suggests that it is controlled by other regulatory inputs as well. In this paper we investigate alternate modes of regulation and roles of σ^E in *Escherichia coli* and demonstrate that σ^E activity is upregulated upon entry into stationary phase in *E. coli* by the alarmone guanosine 3',5'-bispyrophosphate (ppGpp) and not via the RseA-dependent stress signaling pathway. ppGpp is a general signal of starvation stress and is also required for the activation of the σ^S and σ^{54} alternative sigma factors upon entry into stationary phase (27). These results suggest that ppGpp provides a key mechanism for activating alternative sigma factors in concert to provide a coordinated response to nutritional stress.

MATERIALS AND METHODS

Media, strains, and plasmids. Cultures were grown in Luria-Bertani (LB) broth at 30°C with shaking. Bacterial strains used in this work are derivatives of strain SEA001 (MG1655 $\phi\lambda$ [*rpoHp3::lacZ*] Δ *lacX74*, CAG45114 [3]). The *rpoS::Tn10* allele (13) was moved by P1 transduction into strain SEA001 to create strain CAG45133. The *ΔrseA* strain SEA2000 was made by P1 transduction into SEA001 using strain CAG22968 (MC1061 *ΔrseA nadB::Tn10* [12]) as the donor. Tetracycline-resistant transductants were screened for cotransduction of the *ΔrseA* allele by testing for increased σ^E activity and loss of the RseA band on Western blots probed with antibody to RseA. Strains that cannot produce ppGpp (called ppGpp⁰ strains) were constructed by first moving the *ΔrelA251::kan* allele (42) into SEA001 and SEA2000 (*ΔrseA*). The *ΔspoT207::cat* allele (42) was then moved into the resulting *ΔrelA251::kan* transductants by P1 transduction to generate strains SEA2015 and SEA2010. The strains lacking ppGpp were also verified by their failure to grow on M9 minimal medium lacking amino acids. The *ΔdksA::tet* allele (36) was moved into strain SEA001 by P1 transduction to generate strain SEA2051. All P1 transductions were performed with P1 vir, and transductants were isolated by selection on medium containing

the appropriate antibiotic (30). Experiments were performed with strains resulting from at least two independent transductions of the mutant alleles. Strain SEA007 was made by transforming strain SEA001 with the pRseAB plasmid (this plasmid is the same as pLC253 [12]).

Preparation of conditioned media. To determine if σ^E activity is influenced by a secreted molecule, cell-free supernatants were collected from cultures grown to an optical density at 600 nm (OD₆₀₀) of 2.0, the point at which σ^E activity begins to increase. The cultures were centrifuged to remove cells and the supernatant was filtered through a 0.2- μ m filter to remove any remaining cells. This conditioned medium was stored at 4°C. Before the conditioned medium was inoculated with fresh cells, additional tryptone (5 g/liter of medium) and yeast extract (2.5 g/liter of medium) were added to replenish nutrients and the pH was adjusted to that of fresh LB broth. The growth rate in this replenished conditioned medium is comparable to that in fresh LB broth.

Beta-galactosidase assays. Overnight cultures were diluted to an OD₆₀₀ of 0.02 and grown at 30°C with shaking in a gyratory water bath. σ^E activity was assayed by monitoring beta-galactosidase activity from the chromosomally located σ^E -dependent *rpoHp3::lacZ* reporter as previously described (30). For experiments with strains containing RseA, 0.5-ml samples were taken from the cultures. In the strains lacking *rseA*, σ^E activity is considerably higher, and 0.1-ml samples were taken so that beta-galactosidase activity could be measured accurately. Each experiment was repeated a minimum of three times, and most were repeated more than three times. The same overall shape of the curve on the differential rate plots is always observed, while the slopes of the linear phases can vary by up to 10%. In the figures, data compiled from three independent experiments are shown, except with Fig. 3, in which data from two independent experiments are presented.

The level of σ^E activity is determined in these experiments from the slope of the linear portions of the curve on a differential rate plot in which beta-galactosidase activity in a fixed volume of culture is plotted versus the cell density (OD₆₀₀) of the culture. When σ^E activity is constant, the *lacZ* reporter gene will be transcribed and beta-galactosidase produced at a constant rate. The amount of beta-galactosidase activity in each sample will increase as the culture grows, because there will be more cells in successive samples. As such, a straight line with a particular slope will be observed on the differential rate plot, and this slope is a measure of σ^E activity. If σ^E activity, and therefore the rate of production of beta-galactosidase, changes, then the slope will change, reflecting the new rate of synthesis. The differential rate plots essentially reveal new synthesis of beta-galactosidase from the σ^E -dependent promoter beyond what has already accumulated in the cells. This new synthesis is not as evident on the more common plots of Miller units versus time. Miller units reflect the amount of beta-galactosidase activity per cell. When a culture of a strain with the σ^E -dependent reporter is started from a saturated overnight culture, the overall amount of beta-galactosidase per cell is high because σ^E activity is high in stationary phase. However, σ^E activity is low in early exponential phase such that the rate of synthesis of beta-galactosidase from the σ^E -dependent *lacZ* reporter gene is lower than the rate of dilution due to cell division. As a result, the Miller units drop as the culture grows, until the synthesis rate exceeds the dilution rate due to cell division in mid- to late exponential phase. Therefore, it is difficult to assess σ^E activity from a plot of Miller units versus time, whereas it can easily be determined from the differential rate plot.

