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REGULATION OF THE ALTERNATIVE SIGMA FACTOR, $\sigma^E$, DURING NUTRIENT LIMITATION IN \textit{ESCHERICHIA COLI}

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by
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ABSTRACT

Sigma factors in bacteria recognize discrete sets of promoters and turn on transcription of genes necessary for growth and/or survival. In *Escherichia coli*, the housekeeping sigma factor, $\sigma^{70}$, recognizes the majority of promoters for transcription during exponential growth. In addition to $\sigma^{70}$, *E. coli* has other sigma factors, termed alternative sigma factors, which are activated in response to specific environmentally induced and internal stresses. The ability of bacteria to adapt to varying conditions is dependent on the ability of the sigma factors to bind core RNA polymerase and direct transcription of specific genes whose products promote survival. What governs these sigma factors is hence an important area of research. The focus of my thesis work has been the extracytoplasmic function sigma factor, $\sigma^E$, which is the only essential alternative sigma factor in *E. coli*.

$\sigma^E$ activity increases in a growth phase-dependent manner and is subject to two types of regulatory mechanisms. Levels of active $\sigma^E$ are maintained in the cell by the cognate anti-sigma factor, RseA which under non-stress conditions binds to most $\sigma^E$ in the cell. Cell envelope stresses caused by overexpression or disruptions in the proper folding of outer membrane proteins results in a proteolytic cascade that degrades RseA, releasing $\sigma^E$ into the cytoplasm, thereby activating $\sigma^E$-dependent transcription. While $\sigma^E$ is best characterized for its role in maintaining cell envelope homeostasis, $\sigma^E$ activity can also be independently regulated during nutrient limitation in the absence of obvious cell envelope damage.
Entry into stationary phase leads to an increase in $\sigma^E$ activity via the action of the signaling molecule, guanosine tetraphosphate (ppGpp) and its co-regulator, DksA. In my thesis work, I determined the contribution of ppGpp and DksA to upregulation of $\sigma^E$ activity during starvation for specific nutrients or under specific starvation regimes. I report that while ppGpp and DksA act in concert to regulate $\sigma^E$ activity during amino acid starvation and entry into stationary phase in LB, ppGpp regulates $\sigma^E$ in a DksA-independent manner during phosphate starvation and entry into stationary phase in rich defined media (EZ-Rich). The DksA-independent regulation did not fit with models based on any of our previous data including in vitro transcription results where DksA is required to potentiate the effect of ppGpp on $\sigma^E$-dependent transcription. To determine how ppGpp regulates $\sigma^E$ in the absence of DksA, I focused my work on phosphate starvation. I found that ppGpp levels were slightly elevated in the absence of DksA during phosphate starvation. We made an additional finding that the increase in ppGpp during phosphate starvation is not necessarily dependent on regulation by SpoT hydrolase as previously assumed. My data suggests that the combined action of ppGpp and additional unknown regulator(s) results in the observed DksA-independent regulation of $\sigma^E$ activity.

To find additional factors that may compensate for the lack of DksA to regulate $\sigma^E$ activity, we employed a genetic screen. The screen-based approach led to the discovery of a novel regulatory mechanism mediated by the LysR-like regulator, LeuO. LeuO is a DNA-binding transcription factor. Overexpression of LeuO results in overall decreased $\sigma^E$ activity during exponential and stationary phase. I found that LeuO overexpression
leads to lower levels of outer membrane porins (OMPs), specifically those of OmpC, OmpX and OmpL. I propose that LeuO overexpression represses OMP expression, thereby lowering $\sigma^E$ activity via RseA. Additionally, loss of or overexpression of LeuO did not compensate for the lack of DksA to regulate $\sigma^E$ activity; hence LeuO is not part of the ppGpp-dependent, DksA-independent regulation of $\sigma^E$ activity. Overexpression of LeuO also led to filamentation in growing cells. The septal murein-binding protein, DamX, is involved in the observed LeuO-mediated filamentation defect. The filamentation defect was unrelated to the effect of LeuO on $\sigma^E$ activity, hence these are two independent pathways mediated by LeuO. My findings suggest that LeuO-mediated regulation may be a widely used mechanism to control various aspects of cellular physiology.
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“Na chor haryam, na raaj haryam, na bhratra bhajyam na cha bharakaari,
vyaaye krate vardhate eva nityam, vidhya dhanam sarva dhan pradhanam”

The wealth that cannot be stolen, neither confiscated by the state, nor divided in family disputes, the wealth that increases by sharing, that wealth is knowledge and is supreme of all possessions.

-Sanskrit saying from the Subhashita Sangraha

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Chapter 1

Introduction

*Escherichia coli* sigma factors

Bacterial RNA polymerase (RNAP) consists of five subunits ($\alpha_2\beta\beta'\omega$) which comprise the core enzyme. The core RNAP is responsible for transcription of DNA templates; however, it cannot recognize specific promoters by itself. Promoter specificity is conferred by the dissociable sigma (\(\sigma\)) subunit, which recognizes specific DNA elements, assists in DNA melting and subsequently in transcription initiation. The holoenzyme (sigma + core RNAP, denoted E\(\sigma\)) conducts transcription until a stable elongation complex is formed at which point, the sigma dissociates [1,2]. When transcription terminates, the core enzyme returns to the free RNAP pool where it can bind sigma anew and initiate a new round of transcription.

Most bacteria have multiple types of the sigma subunit which can associate with core to form specific holoenzymes that transcribe a diverse set of promoters. In *Escherichia coli*, most transcription during exponential growth is directed by primary sigma factor, \(\sigma^{70}\). Also known as the housekeeping sigma factor, \(\sigma^{70}\) directs transcription of genes whose products are involved in cell growth, protein synthesis and cell division. *E. coli* is also equipped with six alternative sigma factors which redirect RNAP to genes whose products are required for adaptive responses [3,4]. Thus, RNAP can be reprogrammed to transcribe distinct targets forming the basis for how bacteria modulate global transcriptional patterns during different situations.
Alternative sigma factors

All sigmas recognize different sets of promoters and perform unique functions. In *E. coli*, with the exception of $\sigma^N$, all other alternative sigmas are part of the $\sigma^{70}$ family since they share limited sequence and structural homology with $\sigma^{70}$ (reviewed in [3,15]). $\sigma^E (\sigma^{24})$ and $\sigma^H (\sigma^{32})$, both respond to environmental stresses such as ethanol, heat shock and other mutations that lead to the presence of misfolded proteins. Despite similar roles, $\sigma^E$ and $\sigma^H$ are segregated such that $\sigma^E$ transcribes genes whose products alleviate cell envelope stresses while $\sigma^H$ alleviates cytoplasmic stresses [5]. $\sigma^F (\sigma^{28})$, transcribes genes involved in motility and flagellar synthesis [6] while $\sigma^{FecI}$ ($\sigma^{19}$) causes expression of genes for uptake and transport of ferric citrate [7,8]. $\sigma^S (\sigma^{38})$ acts as the master regulator of the general stress response when cells enter stationary phase and transcribes genes whose products promote survival during nutrient poor conditions [9]. $\sigma^N (\sigma^{54})$ directs transcription of genes involved in carbon metabolism, nitrogen metabolism and stress resistance.

A key feature of E$\sigma^N$ is that unlike the other 6 sigma factors, it cannot melt promoter DNA to form open complexes and requires assistance from bacterial enhancer proteins [10-13].

The $\sigma^E$ regulon

Of the six alternative sigma factors in *E. coli*, the extracytoplasmic function (ECF) sigma factor, $\sigma^E$, is the only essential sigma factor for its role in maintaining cell envelope homeostasis [14]. $\sigma^E$ is part of the Group 4 subfamily of sigma factors and represents the most stripped-down version of sigmas possessing only two structurally conserved domains, $\sigma_2$ and $\sigma_4$, which recognize the -10 and -35 promoter element respectively (Figure 1-1, [3]). Group 4, previously called ECF (extracytoplasmic function) sigmas, represents the largest component of the cellular cohort of sigmas, accounting for a large percentage of all known sigmas in bacteria. The number
and type of these sigmas can vary widely even among closely related bacteria and more than one ECF factor is usually found within a single bacterial species [16].

\[ E. coli \sigma^{70} \text{ schematic} \]

Approximately 90 genes from 60 promoters consist of the \( \sigma^E \) regulon in \( E. coli \). Consistent with its role in maintaining cell envelope integrity, most genes transcribed by \( \sigma^E \) include those involved in synthesis, folding and homeostasis of lipopolysaccharides and outer membrane porins. However, genes involved in cell structure and division (\( mreB \) and \( ftsZ \)), DNA recombination and repair (\( recJ, recO, recR \)) as well as others of unknown function are regulated by \( \sigma^E \) [19-21]. \( \sigma^E \) regulates its own operon, which consists of \( rpoE \) (gene encoding \( \sigma^E \)), \( rseA \) and \( rseB \) (negative regulators of \( \sigma^E \)) and \( rseC \) (unknown function). Such an arrangement creates a feedback loop in which \( \sigma^E \) regulates transcription of itself as well as of its inhibitors, thus controlling steady-state levels of \( \sigma^E \) in \( E. coli \).
**Regulation of $\sigma^E$**

$\sigma^E$ is essential for viability in *E. coli* and basal $\sigma^E$ activity is always present in the cell even under non-stress conditions [22,23]. $\sigma^E$ activity increases either due to defects in the cell envelope or as a proactive measure when cells enter stationary phase [24]. To accommodate for slow or sudden environmental changes, bacteria have evolved a sophisticated regulatory system that controls synthesis, activity and availability of various sigmas, including $\sigma^E$.

**Regulation of $\sigma^E$ by anti-sigma factor, RseA**

Many sigma factors including $\sigma^E$, are regulated by stoichiometric association with their cognate anti-sigma factor. The $\sigma^E$-specific anti-sigma factor, RseA, is a single-pass transmembrane protein with a cytoplasmic domain, inner membrane domain and a periplasmic domain [22,25]. Most $\sigma^E$ is sequestered by the RseA cytoplasmic domain during non-stress conditions. Low levels of active $\sigma^E$ ensure viability during growth. RseA inhibits $\sigma^E$ activity by occluding its DNA and core RNAP binding sites [18]. The periplasmic domain of RseA interacts with RseB, the co-anti-sigma factor which blocks proteolysis of RseA [26]. For $\sigma^E$ to be active, RseA must be degraded (Figure 1-2). One of the strongest known signals that trigger degradation of RseA is the presence of misfolded outer membrane porins owing to heat shock, ethanol treatment or overexpression [5,27,28]. Genetic or chemical alterations to membrane lipoproteins can also trigger $\sigma^E$ activity via degradation of RseA [29]. Membrane stress triggers the serine protease, DegS which initiates a proteolytic cascade by first cleaving the the periplasmic domain of RseA. The complex next becomes a substrate for the metalloprotease RseP which degrades the inner membrane spanning region of RseA. The leftover fragment consisting of the cytoplasmic domain of RseA still tethered to $\sigma^E$, is released in the cytoplasm. The SspB adaptor facilitates binding of
the RseA-σ^E complex to ClpXP which degrades the final portion of RseA, releasing σ^E to bind core enzyme and direct transcription [30,31]. RseA thus controls the amount and availability of σ^E in the cell.

Figure 1-2: RseA-dependent regulation of σ^E. Most σ^E is held in an inactive conformation during non-stress conditions by RseA and RseB. Membrane stresses that lead to the presence of misfolded outer membrane porins initiate the RseA degradation pathway via DegS, RseP and ClpXP. Free σ^E binds core RNAP and transcribes genes under Eσ^E control whose products alleviate the stress. See text for details.
**Regulation of $\sigma^E$ by the transcriptional regulators ppGpp and DksA**

Regulation of $\sigma^E$ can occur at two levels: RseA regulates the amount of $\sigma^E$ that is available to bind to core RNAP, whereas activity of available E$\sigma^E$ is enhanced by the alarmone, guanosine tetraphosphate (ppGpp) and co-regulator protein, DksA.

**ppGpp**

The global gene expression regulator, ppGpp, was originally discovered as a small molecule present in unusually large amounts in *E. coli* cells starved for amino acids [32]. The ppGpp-mediated decrease in ribosomal RNA (rRNA) synthesis during amino acid starvation, was termed as ‘stringent response’ [32]. In addition to amino acid starvation, several other growth debilitating conditions such as carbon starvation, phosphate starvation and entry into stationary phase result in ppGpp accumulation. In the cell, ppGpp is synthesized by either of the two synthases, RelA or SpoT, from GTP using ATP as a phosphate donor (Figure 1-3). When cells enter amino acid starvation, ppGpp levels increase owing to increased synthesis by the ribosome-associated RelA enzyme [33]. SpoT is a bifunctional enzyme with a weak ppGpp synthesis capability and a strong ppGpp hydrolase function [34]. In the cell, ppGpp is believed to be synthesized constitutively and under non-stress conditions, SpoT hydrolase degrades it. A general model of the role of SpoT suggests that lowering the activity of SpoT hydrolase leads to ppGpp accumulation during most starvation conditions (except amino acid starvation) such as carbon starvation, phosphate starvation and iron limitation. A strain lacking both RelA and SpoT is unable to produce ppGpp (ppGpp$^o$ strain) and continues to synthesize rRNA; a phenomenon termed as the ‘relaxed response’ [35].
ppGpp synthesis and degradation. ppGpp is synthesized by RelA or SpoT depending on the type of starvation. SpoT hydrolase degrades ppGpp when ppGpp is not required or when levels exceed the requirement. See text for details.

Previous co-crystallization studies indicated that ppGpp binds close to the active center of RNA polymerase and coordinates the Mg$^{2+}$ ion at the active site, thereby regulating transcription [36]. This claim was later refuted by Vrentas et al., when they determined by genetic and biochemical means that the ppGpp binding pocket identified in the co-crystal was not involved in regulation of E. coli rRNA transcription initiation [37]. Recent genetic work from R. Gourse’s lab suggest that ppGpp binds close to the RNAP secondary channel, specifically between β’ residues 612 - 648 (personal communication). The proposed binding region is close to the ω subunit of RNAP, suggesting that while ppGpp binds in the β’ region, it may also interact with the ω subunit. Mutational studies support this possibility since RNAP lacking the ω subunit cannot respond to ppGpp in vitro [38,39].
DksA

DksA, a 17 kDa protein, was first identified as a multicopy suppressor of the temperature sensitivity of dnaKJ mutants [40]. DksA is involved in various cellular functions such as cell division, quorum sensing, and virulence [40-44]. Previous studies have not elucidated the exact molecular mechanisms linking dksA and these phenotypes although it is assumed to be related to DksA’s role in regulating transcription at various promoters. When the structure of DksA was determined at 2Å resolution, it was apparent that DksA shares overall structural similarity to the transcription elongation factor GreA, with a prominent coiled-coil domain in the N-terminus and a Zn finger motif in the C-terminus (Figure 1-4) [45]. Structural data along with immunoprecipitation experiments revealed that DksA binds to RNAP, possibly at the secondary channel like GreA (Figure 1-4) [46,47]. While DksA has an independent role in regulating several aspects of gene expression, it has been best characterized for its apparent synergy with ppGpp in regulating transcription initiation during nutrient limitation. Since DksA is present during all stages of the growth curve and only levels of ppGpp change transiently in response to starvation, DksA is proposed to function by sensitizing the RNAP to the variations in ppGpp levels [48]. A second mechanism postulates that DksA stabilizes the interaction between ppGpp and RNAP to mediate transcription [45,49,50]. DksA has also been shown to compensate for the loss of the ω subunit of RNAP in the response of rRNA promoters to ppGpp [47].
Global transcription regulation by ppGpp and DksA

Since ppGpp and DksA bind directly to RNAP, they can potentially modulate transcription by any associated sigma factor. While ppGpp and DksA inhibit $\sigma^{70}$-dependent transcription of $rrn$ (which encodes rRNA) operons during starvation, they activate $\sigma^{70}$-dependent transcription at other promoters involved in maintenance and survival [48,52-54]. Among the alternative sigma factors, the increase in activities of $\sigma^{{S}}$, $\sigma^{{E}}$ and $\sigma^{{N}}$ are mediated by ppGpp and DksA [55-58]. Additionally, ppGpp and DksA stimulate expression of $rpoS$ (the gene encoding $\sigma^{{S}}$) during stationary phase [59,60].
Models of transcription regulation by ppGpp and DksA

1. Indirect model

ppGpp and DksA are thought to alter the competition among sigma factors for core RNAP such that transcription by alternative sigma factors is favored while transcription by the housekeeping sigma factor at stable RNA operons is repressed. The indirect model is also referred to as “Sigma factor competition model” or “Passive model”.