RESULTS

Growth phase-dependent regulation of σ^E activity. To better understand the regulation and role of σ^E in cells not subjected to sudden stresses that disrupt protein folding, we asked whether σ^E activity was constant throughout the growth of a culture or instead varied with respect to growth phase. σ^E activity was monitored in *E. coli* strain MG1655 commencing immediately after dilution of an overnight culture and continuing until early stationary phase by measuring beta-galactosidase activity produced from a *lacZ* gene under the control of the σ^E -dependent *rpoHp3* promoter (29). In experiments throughout this paper, σ^E activity was determined from the slope of the linear portions of the curve on a differential rate plot in which beta-galactosidase activity in a constant volume of culture is plotted versus the cell density (OD₆₀₀) of the culture. For a complete description of differential rate plots, please see Materials and

FIG. 1. σ^E activity is regulated with respect to growth phase. *E. coli* strain SEA001 (MG1655 with the σ^E -dependent *rpoHp3::lacZ* reporter encoded on the chromosome) was grown in LB broth at 30°C, and beta-galactosidase activity was measured throughout the growth of the culture. The differential rate plot (see Materials and Methods) showing σ^E activity as a function of the optical density of the culture (A) is shown along with the corresponding growth curve (B). Filled circles highlight points during the growth curve when σ^E activity is low, shaded circles show points when σ^E activity begins to increase, and open circles mark the large increase in σ^E activity upon entry into stationary phase.

Methods. The observed differential rate plot could be divided into three linear regions corresponding to early exponential phase ($OD_{600} \approx 0.02$ to 0.3), mid- to late exponential phase ($OD_{600} \approx 0.3$ to 1.8), and entry into stationary phase ($OD_{600} > 1.8$), indicating that σ^E activity was not constant throughout the growth of a culture and changed with respect to growth phase (Fig. 1). σ^E activity was lowest in early exponential phase, as can be seen by the relatively small increase in beta-galactosidase activity in a 0.5-ml aliquot of the culture, even though cell density was increasing (Fig. 1). As the culture progressed through exponential phase, σ^E activity began to increase. In mid-exponential phase, σ^E activity was 3.6-fold higher than in early exponential phase (Fig. 1). σ^E activity is the highest during the transition from exponential phase into stationary phase. Activity was 19.5-fold higher upon entry into stationary phase than in early exponential phase and remained high in early stationary phase (Fig. 1). A similar pattern of

growth phase-dependent activation of σ^E was observed in a variety of growth media, including LB broth, LB broth supplemented with glucose, and M9 glucose minimal medium without amino acids and supplemented with amino acids (data not shown). In addition, the growth phase-dependent regulation of σ^E activity was not specific to the *rpoHp3* promoter, as the σ^E -dependent *fkpA* promoter was regulated in a similar manner (data not shown).

The low levels of σ^E activity in early exponential phase could be a function of growth in early exponential phase or recovery from stationary phase following dilution of the overnight culture. To distinguish between these possibilities, we compared the σ^E activity during early exponential phase in cultures inoculated with cells in stationary phase to that in cultures inoculated with cells in exponential phase. Cultures started with either set of cells had similar small amounts of σ^E activity (data not shown). Thus, the low level of σ^E activity is particular to cells at a low cell density in fresh medium and is not due to the inhibition of σ^E during recovery from stationary phase.