Two basic hypotheses have been proposed to explain how ppGpp alters the competition:

(1) ppGpp has been proposed to promote association of alternative sigma factors with RNAP at the expense of Eσ70 formation. Support for this model comes from experiments that showed that varying the level of a particular holoenzyme (either by generating low-affinity sigma factor mutants or by regulated expression) increased transcription by other holoenzymes. For example, mutant σS is unable to compete with σ70 resulting in an increase in σ70-dependent transcription and decrease in σS-dependent transcription at cognate promoters [61]. Similarly, overexpression of the σ70-specific anti-sigma factor Rsd stimulates EσE activity as σ70 is sequestered [56]. Thus, it can be argued that processes that disfavor formation of Eσ70 result in redistribution of RNAP from housekeeping genes to survival-associated genes.

(2) During periods of rapid growth, transcription at σ70-dependent rRNA and tRNA genes sequesters ~60-70% of core RNAP, reducing the amount of core RNAP available to bind alternative sigmas. Therefore, levels of alternative holoenzymes are low and consequently transcription from their regulon promoters is also low [62]. Growth arrest during nutrient
limitation leads to an increase in ppGpp levels, which along with DksA, is postulated to inhibit transcription initiation at stable RNA operons. This releases a large amount of core RNAP previously involved in transcribing stable RNA genes, which is now available all sigmas including alternative sigmas leading to enhanced output from their cognate promoters. (Figure 1-5). Support for this hypothesis comes from experiments that indicate that mutations in β and β’ subunits that bypass the requirement for ppGpp for activation of transcription by alternative sigma factors do not affect Eσ70 stability but reduce transcription by Eσ70 at stable RNA promoters [56,63-65].

2. Direct Model

Direct negative effects of ppGpp on promoters have been detected in vitro, most of which involve regulation of σ70-dependent rrn promoters [38,48,66]. Previous work from our lab demonstrated that ppGpp and DksA together increased transcription of σE-dependent promoters both in vivo and in vitro [56]. σE is the only sigma factor other than σ70 that has been shown to be directly regulated by ppGpp and DksA (Figure 1-5). However, because the in vivo effects exceed the effects of ppGpp and DksA on transcription by EσE in vitro [56], there are likely to be other factors that work with ppGpp and DksA to directly enhance transcription from σE-dependent promoters.
Figure 1-5: Models of transcription regulation by ppGpp and DksA. (Left) Direct model. Low level $\sigma^E$ activity during rapid growth ensures viability. Nutrient limitation increases $E\sigma^E$ activity, mediated by ppGpp and DksA. (Right) Indirect model. Most core RNAP is held at ribosomal operons during rapid growth in rich media. Nutrient limitation leads to ppGpp accumulation which along with DksA binds to core RNAP. Transcription at $rrn$ promoters decreases (broken arrows), whereas transcription at alternative sigma factor regulon genes increases (solid arrows). See text for details.
Kinetic basis for the varying effect of ppGpp and DksA on promoters

It has been proposed that whether ppGpp and DksA stimulate or repress transcription depends on the kinetic properties of a given promoter [53]. The DNA elements that control the kinetics include the -35 and -10 sequence, the spacer length between the -35 and -10 sequence, presence of a GC or AT-rich sequence between the transcription start site and -10 sequences and the DNA supercoiling status [54,67,68]. The kinetic model suggests that at positively regulated promoters, the open complex has lower free energy than the closed complex making it stable. ppGpp and DksA further lower the energy barrier between the two complexes, decreasing activation energy and stimulating transcription initiation. In contrast, at negatively regulated promoters, the open complex has higher free energy than the closed complex, and the resulting open complex is unstable. ppGpp and DksA further lower the energy barrier in favor of dissociation, inhibiting transcription initiation (Figure 1-6, [69,70]).

Figure 1-6: Kinetic basis for ppGpp and DksA-dependent regulation. (a) Binding of ppGpp and DksA lowers the free energy ($\Delta G$) of an intermediate and/or transition state ([RP]$^\dagger$) (dashed lines) between intermediates (labeled RP$_1$ and RP$_2$). At positively regulated promoters, the RP$_2$ complex might have lower free energy than RP$_1$, making it more stable, which would make the promoter insensitive to inhibitory effects of ppGpp and DksA. If there were also a high energy
barrier between the two intermediates, ppGpp and DksA could lower this barrier, thereby decreasing the activation energy and increasing transcription initiation. (b) At negatively regulated promoters, if RP₂ has higher free energy than RP₁, as for promoters that do not form stable open complexes at equilibrium, then the destabilizing effects of ppGpp and DksA would lower the barrier between the two states, in favor of dissociation, thereby discouraging RP₂ formation and inhibiting transcription (Image from [70]).

Role of nucleotide substrates in regulation of transcription

In addition to ppGpp and DksA, a third regulatory molecule that plays an important role in regulating transcription is the concentration of the initiating nucleoside triphosphate (iNTP). iNTP concentration serves as a regulator of rnm transcription in a manner that changes with growth rate. Higher concentrations of iNTP are required at unstable open complexes for initiation to occur successfully [71]. The higher iNTP requirement is easily fulfilled during growth in rich media like LB but not during starvation, making iNTP concentrations the rate-limiting step in transcription initiation. High iNTP concentrations can also overcome inhibitory effects of ppGpp [66]. Thus the intrinsic kinetic properties of promoters, the stringent regulators ppGpp and DksA and the iNTP concentrations, all affect transcription.

The various regulation strategies described here are not mutually exclusive and while no single model can account for all the observed effects on gene expression changes during various types of starvation, two or more mechanisms might converge to ultimately account for the final outcome for a given promoter.
Regulation of $\sigma^E$ by ppGpp and DksA

Our lab has previously shown that $\sigma^E$ activity increases in an RseA-independent, ppGpp and DksA-dependent manner during entry into stationary phase [56]. Other specific starvation conditions that result in ppGpp accumulation also increase $\sigma^E$ activity such as carbon starvation, phosphate starvation and amino acid starvation. Unlike $\sigma^S$, ppGpp does not regulate $\sigma^E$ synthesis but instead affects $\sigma^E$-dependent transcription. We further showed that $rpoD$ (gene encoding $\sigma^{70}$) mutants that lower binding affinity of $\sigma^{70}$ to core RNAP led to nearly constitutive $\sigma^E$ activity, suggesting that mutant $\sigma^{70}$ allows $\sigma^E$ to recruit core RNAP more efficiently. The outcome is analogous to the action of ppGpp hence it seems that one way by which ppGpp works is by tipping competition for core RNAP in favor of alternative sigma factors including $\sigma^E$.

While previous experiments have shed new light on how ppGpp and DksA work on available $\sigma^E$ to increase its activity during entry into stationary phase; it was unclear whether ppGpp and DksA regulated $\sigma^E$ activity similarly during other specific types of starvation. My thesis work started with the observation that ppGpp leads to an increase in $\sigma^E$ activity in a DksA-independent manner during phosphate starvation (See Phosphate homeostasis). The DksA-independent regulation was surprising because previous in vitro work had clearly demonstrated that ppGpp by itself had a very small effect on E$\sigma^E$ activity and that DksA was essential for potentiating the increase in activity. Based on this observation, I hypothesized that “Regulation of $\sigma^E$ activity by the global stress response factor ppGpp, follows a different regulation pattern depending on the nutrient limitation condition and potentially involves cofactors other than DksA.”
I show in my thesis work that regulation of $\sigma^E$ during phosphate starvation is RseA and PhoB - independent. I demonstrated that ppGpp levels are slightly higher (~2.5 fold) in the absence of DksA, which along with an unknown co-regulator, may explain the observed in vivo DksA - independence. I also show that while the dominant form of ppGpp present during phosphate starvation is the tetraphosphate form, its precursor form, pppGpp, can also regulate $\sigma^E$ activity. Further, I found that ppGpp levels can be regulated even if SpoT hydrolase is inactive suggesting that ppGpp accumulation is not necessarily a result of lowered degradation by SpoT. I also demonstrate that overexpression of dksA can compensate for the lack of ppGpp during phosphate starvation to regulate $\sigma^E$, suggesting that high levels of DksA might mimic the effects of ppGpp on $E\sigma^E$. Finally, I suggest that the additional co-regulator that assists the ppGpp -dependent increase in $\sigma^E$ activity could be polyphosphate which is hypothesized to have regulatory roles during certain types of starvation including phosphate starvation. Further experiments will be needed to determine this possibility. My thesis work leads me to conclude that DksA and ppGpp have independent and complementary roles in regulating $\sigma^E$ activity during various types of starvation and that iNTP and additional regulators such as polyphosphate may be part of the observed regulation.