Role of RseA in the growth phase-dependent regulation of σ^E activity. RseA is the primary regulator of σ^E activity during the stress response and is the only known direct regulator of σ^E activity (12, 32). To determine if the RseA-dependent pathway that regulates σ^E during the stress response regulates σ^E during the growth of a culture, σ^E activity was measured in a strain lacking RseA. If RseA is the sole regulator of σ^E , σ^E should be unregulated in the $\Delta rseA$ strain and transcribe the *lacZ* reporter gene at a constant rate throughout the growth curve. As a result, σ^E -dependent beta-galactosidase activity in a 100- μ l aliquot of culture should increase with respect to cell density in a linear fashion, resulting in a straight line on the differential rate plot. σ^E activity was elevated in the $\Delta rseA$ strain compared to that of the wild-type strain throughout the growth curve (Fig. 2). This result was expected since RseA, the main inhibitor of σ^E , had been removed from this strain. However, even though σ^E activity was significantly higher, it was still regulated with respect to the growth phase of the culture (Fig. 2). σ^E activity in the $\Delta rseA$ strain was again lowest in early exponential phase (Fig. 2) and then increased 2.4-fold during mid-exponential phase (Fig. 2). σ^E activity was the highest in early stationary phase (Fig. 2), increasing fivefold between early exponential phase and entry into stationary phase. The result that σ^E activity is still regulated with respect to growth phase in the absence of RseA indicates that RseA is not the sole regulator of σ^E activity and provides the first observation of the RseA-independent regulation of σ^E . These results also suggest that the system responsible for activating σ^E during entry into stationary phase does not respond to the overall amount of σ^E activity in the cell, since the strain lacking RseA has significantly higher levels of σ^E activity than wild-type cells, yet still exhibits growth phase-dependent activation of σ^E .

σ^E is not regulated by a cell density-dependent extracellular signal. The observations that σ^E activity is low in dilute cultures and increases as the density of the culture increases suggest that σ^E could be regulated by a secreted factor that accumulates as the culture grows. If a secreted molecule regulates σ^E activity, then cell-free supernatants harvested from cultures with high σ^E activity should be enriched with this molecule and σ^E activity should be higher during exponential phase in cultures grown in conditioned medium than in those

FIG. 2. RseA is not required for the regulation of σ^E activity with respect to growth phase. The $\Delta rseA$ strain SEA2000 was grown in LB broth at 30°C, and the beta-galactosidase activity of the σ^E -dependent $\lambda poHp3::lacZ$ reporter was measured throughout the growth of the culture. The differential rate plot showing σ^E activity as a function of the optical density of the culture (A) is shown along with the corresponding growth curve (B). Filled circles highlight points during the growth curve where σ^E activity is low, shaded circles show points where σ^E activity begins to increase, and open circles mark the increase in σ^E activity upon entry into stationary phase.

grown in fresh LB broth. To test this hypothesis, we isolated cell-free supernatants from cultures during entry into stationary phase, the point at which σ^E activity increases. To eliminate effects due to the change in pH and depletion of nutrients from the medium as a culture grows, the pH of the cell-free supernatant was adjusted to that of fresh media and nutrients were replenished by the addition of yeast extract and tryptone. The level of σ^E activity in this replenished conditioned medium was similar to that of cells grown in fresh medium (Fig. 3).

In the course of these experiments, we also observed that the increase in σ^E activity upon entry into stationary phase was not a function of the density of a culture. Cultures grown in conditioned medium not supplemented with additional nutrients entered stationary phase at a lower cell density than cultures grown in fresh medium. In these cultures, σ^E activity increased at the transition into stationary phase, even though the cell density was low (data not shown). Therefore, the increase of

FIG. 3. σ^E activity is not affected by growth in conditioned medium. Conditioned medium from a culture of SEA001 at an OD_{600} of 2.0 was prepared as described in Materials and Methods. Overnight cultures were diluted into this conditioned medium (open circles) or fresh LB broth (crosses), and then cultures were grown at 30°C. σ^E -dependent beta-galactosidase activity was measured. The growth rate was not affected by the conditioned medium (data not shown).

σ^E activity upon entry into stationary phase is related to the availability of nutrients, not the density of a culture.

ppGpp, not σ^S , is required for activation of σ^E upon entry into stationary phase. The alternative sigma factor σ^S is responsible for transcribing a large number of genes upon entry into stationary phase. To determine if σ^S contributes to the growth phase-dependent regulation of σ^E , we examined σ^E activity in a strain lacking the *rpoS* gene. σ^E activity was unaffected in this strain, indicating that σ^S does not play a role in the regulation of σ^E (data not shown).

Another major regulator of gene expression upon entry into stationary phase is the alarmone ppGpp. ppGpp is best known as the primary effector of the bacterial response to amino acid starvation—the stringent response. Following amino acid starvation, ppGpp directly inhibits the transcription of stable RNA (rRNA and tRNA) genes and increases the transcription of genes encoding several enzymes and transporters needed for amino acid biosynthesis (9, 36, 37). The levels of ppGpp in the cell increase during most nutritional stresses, in addition to increasing during amino acid starvation, including at the transition into stationary phase (9, 33). The alternative sigma factors σ^S and σ^{54} are activated upon entry into stationary phase, much like σ^E , and ppGpp is required for their activation (15, 21, 24, 25). To determine if σ^E is regulated by ppGpp in a manner similar to that of σ^S and σ^{54} , we examined σ^E activity in cells that cannot produce ppGpp (this strain is referred to as ppGpp^o) due to inactivation of the *relA* and *spoT* genes, whose products are responsible for the cellular synthesis of ppGpp. σ^E activity was relatively unchanged throughout exponential phase in the ppGpp^o strain compared to that of a wild-type strain, increasing 2.6-fold between early exponential phase and mid-exponential phase in the ppGpp^o strain (Fig. 4). However, at the transition into stationary phase, σ^E activity did not increase but instead decreased compared to the level of activity found during mid-exponential phase (Fig. 4). This result demonstrates that ppGpp is required for the increase in σ^E activity

FIG. 4. The increase in σ^E activity upon entry into stationary phase is dependent on ppGpp and partially dependent on *dksA*. The wild-type strain (open circles) and $\Delta dksA::Tet$ (crosses) and ppGpp^o (*relA251::kan spoT207::cam*; filled diamonds) variants were grown at 30°C, and beta-galactosidase activity was measured throughout the growth curve. The differential rate plot of σ^E -dependent beta-galactosidase activity (A) and the growth curve (B) are shown.

during entry into stationary phase. The requirement of ppGpp for this increase in σ^E activity is not specific to the *rpoHp3* promoter, as a similar effect was observed for the σ^E -dependent *flkA* promoter (data not shown).

Since ppGpp is required for the increase in σ^E activity upon entry into stationary phase, it is possible that ppGpp is required for the increase in σ^E activity during the cell envelope stress response. Therefore, we examined the activation of σ^E in response to envelope stress induced by the overproduction of the outer membrane porin OmpC in a strain lacking ppGpp. We found that σ^E activity still increased following OmpC overproduction in the ppGpp^o strain (data not shown). As such, the signaling pathway activating σ^E via ppGpp is distinct from the stress-signaling pathway.

Recently, *in vivo* and *in vitro* experiments demonstrated that the protein DksA can potentiate the activity of ppGpp. DksA enhances both the ppGpp-dependent inhibition of rRNA synthesis and the ppGpp-dependent activation of transcription of amino acid biosynthetic genes (36–38). To determine if DksA also contributes to the regulation of σ^E by ppGpp, we examined the growth phase-dependent regulation of σ^E activity in a

FIG. 5. ppGpp does not require RseA to regulate σ^E activity. $\Delta rseA$ (open circles) and $\Delta rseA$ ppGpp^o (*relA251::kan spoT207::cam*; filled diamonds) variants were grown at 30°C, and beta-galactosidase activity was measured throughout the growth curve. The differential rate plot of σ^E -dependent beta-galactosidase activity (A) and the growth curve (B) are shown.

strain lacking *dksA*. In this strain, σ^E activity was not activated to the same extent upon entry into stationary phase as in wild-type cells, but it did increase slightly at this time compared to the level of activation of the ppGpp^o strain, in which no increase was observed (Fig. 4).

Activation of σ^E by ppGpp does not require RseA. The above results demonstrate that ppGpp is required for the increase in σ^E activity during entry into stationary phase. One possible explanation for this result is that in cells lacking ppGpp, the levels of extracytoplasmic stress are decreased and the effect of ppGpp is mediated through the RseA-dependent stress-signaling pathway. To address this hypothesis, we constructed a strain lacking both RseA and ppGpp. In this $\Delta rseA$ ppGpp^o strain, σ^E activity was similar to that in the $\Delta rseA$ strain throughout exponential phase. However, during entry into stationary phase, σ^E activity remained at the level found during mid-exponential phase and failed to increase further, indicating that ppGpp acts independently of RseA to control σ^E activity (Fig. 5). Our data also suggest that ppGpp and RseA are not the only regulators of σ^E . σ^E activity was not fully unregulated in the strain lacking both ppGpp and RseA; it

FIG. 6. σ^E activity is required for growth. MG1655(pRseAB) (this plasmid contains *rseA* and *rseB* under the control of the IPTG-inducible pTrc promoter) were grown at 30°C. To maintain the cultures in exponential phase at a low cell density where σ^E activity was low, the cultures were diluted into fresh prewarmed LB broth to an OD₆₀₀ of 0.02 when they reached an OD₆₀₀ of ~0.2. IPTG was added to one culture after 100 min of growth to induce the overexpression of *rseA* and *rseB* (filled symbols) but was not added to the control culture (open symbols). The growth curves for both cultures are shown. Representative data are shown.

increased 2.8-fold between early exponential phase and mid-exponential phase.