**Phosphate homeostasis**

While $\sigma^E$ activity increases following entry into various types of starvations, I focus my work in this study mainly on regulation of $\sigma^E$ activity during phosphate starvation. In terms of cellular content, phosphorus the third most abundant element in *E. coli*. It is a component of membrane lipids, complex sugars and nucleic acids. Phosphate is also involved in energy metabolism and signal transduction. Phosphorylated compounds from the extracellular environment are
transported through the PhoE porin to the periplasm. Inorganic phosphate (Pi) then makes it way from alkaline phosphatase in the periplasm to the inner membrane proteins PstC and PstA which finally transport it to the cytoplasm where the Pi gets incorporated into various biomolecules. The PhoBR system is activated when the extracellular concentration of phosphate falls <4 μM. Phosphorylated PhoB binds to promoter DNA at the ‘Pho Box’ and enhances transcription of members of the phosphate (Pho) regulon. The Pho genes are mainly involved in acquisition and metabolism of phosphate [72,73]. In addition to activation of the Pho regulon, two other stress response factors, ppGpp and polyphosphate accumulate in the cell in response to phosphate limitation. While ppGpp modulates gene expression to adapt to limiting phosphate, poly P is thought to function both as a phosphate reservoir and as a regulatory factor.

The *E. coli* outer membrane is composed of lipopolysaccharides (LPS), including lipid A. LPS maintains outer membrane integrity by forming a protective barrier against various environmental stresses [74-76]. In the absence of sufficient phosphate, bacteria can modify their phospholipids by substituting them with phosphorus-free lipids [73,77]. The Pho regulon has been implicated in modifications of lipid A structure and cell surface perturbations in *E. coli* [78]. Changes in Lipid A structure makes cells more susceptible to host complement factors, antibiotics and antimicrobial peptides [79,80,78]. In addition, phosphate starvation also leads to an imbalance in cyclopropane and unsaturated fatty acids and increases outer membrane permeability [81]. Since membrane integrity is necessary for resistance to environmental stresses, and the major role of σE is to maintain cell envelope homeostasis [14]; we believe that σE activity increases following entry into phosphate starvation either as a proactive measure before envelope damage occurs or in response to unknown outer membrane modifications.
Chapter 2

Regulation of the *Escherichia coli* alternative sigma factor, $\sigma^E$, by the global stress response factors ppGpp and DksA during nutrient limitation conditions

Abstract

During stress, the housekeeping sigma factor in *Escherichia coli* is replaced by an alternative sigma factor that binds to core RNA polymerase and directs transcription of genes whose products alleviate the particular stress. The extracytoplasmic function sigma factor, $\sigma^E$, is an essential alternative sigma factor in *E. coli*. $\sigma^E$ is best characterized for its role in responding to cell envelope stress, which relies on inhibition by the anti-sigma factor, RseA. $\sigma^E$ activity can also be independently regulated in the absence of obvious cell envelope damage. Nutrient limitation leads to an increase in $\sigma^E$ activity via the action of the alarmone guanosine tetraphosphate (ppGpp) and the coregulator protein, DksA. In this chapter, we investigate regulation of $\sigma^E$ during specific starvations. We report that while ppGpp is always required for the increase in $\sigma^E$ activity following entry into specific starvation, the requirement for DksA varies. We focus our studies on regulation of $\sigma^E$ during phosphate starvation which occurs in a ppGpp-dependent and DksA-independent manner. In contrast, another sigma factor, $\sigma^S$, requires both DksA and ppGpp whereas $\sigma^{70}$ is regulated in a ppGpp-independent and DksA-dependent manner during phosphate starvation. Our observations suggest that ppGpp and DksA do not always act in concert to regulate transcription by various sigma factors and that additional factors may assist in regulation. Our data also indicate that ppGpp synthesis can be regulated even if SpoT hydrolase is inactive suggesting that alternate mechanisms exist to maintain ppGpp levels in the cell.
Introduction

Sigma factors are the subunit of bacterial RNA polymerase (RNAP) responsible for specific promoter recognition. In Escherichia coli, the housekeeping sigma factor, $\sigma^{70}$, binds to core RNA polymerase (E$\sigma^{70}$) to transcribe genes whose products are required for optimal cell growth, protein synthesis and cell division. E. coli switches $\sigma^{70}$-dependent transcription to transcription using alternative sigma factors when conditions change for the worse, either owing to nutritional deficiency or other environmental stresses such as changes in temperature, pH, osmotic stresses, etc. Depending on the type of stress, the appropriate alternative sigma factor binds to core polymerase and transcribes a specific set of genes whose products alleviate the stress.

$\sigma^E$ is an alternative sigma factor that responds to extracytoplasmic stress caused by the presence of misfolded outer membrane proteins, heat shock and mutations in genes encoding chaperones required for outer membrane protein folding [5,27,82]. The levels of available $\sigma^E$ are dependent on the degradation rate of the cognate anti-sigma factor RseA which under non-stress conditions binds to most $\sigma^E$ and prevents its association with core RNAP [22,25]. Independent from its role in maintaining cell envelope homeostasis, $\sigma^E$ activity also increases in response to nutrient limitation. During entry into stationary phase, E$\sigma^E$ activity is regulated by the action of two key factors: the signaling molecule, guanosine tetraphosphate (ppGpp) and its coregulator protein, DksA [24].

In E. coli, ppGpp is synthesized by two enzymes: RelA and SpoT, homologs of which are found in many other bacterial species [83]. RelA is a strong ppGpp synthase, while SpoT is a weaker
synthase. SpoT is specialized for its hydrolase activity which degrades ppGpp. The levels of ppGpp in the cell are believed to be maintained by regulation of the opposing synthase and hydrolase activities. ppGpp levels rise during amino acid starvation due to increased synthesis by RelA [33]. In contrast, decreased hydrolysis by SpoT is thought to lead to higher levels of ppGpp following phosphate, carbon, fatty acid and iron limitation [84-87]. A strain lacking both RelA and SpoT is unable to produce ppGpp and is termed as a ppGpp$^0$ strain.

The DnaK multi-copy suppressor, DksA, has been best studied for its role in assisting ppGpp-dependent regulation at various promoters. The pleiotropic effects of DksA and ppGpp on various sigma factors are mediated by their ability to bind RNAP and directly affect transcription initiation by any associated sigma factor [36,48]. DksA is believed to be present at a constant level from exponential to stationary phase; hence, it may function by sensitizing the promoter to small changes in ppGpp levels by altering the kinetics of open complex formation [48]. A second mechanism postulates that DksA stabilizes the interaction between ppGpp and RNAP to mediate transcription [45,49,50].

Regulation of $\sigma^E$ activity by ppGpp and DksA is proposed to occur via two simplistic models. During exponential growth, the majority of cellular transcription occurs on the ribosomal $rrn$ operons to supply the cell with sufficient ribosomes. A nutritional downshift such as entry into stationary phase leads to ppGpp- and DksA-mediated repression of $\sigma^{70}$ activity at $rrn$ operons, particularly at $rrn$ P1 promoters [88]. During this time, stable RNA synthesis slows as cells adjust to unfavorable conditions by ceasing energy-consuming activities [62]. The subsequent increase in the amount of free core polymerase previously bound at the $rrn$ operons allows
alternative sigma factors like $\sigma^E$ to better compete with $\sigma^{70}$ for core polymerase, thereby indirectly increasing E$\sigma^E$-mediated transcription [45,48,64,89]. In addition to this indirect mode of regulation, ppGpp and DksA can also directly enhance E$\sigma^E$-dependent transcription in vitro [56]. Both the direct and indirect model require the concerted action of ppGpp and DksA.