The essential role of σ^E is not restricted to entry into stationary phase. Our finding that σ^E activity was low in early exponential phase and increased at the transition into stationary phase suggested that the essential role for σ^E in the cell is during entry into stationary phase. According to this model, σ^E should not be essential in a culture maintained in exponential phase where σ^E activity is low. To test this hypothesis, we utilized a strain carrying a plasmid encoding the inhibitors of σ^E , RseA and RseB, under the control of an IPTG (isopropyl- β -D-thiogalactopyranoside)-inducible promoter. We inhibited σ^E activity by overproducing RseA and RseB and prevented the culture from entering stationary phase by diluting it with fresh prewarmed medium whenever the OD₆₀₀ reached 0.2 to 0.3. We found that σ^E activity was essential for continued cell growth even at these low cell densities. Within three generations following the overexpression of *rseA* and *rseB*, the culture stopped growing and the cell density began to drop, indicating lysis (Fig. 6). Similar results were obtained when σ^E activity was blocked through the stabilization of RseA following depletion of the protease DegS (data not shown). These effects are not likely to be due to toxicity associated with the overexpression of RseA, as overexpression of RseA has no effect in a mutant strain that does not require σ^E for viability (11). These results clearly demonstrate that σ^E plays an essential role throughout exponential phase, even though σ^E activity is not maximal during this phase of growth.

DISCUSSION

The alternative sigma factor σ^E in *E. coli* has been studied primarily for its role in response to protein misfolding in the cell envelope induced by stresses such as a heat shock (1, 4). Previous work addressing the regulation of σ^E activity during the stress response showed that σ^E activity is primarily con-

trolled by the regulated proteolysis of the anti-sigma factor RseA (2, 3). The work presented here is the first reported instance of regulation of σ^E activity that does not involve RseA and a signal originating in the cell envelope. The alarmone ppGpp, instead of RseA, is required for the increase of σ^E activity upon entry into stationary phase. Such regulation can serve to adjust the amount of σ^E -dependent transcription in the cell to meet cellular needs in the absence of a cell envelope signal that alters the degradation rate of RseA.

Role of RseA in the regulation of σ^E activity. Does RseA have any role in the growth phase-dependent regulation of σ^E ? RseA is clearly required to set the overall amount of σ^E activity in the cell, since σ^E activity is significantly higher in a strain lacking RseA at all points in the growth curve. The observation that the increase in σ^E activity between early exponential phase and entry into stationary phase is greater in wild-type cells than in cells lacking RseA (19-fold increase in the wild type; 5-fold increase in $\Delta rseA$ cells) could reflect a contribution by RseA to the growth phase-dependent regulation of σ^E . Previous studies indicate that the amount of σ^E activity in the cell is correlated with the rate of proteolysis of RseA, such that when RseA is rapidly degraded, σ^E activity is high and when RseA is more stable, σ^E activity is low (3). If RseA does contribute to the growth phase-dependent regulation of σ^E , then we predict that the degradation rate of RseA is lower in cells during early exponential phase than during entry into stationary phase. However, the stability of RseA changes very little throughout the growth curve (S. Ades, unpublished observations). Therefore, if RseA does more than determine the overall level of σ^E activity during growth of a culture, it does so through a novel pathway, not via the proteolytic pathway. Another explanation for the difference in activation, which we favor, is that there is a smaller overall increase in σ^E activity during entry into stationary phase in the $\Delta rseA$ strain than in the wild-type strain. Although further experiments are required to resolve this issue, we propose two mechanisms that can explain why σ^E would not be activated to the same extent in cells lacking RseA. σ^E protein levels are much higher in the $\Delta rseA$ strain than in a strain with RseA (unpublished observation). With the increased cellular level of σ^E and the additional activation of σ^E by ppGpp, the σ^E -dependent promoter that directs the transcription of the *lacZ* reporter gene may be saturated during entry into stationary phase, so that no further increase in transcription initiation is possible. Alternatively, the reason may lie with the mode of action of ppGpp. One model proposed for the mechanism of action of ppGpp in activating the alternative sigma factors posits that ppGpp alters the competition among sigma factors for core RNA polymerase in favor of the alternative sigma factors (21). If this proves to be true for σ^E , then since the levels of σ^E are significantly higher in the absence of RseA, σ^E may already be better able to compete for core RNA polymerase. As such, σ^E may not be as sensitive to activation by ppGpp during entry into stationary phase in the $\Delta rseA$ strain.

RseA-independent regulation of σ^E . Entry into stationary phase is characterized by significant changes in cellular physiology and gene expression (18, 20, 34). Several regulatory networks act to effectuate these changes (18). To date, we have tested the role of three of these regulators in controlling σ^E activity. σ^E is not regulated by the general stress factor σ^S , as

there was no effect on σ^E activity in a strain lacking *rpoS*. σ^E also does not appear to be regulated by a secreted factor that accumulates as the density of the culture increases. However, we cannot formally rule this out, since such a factor could have been unstable in our conditioned media or the cells may require a receptor to respond to such a signal that itself is expressed only during entry into stationary phase. The third factor tested, ppGpp, is required for the activation of σ^E .

Cells lacking ppGpp have many defects, particularly during entry into stationary phase, and it is possible that σ^E activity is affected indirectly by a cellular stress due to these pleiotropic effects via the stress-signaling pathway, rather than through a direct ppGpp-dependent regulatory interaction (9). We feel that this possibility is unlikely for several reasons. First, one might expect that defects in cells lacking ppGpp would cause an increase in cell envelope stress, resulting in an increase in σ^E activity. However, σ^E activity decreases during entry into stationary phase in the ppGpp^o strain, rather than increases. It is possible that cell envelope stress is lower during entry into stationary phase in cells lacking ppGpp than in wild-type cells and that σ^E activity decreases because lower levels of cell envelope stress lead to the stabilization of RseA (3). If this model were correct, then RseA should be required for the growth phase-dependent activation of σ^E . In fact, we demonstrated that ppGpp acts independently of the RseA-dependent stress-signaling pathway.

The activity of the alternative sigma factors σ^S and σ^{54} increases during entry into stationary phase in a ppGpp-dependent manner, and in this paper we show that σ^E can be added to the list (21, 25). ppGpp provides a means to coordinately activate several stress responses at once, as opposed to activating each pathway individually through its dedicated signaling pathway. This mechanism is likely to be important for survival in the environment. For instance, it would be advantageous for the cell to coordinately activate multiple stress responses to prepare for harsher conditions should they arise in conjunction with nutritional stress.

How might ppGpp regulate σ^E activity? A priori, ppGpp could alter the levels of σ^E in the cell and/or the activity of σ^E either directly or indirectly. Studies of the regulation of σ^S and σ^{54} indicate that regulation of the activity of the sigma factors is a key point in common. ppGpp regulates both the expression and the activity of σ^S but regulates only the activity, not the expression, of σ^{54} (15, 21, 24, 25). As mentioned above, one of the models to explain the activation of alternative sigma factors by ppGpp suggests that ppGpp acts indirectly by altering the competition among sigma factors for core RNA polymerase, disfavoring σ^{70} (21, 28). This model can also explain how ppGpp activates σ^E . However, further experiments are required to determine if this model applies to σ^E as well.

Role of σ^E in stationary phase. Our observations that σ^E activity increased upon entry into stationary phase initially led us to believe that the essential role for σ^E is to enable this transition. Although we found that σ^E activity is required for growth in exponential phase, σ^E is still likely to play a critical role during entry into stationary phase. As cells enter stationary phase, large changes in cellular metabolism, morphology, and gene expression occur that render the cells more stress resistant and able to survive under adverse conditions (20, 34). Morphological changes in the cell envelope are extensive and

include increased lipopolysaccharide in the outer membrane, increased cross-linking of the peptidoglycan layer both within the peptidoglycan and between the peptidoglycan and outer membrane, accumulation of osmoprotectants, such as membrane-derived oligosaccharides, and changes in the phospholipid and fatty acid contents in the inner membrane (20, 34). The regulatory mechanisms that underlie these changes are not fully understood. σ^E could directly control some of these changes, or it could be needed to respond to stresses that arise as the cell envelope is remodeled. Additionally, σ^E could be activated to better prepare the bacterium for stresses that it may encounter later in stationary phase. By activating stress responses upon entry into stationary phase, the cell can essentially preload itself with stress proteins should they be needed.