We have previously shown that $\sigma^E$ activity increases in an RseA-independent, ppGpp- and DksA-dependent manner during entry into stationary phase in LB. Other specific starvation conditions that result in ppGpp accumulation also lead to high $\sigma^E$ activity such as carbon starvation, phosphate starvation and amino acid starvation. Unlike $\sigma^S$, ppGpp does not regulate $\sigma^E$ synthesis but in concert with DksA, can directly stimulate E$\sigma^E$-dependent transcription in vitro. We further showed that mutant $\sigma^{70}$ with a lower binding affinity for core RNAP results in increased E$\sigma^E$ activity. The outcome is analogous to the action of ppGpp hence in addition to the direct regulation; ppGpp may also work by tipping the competition for core RNAP in favor of alternative sigma factors including $\sigma^E$ [56].

In this chapter, I further investigate regulation of $\sigma^E$ by ppGpp and DksA during various nutrient limitation conditions. In rich medium, entry into stationary phase is complex due to a number of changes in the growth medium compared to entry into stationary phase due to starvation for a specific nutrient. Therefore, I examined the contribution of ppGpp and/or DksA to the increase in $\sigma^E$ activity during various nutrient limitation conditions. Transcription by E$\sigma^E$ was assayed by measuring $\beta$-galactosidase activity produced by the chromosomally encoded single-copy rpoH P3-lacZ transcriptional fusion inserted at the $\lambda$ att site. The P3 promoter of rpoH (which encodes $\sigma^H$) is transcribed by $\sigma^E$, and has been used extensively to monitor $\sigma^E$ activity [5]. I found that while
ppGpp was always required to stimulate $\sigma^E$ activity following entry into any nutrient limitation condition tested; the requirement for DksA varied.

In focusing on phosphate starvation, I found that $\sigma^E$ activity is ppGpp-dependent but DksA-, RseA- and PhoB-independent. In contrast, DksA, but not ppGpp, was required for regulation of $\sigma^{70}$-dependent transcription at $rrnB$ P1 promoters during phosphate starvation. In examining the contribution of RelA and SpoT to regulation of $\sigma^E$ activity, I found that ppGpp synthesized from either source was sufficient to elevate $\sigma^E$ activity following phosphate starvation. In addition, ppGpp accumulated even in the absence of a functional SpoT hydrolase, suggesting that alternate mechanisms exist for optimizing ppGpp levels in the cell. The data presented here indicate that DksA and ppGpp have independent and complementary roles in regulating sigma factor activity during various nutrient limitation conditions and that additional factor(s) contribute to the regulation.
Results

Regulation of $\sigma^E$ by ppGpp varies depending on the nutrient limitation condition

$\sigma^E$ activity was monitored in cultures grown in LB, EZ-Rich (MOPS-based rich defined media, see Materials and Methods), low phosphate (0.13mM) or limiting amino acid (0.06mM isoleucine). Under all conditions examined, $\sigma^E$ activity increased in the wild type strain, but not in a ppGpp$^o$ strain, when growth was slowed due to depletion of the specific nutrients or entry into stationary phase in rich medium. The requirement for DksA however, varied. $\sigma^E$ activity was completely DksA -dependent during amino acid starvation and partly DksA-dependent during entry into stationary phase in LB (Figure 2-1). However, $\sigma^E$ activity was regulated in a DksA -independent manner during phosphate starvation and entry into stationary phase in EZ-Rich (Figure 2-1). The results were not unique to the rpoH P3-lacZ reporter, since the $\sigma^E$-dependent rybB-lacZ reporter was also regulated in a similar manner under different starvation regimes (data not shown).

To determine whether the DksA -independent regulation was specific to $\sigma^E$, activity of the stationary phase sigma factor, $\sigma^S$, was also determined under the various starvation conditions as described above in wild type, $\Delta dksA$ and ppGpp$^o$ strains carrying the $\sigma^S$ -dependent bolA-lacZ transcriptional fusion reporter. In contrast to $\sigma^E$, $\sigma^S$ activity remained ppGpp- and DksA -dependent under all conditions examined (Figure 2-1). The results suggest that regulation by ppGpp is not equivalent for at least two alternative sigma factors, $\sigma^E$ and $\sigma^S$, under the same starvation conditions.
σE activity in LB

σE activity in low amino acid media

σE activity in EZ-Rich
**σ^E activity in low phosphate media**

**σ^S activity in LB**

**σ^S activity in low amino acid media**
**σ^S activity in EZ-Rich**

**σ^S activity in low phosphate media**

**Figure 2-1:** Regulation of σ^E and σ^S by ppGpp and DksA during various nutrient limitation conditions. Samples were assayed for σ^E and σ^S activity by monitoring β-galactosidase activity produced from the single-copy *rpoH* P3-*lacZ* and *bolA-lacZ* transcriptional fusion reporters respectively. All strains were grown at 30°C in either LB, limiting amino acid media, EZ-Rich or low phosphate media as indicated. Differential rate plots (right) showing σ^E activity in wild type (SEA001, diamonds), ΔdksA (SEA6020, crosses) and ppGpp^o^ (SEA2010, triangles) strains is shown along with differential rate plots for σ^S activity in the wild type (SEA6328, diamonds), ΔdksA (SEA6329, crosses) and ppGpp^o^ (SEA6401, triangles) at various nutrient limitation conditions. Growth curves are shown on the left. One representative data set from at least two independent repeats is shown.
Regulation of $\sigma^E$ under non-stress conditions by ppGpp and DksA

To uncouple the effect of increase in ppGpp levels following starvation from the requirement of DksA to regulate $\sigma^E$ activity, I examined $\sigma^E$ activity during exponential phase (non-stress condition) in a $\Delta dksA$ strain and wild type strain carrying a plasmid with a truncated $relA'$ which constitutively produces ppGpp without associating with ribosomes upon IPTG induction [90]. When this variant $relA'$ gene was overexpressed, $\sigma^E$ activity did not increase in the absence of DksA compared to a wild-type strain. Therefore, in the absence of a starvation signal, DksA is necessary for the increase in $\sigma^E$ activity regardless of ppGpp levels (Figure 2-2). This result suggests that an additional factor present during nutrient limitation could account for the DksA-independent regulation of $\sigma^E$ activity.

Figure 2-2: Increase in $\sigma^E$ activity during non-stress conditions requires DksA. $\sigma^E$ activity in the wild type (SEA6483, diamonds) is shown in comparison to a $\Delta dksA$ strain (SEA6487, circles). $\sigma^E$ activity was measured during exponential phase from $\sigma^E$-dependent $rybB$-$lacZ$ reporter following gratuitous induction of ppGpp. IPTG was added (indicated by the arrow) to a final concentration of 20 $\mu$M to induce active $relA'$ from pALS13 in both strains. Strains were grown in EZ-Rich media at 30°C. The differential rate plot showing $\sigma^E$ activity as a function of the optical density of the culture (B) is shown along with the corresponding growth curve (A). Data compiled from two independent experiments are shown.

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ppGpp does not affect $\sigma^E$ synthesis

To determine whether ppGpp stimulates $\sigma^E$ activity during phosphate starvation by increasing the synthesis of $\sigma^E$ during phosphate starvation, we measured $\sigma^E$ levels by Western blotting in wild type and ppGpp$^0$ strains. $\sigma^E$ levels were similar in both strains following entry into phosphate starvation, suggesting that ppGpp does not affect production of $\sigma^E$. In both cases, $\sigma^E$ levels increased by ~2-2.5-fold following entry into phosphate starvation.