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REFERENCES

- Ades, S. E. 2004. Control of the alternative sigma factor σ^E in *Escherichia coli*. *Curr. Opin. Microbiol.* **7**:157–162.
- Ades, S. E., L. E. Connolly, B. M. Alba, and C. A. Gross. 1999. The *Escherichia coli* sigma(E)-dependent extracytoplasmic stress response is controlled by the regulated proteolysis of an anti-sigma factor. *Genes Dev.* **13**:2449–2461.
- Ades, S. E., I. L. Grigorova, and C. A. Gross. 2003. Regulation of the alternative sigma factor sigma(E) during initiation, adaptation, and shutoff of the extracytoplasmic heat shock response in *Escherichia coli*. *J. Bacteriol.* **185**:2512–2519.
- Alba, B. M., and C. A. Gross. 2004. Regulation of the *Escherichia coli* sigma-dependent envelope stress response. *Mol. Microbiol.* **52**:613–619.
- Alba, B. M., J. A. Leeds, C. Z. Lu, and C. A. Gross. 2002. DegS and YaeL participate sequentially in the cleavage of RseA to activate the sigma(E)-dependent extracytoplasmic stress response. *Genes Dev.* **16**:2156–2168.
- Arsene, F., T. Tomoyasu, and B. Bukau. 2000. The heat shock response of *Escherichia coli*. *Int. J. Food Microbiol.* **55**:3–9.
- Bang, I. S., J. G. Frye, M. McClelland, J. Velayudhan, and F. C. Fang. 2005. Alternative sigma factor interactions in *Salmonella*: σ^E and σ^{H1} promote antioxidant defences by enhancing σ^S levels. *Mol. Microbiol.* **56**:811–823.
- Campbell, E. A., J. L. Tupy, T. M. Gruber, S. Wang, M. M. Sharp, C. A. Gross, and S. A. Darst. 2003. Crystal structure of *Escherichia coli* sigmaE with the cytoplasmic domain of its anti-sigma RseA. *Mol. Cell* **11**:1067–1078.
- Cashel, M., D. R. Gentry, V. J. Hernandez, and D. Vinella. 1996. The stringent response, p. 1458–1496. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli and Salmonella: cellular and molecular biology*, 2nd ed., vol. 1. ASM Press, Washington, D.C.
- Dartigalongue, C., D. Missiakas, and S. Raina. 2001. Characterization of the *Escherichia coli* sigma E regulon. *J. Biol. Chem.* **276**:20866–20875.
- De Las Penas, A., L. Connolly, and C. A. Gross. 1997. σ^E is an essential sigma factor in *Escherichia coli*. *J. Bacteriol.* **179**:6862–6864.
- De Las Penas, A., L. Connolly, and C. A. Gross. 1997. The sigmaE-mediated response to extracytoplasmic stress in *Escherichia coli* is transduced by RseA and RseB, two negative regulators of sigmaE. *Mol. Microbiol.* **24**:373–385.
- Dukan, S., and D. Touati. 1996. Hypochlorous acid stress in *Escherichia coli*: resistance, DNA damage, and comparison with hydrogen peroxide stress. *J. Bacteriol.* **178**:6145–6150.
- Flynn, J. M., I. Levchenko, R. T. Sauer, and T. A. Baker. 2004. Modulating substrate choice: the SspB adaptor delivers a regulator of the extracytoplasmic-stress response to the AAA+ protease ClpXP for degradation. *Genes Dev.* **18**:2292–2301.
- Gentry, D. R., V. J. Hernandez, L. H. Nguyen, D. B. Jensen, and M. Cashel. 1993. Synthesis of the stationary-phase sigma factor sigma s is positively regulated by ppGpp. *J. Bacteriol.* **175**:7982–7989.
- Gruber, T. M., and C. A. Gross. 2003. Multiple sigma subunits and the partitioning of bacterial transcription space. *Annu. Rev. Microbiol.* **57**:441–466.
- Guisbert, E., C. Herman, C. Z. Lu, and C. A. Gross. 2004. A chaperone