**Figure 2-3:** ppGpp does not affect steady-state levels of $\sigma^E$. Wild type (SEA001, diamonds) and ppGpp$^0$ strain (SEA2010, circles) were grown in low phosphate media and protein samples extracted during exponential phase, transition to phosphate starvation and after entry into phosphate starvation as indicated by the time points 1-7 in (A). Samples were also extracted from the $\Delta rseA$ strain (SEA6462, positive control) and $\Delta rpoE$ suppressor+ strain (SEA4041, negative control). Following protein extraction, the samples were Western blotted to determine $\sigma^E$ levels. Western blots probed with anti-$\sigma^E$ antibody are displayed in (B). Equal amounts of protein extracts, as determined by BCA assay were loaded in each lane. The $\sigma^E$ band is indicated with an arrowhead. A cross-reacting band, which is present in strains lacking the $rpoE$ gene, runs directly above the $\sigma^E$ band. A representative blot is shown and similar results were obtained in three separate experiments.
**Unknown additional factor may compensate for the lack of DksA**

The observed DksA-independent effect during phosphate starvation may be attributed to the presence of another regulator that functions with ppGpp to regulate transcription. This factor may be a structural or functional homolog of DksA. In *E. coli*, known structural homologs of DksA include the Gre factors (GreA and GreB) which are anti-pausing, transcription elongation factors. Despite a lack of sequence homology, owing to the structural similarity, the Gre factors and DksA bind to the same secondary channel on RNA polymerase and thereby affect transcription [91]. Another factor, Rnk (regulator of nucleoside kinase) bears high sequence similarity and is structurally homologous to the Gre factors. Rnk is not functionally homologous to the Gre factors, but can compete with GreA, GreB and DksA in binding to RNA polymerase *in vitro* [92]. Finally, overexpression of an uncharacterized ORF, *ybiI* has been shown to alleviate some of the amino acid auxotrophies of a Δ*dksA* strain [93]. Thus, YbiI is a potential functional homolog of DksA. We determined σ^E^ activity in strains carrying deletions of *greA*, *greB*, *rnk*, *ybiI* either alone or in combination with *dksA* during phosphate starvation. Our results indicated that none of these factors modulate σ^E^ activity during phosphate starvation (Figure 2-4).

Assuming that the mechanism of action of the compensatory factor is unlike that of DksA, we determined the effect of another factor, Crl, that has been shown to positively influence transcription by promoting $E_{\sigma^S}$, $E_{\sigma^{70}}$ and $E_{\sigma^H}$ holoenzyme formation [94]. Our results indicate that σ^E^ activity increases following phosphate starvation regardless of the presence or absence of Crl suggesting that Crl is not the DksA compensatory factor (Figure 2-4).
None of the known homologs of DksA or the transcription factors Crl compensate for DksA to regulate $\sigma^E$ activity during phosphate starvation. $\sigma^E$ activity following entry into phosphate starvation in the wild-type (SEA001), $\Delta$dksA (SEA6020), $\Delta$coreA (SEA7029), $\Delta$coreB (SEA7030), $\Delta$coreA $\Delta$dksA (SEA7032), $\Delta$coreB $\Delta$dksA (SEA7033), $\Delta$ybiI (SEA7062), $\Delta$ybiI $\Delta$dksA (SEA7063), $\Delta$rnk (SEA7060), $\Delta$rnk $\Delta$dksA (SEA7061), $\Delta$crl (SEA7149) and $\Delta$crl $\Delta$dksA (SEA7152) strains is shown. All strains were grown in low phosphate media at 30°C. Samples were assayed for $\sigma^E$ activity by monitoring $\beta$-galactosidase activity produced from the rpoH P3-lacZ reporter. Data represents the average compiled from three independent experiments. Error bars represents the standard deviation between the individual data sets. Sigma factor activity in the mutant strains is shown relative to that in the wild-type strain which is normalized to 1.

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Regulation of $\sigma^{70}$ during phosphate starvation depends on DksA but not ppGpp

Regulation of transcription initiation by ppGpp at ribosomal rrn promoters, using rrnB P1 as a representative promoter, has been studied extensively during amino acid starvation, during growth in LB and at different growth rates. Whether an increase in ppGpp levels necessarily leads to a decrease in $\sigma^{70}$ activity at rrn promoters during starvation conditions other than amino acid starvation has not been studied as extensively. To examine the contribution of ppGpp to regulation of $\sigma^{70}$ activity at rrn promoters, we subjected strains carrying the $\sigma^{70}$-dependent rrnB P1-lacZ transcriptional fusion reporter to phosphate starvation. Measurements of reporter activity
suggested that in the wild type and ppGpp\(^{-}\) strain, \(\sigma^{70}\) activity did not increase following entry into phosphate starvation. However, \(rrnB\) P1 promoter activity continued to increase in the \(\Delta dksA\) strain, suggesting that DksA was necessary for regulation of \(\sigma^{70}\) activity (Figure 2-5).

![Figure 2-5: Regulation of \(\sigma^{70}\) activity at \(rrnB\) P1 promoter during phosphate starvation and stationary phase in EZ-Rich is ppGpp-independent. The wild type (SEA6583), \(\Delta dksA\) (SEA7025) and ppGpp\(^{0}\) strain (SEA7027) were grown in EZ-Rich and low phosphate media at 30\(^{\circ}\)C. \(\sigma^{70}\) activity was measured throughout growth from \(\sigma^{70}\)-dependent \(rrnB\) P1-\(lacZ\) transcriptional fusion reporter. The differential rate plot showing \(\sigma^{70}\) activity as a function of the optical density of the culture (B, C) is shown along with the corresponding growth curve (A). A representative data set is shown and similar results were obtained in three separate experiments.](image-url)
The *rrnB* P1 promoter used in this experiment is not a full-length promoter, but only contains the upstream sequences (-66 to +1) that are required for the response to ppGpp [38]. Assuming that the lack of the full-length promoter region and associated regulatory regions were responsible for the unexpected ppGpp-independent regulation, we determined σ^{70} activity following phosphate starvation in strains carrying the full-length *rrnE* promoter fused to *lacZ*. Transcription at *rrnE* was regulated in a ppGpp-independent manner, suggesting that ppGpp is not required to regulate σ^{70} activity at *rrn* genes following phosphate starvation (Figure 2-6).

![Figure 2-6: Regulation of σ^{70} activity at full-length *rrnE* promoter during phosphate starvation and stationary phase in EZ-Rich is ppGpp-independent. The wild type (SEA7043), ΔdksA (SEA7044) and ppGpp⁰ strain (SEA7046) were grown in EZ-Rich and low phosphate media at 30°C. σ^{70} activity was measured throughout growth from σ^{70}-dependent *rrnE-lacZ* transcriptional reporter. The differential rate plot showing σ^{70} activity as a function of the optical density of the culture (B,C) is shown along with the corresponding growth curve (A). A representative data set is shown and similar results were obtained in three separate experiments.](image-url)

Figure 2-6
While β-galactosidase assays are a useful tool to measure responses of lacZ-based reporters to changes in steady-state growth, they can be misleading during non-steady state situations such as nutritional downshifts because β-galactosidase is a stable enzyme that accumulates in the cell over time even if reporter activity decreases. To directly examine rrn promoter activity, we analyzed the short-lived RNA products themselves by measuring levels of the rrnB P1 transcript synthesized by primer extension on rrnB P1-lacZ [38] following phosphate starvation. In the wild type strain, rrnB P1 promoter activity decreased such that it was barely detectable after entry into phosphate starvation. In contrast, the level of rrnB P1 transcript formed did not decrease in the ΔdksA strain. Similar to the wild-type strain, rrn promoter activity did not increase during phosphate starvation in the ppGpp⁰ strain (Figure 2-7).

The data shown in figures 2-5, 2-6 and 2-7 indicates that contrary to regulation of σ⁰; DksA, but not ppGpp, is required for the decrease in σ⁰⁺⁺-dependent transcription at rrnB P1 and rrnE during phosphate starvation. Consistent with previously published data, our control experiments under amino acid starvation indicate that regulation at rrnB P1 remains both ppGpp- and DksA - dependent (Figure 2-7).
Figure 2-7: DksA but not ppGpp is required for shut-off of transcription at the $\sigma^{70}$-dependent $rrnB$ P1 promoter during phosphate starvation. $rrnB$ P1 promoter activity was assayed by primer extension. P1 transcript produced from single-copy $rrnB$ P1-lacZ transcriptional reporter fusion is indicated by arrowheads. Wild type (SEA6583), $\Delta$dksA (SEA7025) and ppGpp$^0$ strains (SEA7027) were grown in EZ-Rich for >4 doublings to an OD$_{600}$ of 0.2. Cells were then shifted to low phosphate media (A), amino acid starved media by addition of 1mg/ml final concentration of serine hydroxamate, SH (B) or back into EZ-Rich (C). Addition of SH triggers immediate starvation hence RNA samples were extracted within 5-15 minutes of SH addition. In contrast, when the cells are shifted to low phosphate media, starvation is gradual, hence RNA samples are extracted 15-90 minutes following the downshift. The relevant regions of the gels from a representative experiment are shown.

Regulation of $\sigma^E$ activity during phosphate starvation is RseA-independent

We have previously shown that $\sigma^E$ activity is regulated in a growth phase-independent manner; activity is low during exponential phase and increases during entry into stationary phase [24]. Hence, when $\beta$-galactosidase activity in a given volume of cells is plotted against the culture density (differential rate plot, also see [24]), the slope is non-linear. To determine if the RseA-dependent pathway that regulates $\sigma^E$ during extracytoplasmic stress also regulates $\sigma^E$ during phosphate starvation, $\sigma^E$ activity was measured in a $\Delta$rseA strain grown in limiting phosphate. If RseA contributes to the regulation of $\sigma^E$ activity during phosphate starvation, then in a strain lacking RseA, $\sigma^E$ activity should be mostly unregulated resulting in a single linear slope as activity increases with respect to culture density. Even though $\sigma^E$ activity is generally higher in a $\Delta$rseA strain compared to a wild-type strain (data not shown, [24]), I found that activity still increased by ~10-fold during entry into phosphate starvation, indicating that regulation of $\sigma^E$ activity is RseA-independent (Figure 2-8).
Figure 2-8: Regulation of $\sigma^E$ activity during phosphate starvation is RseA-independent. The $\Delta rseA$ strain (SEA6462) was grown in EZ-Rich and in low phosphate media at 30°C, and $\beta$-galactosidase activity of the $\sigma^E$-dependent $rpoH$ P3-lacZ reporter was measured throughout the growth of the culture. The differential rate plot showing $\sigma^E$ activity as a function of the optical density of the culture (B) is shown along with the corresponding growth curve (A). Representative data set from at least two independent repeats is shown.

Regulation of $\sigma^E$ during phosphate starvation is PhoB-independent

The transcription regulator, PhoB, is activated in response to phosphate starvation and regulates many genes whose products are involved in the acquisition and metabolism of phosphate [95,96]. To determine whether PhoB was also involved in regulation of $\sigma^E$, we examined $\sigma^E$ activity in $\Delta phoB$ strain following phosphate starvation. We found that $\sigma^E$ activity in $\Delta phoB$ strain increased as in the wild-type strain during phosphate starvation, indicating that neither PhoB nor other members of the Pho regulon control $\sigma^E$ activity (Figure 2-9).
Figure 2-9: Regulation of $\sigma^E$ activity during phosphate starvation is PhoB-independent. The wild type strain (SEA001, diamonds) and $\Delta$phoB strain (SEA7166, crosses) were grown in low phosphate media at 30°C, and $\beta$-galactosidase activity of the $\sigma^E$-dependent $rpoH$ P3-lacZ reporter was measured throughout the growth of the culture. The differential rate plot showing $\sigma^E$ activity as a function of the optical density of the culture (B) is shown along with the corresponding growth curve (A). Representative data set from two independent repeats is shown.

Alkaline phosphatase activity is ppGpp-dependent and DksA-independent

Alkaline Phosphatase (AP), encoded by phoA, is a nonspecific periplasmic phosphomonoesterase that is expressed only when environmental phosphate is limiting. While the exact mechanism of action is not known, the increase in alkaline phosphatase activity is ppGpp-dependent in vivo [86]. To determine whether other cellular processes occurring during phosphate starvation could also be regulated like $\sigma^E$ in a ppGpp-dependent and DksA-independent manner, we analyzed AP activity in a $\Delta$dksA strain. Consistent with previous observations, AP activity was completely dependent on ppGpp. However, AP activity increased even in the absence of DksA (Figure 2-10), indicating that DksA is not an absolute requirement for all phosphate starvation induced ppGpp-dependent processes.
Figure 2-10: Alkaline phosphatase activity is ppGpp-dependent and DksA-independent during phosphate starvation. The wild type strain (SEA6583), ΔdksA strain (SEA7025) and ppGppο strain (SEA7027) were grown in low phosphate media at 30°C, and alkaline phosphatase activity was measured throughout growth. Alkaline phosphatase activity plot (B) is shown along with the corresponding growth curve (A). Data compiled from two independent experiments are shown.

Intracellular levels of ppGpp and other nucleotides during phosphate starvation

The DksA-independent regulation of σE activity during phosphate starvation could be due to differences in the production of ppGpp in the ΔdksA strain compared to the wild-type strain. To address this question, we measured ppGpp levels during phosphate starvation in both wild type and ΔdksA strain. The results shown in Figure 2-11A and 2-11C indicate that as cells enter starvation, there is an increase in ppGpp levels that correlates with the increase in σE activity shown in Figure 2-11B. In addition, ppGpp levels in the ΔdksA strain are slightly higher (~2.5-fold, Figure 2-11D) than in the wild-type strain during phosphate starvation. After the initial increase, ppGpp levels go down, but do not completely disappear. Our TLC data also indicates a decrease in ATP levels (~6.5-fold), GTP levels (~4.2-fold) and CTP levels (~6.3-fold) in the wild type strain following phosphate starvation (Figure 2-11C and 2-11E). We also noted a similar decrease in NTP levels in the ΔdksA strain (Figure 2-11C and 2-11F). ATP and CTP levels in the ppGppο strain were comparable to those
seen in the wild-type strain, whereas levels of GTP were slightly higher (~25% more) in the ppGpp° strain compared to wild-type (Figure 2-11C, data not shown).
Figure 2-11: $\sigma^E$ activity and nucleotide levels following phosphate starvation.

(A, B) Wild type strain (SEA001, diamonds), $\Delta$dksA strain (SEA6020, crosses) and ppGpp$^o$ strain (SEA2010, circles) were grown at 37°C in EZ-Rich. Cells were shifted to low phosphate media at OD$_{600}$ of 0.2 (indicated by the arrow). $\beta$-galactosidase activity of the $\sigma^E$-dependent rpoH P3-lacZ reporter was measured throughout growth. The amount of $\beta$-galactosidase activity per cell (equivalent to Miller units, see Materials and Methods) as a function of the time of growth of the culture (B) is shown along with the corresponding growth curve (A). (C) One-dimensional thin-layer chromatograms of labeled cell extracts. Wild-type, $\Delta$dksA and ppGpp$^o$ strains described in (A,B) were subject to phosphate starvation and nucleotides extracted as described in Materials and Methods. The numbers indicate time after the shift to low phosphate media. A representative chromatogram from three independent data sets is shown. (D) Average (p)ppGpp amounts during phosphate starvation in wild-type and $\Delta$dksA strains are shown. Averages and standard deviations are from three independent experiments (E, F) Average ATP (squares), GTP (circles) and CTP (triangles) amounts following phosphate starvation in wild type and $\Delta$dksA strains are shown. NTP levels are normalized to the respective NTP levels at the first time point (t=15’) that was extracted following the shift to low phosphate media. Averages and standard deviations are from three independent experiments.

Either form of (p)ppGpp can regulate $\sigma^E$ activity

In *E. coli*, ppGpp is synthesized from the precursor pppGpp (collectively called (p)ppGpp) by the action of the enzyme guanosine pentaphosphate exopolyphosphatase (GppA) [97]. To determine whether $\sigma^E$ activity is regulated by a particular form of (p)ppGpp or by a certain ratio of ppGpp to pppGpp, we measured $\sigma^E$ activity in a $\Delta$gppA strain that only makes pppGpp. Our data indicates that when the cells only synthesize pppGpp in response to phosphate limitation, $\sigma^E$ activity increases to the same extent as in a wild-type strain, indicating that the pentaphosphate form alone is sufficient to regulate $\sigma^E$ activity (Figure 2-12A). As an alternative approach, we extracted nucleotide samples from a wild-type and $\Delta$dksA strain subject to phosphate starvation (Figure 2-12B). While low levels of pppGpp is present initially when cells are shifted to low phosphate media (t=30’ in Figure 2-12B), almost all pppGpp is converted to ppGpp when cells are in phosphate starvation (t=60’, 90’). Together, these data indicate that during phosphate starvation, the tetraphosphate form
predominates; however, in its absence, the pentaphosphate form functions just as well to regulate $\sigma^E$ activity.