- network controls the heat shock response in *E. coli*. *Genes Dev.* **18**:2812–2821.
18. Hengge-Aronis, R. 1996. Regulation of gene expression during entry into stationary phase, p. 1497–1512. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
  19. Hengge-Aronis, R. 2002. Signal transduction and regulatory mechanisms involved in control of the sigma(S) (RpoS) subunit of RNA polymerase. *Microbiol. Mol. Biol. Rev.* **66**:373–395.
  20. Huisman, G. W., D. A. Siegele, M. M. Zambrano, and R. Kolter. 1996. Morphological and physiological changes during stationary phase, p. 1672–1682. In F. C. Neidhardt, R. Curtiss III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. ASM Press, Washington, D.C.
  21. Jishage, M., K. Kvint, V. Shingler, and T. Nystrom. 2002. Regulation of sigma factor competition by the alarmone ppGpp. *Genes Dev.* **16**:1260–1270.
  22. Kanehara, K., K. Ito, and Y. Akiyama. 2002. YaeL (EcfE) activates the sigma(E) pathway of stress response through a site-2 cleavage of anti-sigma(E), RseA. *Genes Dev.* **16**:2147–2155.
  23. Kanehara, K., K. Ito, and Y. Akiyama. 2003. YaeL proteolysis of RseA is controlled by the PDZ domain of YaeL and a Gln-rich region of RseA. *EMBO J.* **22**:6389–6398.
  24. Kvint, K., A. Farewell, and T. Nystrom. 2000. RpoS-dependent promoters require guanosine tetraphosphate for induction even in the presence of high levels of sigma(S). *J. Biol. Chem.* **275**:14795–14798.
  25. Laurie, A. D., L. M. Bernardo, C. C. Sze, E. Skarfstad, A. Szalewska-Palasz, T. Nystrom, and V. Shingler. 2003. The role of the alarmone (p)ppGpp in sigmaN competition for core RNA polymerase. *J. Biol. Chem.* **278**:1494–1503.
  26. Levchenko, I., R. A. Grant, J. M. Flynn, R. T. Sauer, and T. A. Baker. 2005. Versatile modes of peptide recognition by the AAA+ adaptor protein SspB. *Nat. Struct. Mol. Biol.* **12**:520–525.
  27. Magnusson, L. U., A. Farewell, and T. Nystrom. 2005. ppGpp: a global regulator in *Escherichia coli*. *Trends Microbiol.* **13**:236–242.
  28. Magnusson, L. U., T. Nystrom, and A. Farewell. 2003. Underproduction of sigma 70 mimics a stringent response. A proteome approach. *J. Biol. Chem.* **278**:968–973.
  29. Mecsas, J., P. E. Rouviere, J. W. Erickson, T. J. Donohue, and C. A. Gross. 1993. The activity of sigma E, an *Escherichia coli* heat-inducible sigma-factor, is modulated by expression of outer membrane proteins. *Genes Dev.* **7**:2618–2628.
  30. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
  31. Missiakas, D., J. M. Betton, and S. Raina. 1996. New components of protein folding in extracytoplasmic compartments of *Escherichia coli* SurA, FkpA and Skp/OmpH. *Mol. Microbiol.* **21**:871–884.
  32. Missiakas, D., M. P. Mayer, M. Lemaire, C. Georgopoulos, and S. Raina. 1997. Modulation of the *Escherichia coli* sigmaE (RpoE) heat-shock transcription-factor activity by the RseA, RseB and RseC proteins. *Mol. Microbiol.* **24**:355–371.
  33. Murray, H. D., D. A. Schneider, and R. L. Gourse. 2003. Control of rRNA expression by small molecules is dynamic and nonredundant. *Mol. Cell* **12**:125–134.
  34. Nystrom, T. 2004. Stationary-phase physiology. *Annu. Rev. Microbiol.* **58**:161–181.
  35. Onufryk, C., M. L. Crouch, F. C. Fang, and C. A. Gross. 2005. Characterization of six lipoproteins in the sigmaE regulon. *J. Bacteriol.* **187**:4552–4561.
  36. Paul, B. J., M. M. Barker, W. Ross, D. A. Schneider, C. Webb, J. W. Foster, and R. L. Gourse. 2004. DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. *Cell* **118**:311–322.
  37. Paul, B. J., M. B. Berkmen, and R. L. Gourse. 2005. DksA potentiates direct activation of amino acid promoters by ppGpp. *Proc. Natl. Acad. Sci. USA* **102**:7823–7828.
  38. Perederina, A., V. Svetlov, M. N. Vassilyeva, T. H. Tahirov, S. Yokoyama, I. Artsimovitch, and D. G. Vassilyev. 2004. Regulation through the secondary channel—structural framework for ppGpp-DksA synergism during transcription. *Cell* **118**:297–309.
  39. Rezuchova, B., H. Miticka, D. Homerova, M. Roberts, and J. Kormanec. 2003. New members of the *Escherichia coli* sigmaE regulon identified by a two-plasmid system. *FEMS Microbiol. Lett.* **225**:1–7.
  40. Rhodius, V. A., W. C. Suh, G. Nonaka, J. West, and C. A. Gross. 2005. Conserved and variable functions of the sigma(E) stress response in related genomes. *PLoS Biol.* **4**:e2.
  41. Testerman, T. L., A. Vazquez-Torres, Y. Xu, J. Jones-Carson, S. J. Libby, and F. C. Fang. 2002. The alternative sigma factor sigmaE controls antioxidant defences required for *Salmonella* virulence and stationary-phase survival. *Mol. Microbiol.* **43**:771–782.
  42. Vinella, D., M. Cashel, and R. D'Ari. 2000. Selected amplification of the cell division genes ftsQ-ftsA-ftsZ in *Escherichia coli*. *Genetics* **156**:1483–1492.
  43. Walsh, N. P., B. M. Alba, B. Bose, C. A. Gross, and R. T. Sauer. 2003. OMP peptide signals initiate the envelope-stress response by activating DegS protease via relief of inhibition mediated by its PDZ domain. *Cell* **113**:61–71.