Figure 2-12: $\sigma^E$ activity can be regulated by either ppGpp or pppGpp. (A,B) $\sigma^E$ activity in low phosphate media in the wild-type (SEA001, diamonds) and $\Delta gppA$ (SEA7194, crosses) strains at 30°C is shown. Samples were assayed for $\sigma^E$ activity by monitoring $\beta$-galactosidase activity produced from the rpoH P3-lacZ reporter. The differential rate plot showing $\sigma^E$ activity as a function of the optical density of the culture (B) is shown along with the corresponding growth curve (A). Representative data set from two independent repeats is shown. (C) One-dimensional thin-layer chromatogram of labeled cell extracts. Wild type (SEA001), $\Delta dksA$ (SEA6020), ppGpp° (SEA2010, negative control) and strain with high levels of (p)ppGpp synthesized from relA gene on the pALS13 plasmid (SEA7195, positive control) were labeled with $^{32}$Pi, subjected to phosphate starvation and nucleotides extracted as described in Materials and Methods. The numbers indicate time after the shift to low phosphate media. A representative chromatogram from three independent data sets is shown.
The enzymatic activity of SpoT is required for regulation of $\sigma^E$ activity

While the cellular location of SpoT is still under debate, SpoT was shown to interact with a membrane protein involved in fatty acid biosynthesis [98]. If SpoT is membrane-bound, then it could directly interact with another membrane-bound protein, the $\sigma^E$ anti-sigma factor RseA. Thus, SpoT could modulate $\sigma^E$ activity independently of ppGpp synthesis. In the $\Delta$relA spoT synthase-strain, the point mutation E319Q in SpoT specifically impairs the enzymatic activity of SpoT synthase while maintaining the structure of the protein [99]. $\sigma^E$ activity did not increase in this strain following phosphate starvation suggesting that synthesis of ppGpp is required for the increase in $\sigma^E$ activity (Figure 2-13).

**Figure 2-13:** The enzymatic activity of SpoT is required to regulate $\sigma^E$ activity. $\sigma^E$ activity following entry into phosphate starvation in the wild type (SEA001, diamonds), $\Delta$relA spoT synthase- (SEA6565, triangles) and ppGpp$^o$ (SEA2010, crosses) strains is shown. Strains were grown in low phosphate media at 30°C. Samples were assayed for $\sigma^E$ activity by monitoring $\beta$-galactosidase activity produced from the rpoH P3-lacZ reporter. The differential rate plot showing $\sigma^E$ activity as a function of the optical density of the culture (B) is shown along with the corresponding growth curve (A). Representative data set from three independent repeats is shown.
Regulation of $\sigma^E$ is independent of the source of ppGpp

In contrast to amino acid starvation in which ppGpp levels increase due to increased synthesis by RelA, ppGpp levels are thought to increase during other types of starvation owing to a decrease in ppGpp hydrolysis by SpoT. Since ppGpp leads to an increase in $\sigma^E$ activity in a DksA-dependent manner during amino acid starvation (RelA-mediated) and in a DksA-independent manner during phosphate starvation (hypothesized to be SpoT-mediated); we analyzed whether the requirement for DksA depended on the source of ppGpp. Strains that selectively synthesize ppGpp from either RelA ($spoT$ synthase-, [99]) or SpoT ($\Delta relA spoT$ synthase+ hydrolase-, R39A point mutation renders SpoT hydrolase non-functional, [99]) were used.

We found that the increase in $\sigma^E$ activity in the $spoT$ synthase- mutant strain during phosphate starvation is comparable to that of the wild-type strain (Figure 2-14A). $\sigma^E$ activity also increased in the $\Delta relA$ strain, indicating that ppGpp synthesized from either source can regulate $\sigma^E$ activity during phosphate starvation (Figure 2-14A). In the $\Delta relA$ strain, maximal ppGpp accumulation was slightly delayed; consistent with the idea that SpoT is a weaker synthase (Figure 2-14B, 2-14C).

Role of SpoT hydrolase in regulation of ppGpp levels

Our data that the increase in $\sigma^E$ activity can be mediated by either source of ppGpp are consistent with the model that ppGpp levels increase due to decreased SpoT hydrolase activity during phosphate starvation. This model has been difficult to test because in the presence of the strong ppGpp synthase RelA, $spoT$ cannot be deleted. However, a variant of SpoT was recently constructed in which the hydrolase is inactivated by a point mutation. In the $\Delta relA spoT$ hydrolase- strain, ppGpp is synthesized from the weak SpoT synthase. To determine the contribution of SpoT
hydrolase during phosphate starvation, we measured $\sigma^E$ activity in the $\Delta relA$ spoT hydrolase- strain. Even though $\sigma^E$ activity was reduced by ~2-fold in this strain compared to a wild-type strain, it represented an increase in activity following entry into phosphate starvation (Figure 2-14A). To determine whether the increase in $\sigma^E$ activity correlated with a change in ppGpp levels, nucleotides were extracted from the $\Delta relA$ spoT hydrolase- strain growing in parallel cultures in EZ-Rich and in phosphate limited media. In rich media, the strain continued to grow exponentially, transitioning into stationary phase at OD$_{600}$ of ~1.5 at which point ppGpp is visible (Time point 90’, Figure 2-14D). In comparison, ppGpp in the $\Delta relA$ spoT hydrolase- strain increased within 30 minutes of being transferred to low phosphate media (Figure 2-14D). The overall amount of ppGpp synthesized during phosphate starvation in $\Delta relA$ spoT hydrolase- strain appears modest, but it represents a ~25-fold increase compared to when the strain was not subject to phosphate starvation (after normalizing the increase in cell density of the two cultures) (Figure 2-14D, 2-14E). This result suggests that ppGpp levels can be regulated, even if SpoT hydrolase is non-functional.
Figure 2-14: Phosphate starvation can be regulated by RelA or SpoT.

(A,B) σ^E activity in the wild type (SEA001, diamonds), ΔrelA (SEA6513, squares), spoT synthase- (SEA6575, triangles) and ΔrelA spoT hydrolase- (SEA7019, crosses) strains after entry into phosphate starvation is shown. All strains were grown in low phosphate media at 30°C. Samples were assayed for σ^E activity by monitoring β-galactosidase activity produced from the rpoH P3-lacZ reporter. The differential rate plot showing σ^E activity as a function of the optical density of the culture (B) is shown along with the corresponding growth curve (A). Representative data set from three independent repeats is shown.

(C) One-dimensional thin-layer chromatogram of labeled cell extracts. Strains described in (A) were labeled with ^32Pi, subject to phosphate starvation and nucleotides extracted as described in Materials and Methods at 37°C. The numbers indicate time after cells were shifted to low phosphate media. A representative chromatogram from three independent data sets is shown.

(D) Average (p)ppGpp amounts during phosphate starvation in wild type, ΔrelA, spoT synthase- and ΔrelA spoT hydrolase- are shown. (p)ppGpp levels are normalized to GTP levels in the same sample. Averages and standard deviations are from three independent experiments.

(E) One-dimensional thin-layer chromatogram of labeled cell extracts. Nucleotide samples were extracted at time points indicated from duplicate samples of ΔrelA spoT hydrolase- strain grown under no starvation (squares) and phosphate starvation (circles). The numbers indicate time after the shift to new media at 37°C. A representative chromatogram from three independent data sets is shown.
Average (p)ppGpp amounts in the ΔrelA spoT hydrolase- strain are shown. (p)ppGpp levels are normalized to the increase in cell density (OD$_{600}$). Averages and standard deviations are from three independent experiments.

Regulation of $\sigma^E$ activity by DksA

Overexpression of dksA can compensate for the lack of ppGpp during amino acid auxotrophy, cell-cell aggregation, filamentation ($\sigma^{70}$-mediated), motility ($\sigma^F$-mediated) and stationary phase morphology ($\sigma^S$-mediated) [100]. Therefore, we examined whether overexpression of dksA could compensate for the lack of ppGpp in regulating $\sigma^E$ activity as well. Wild type dksA was overexpressed from an IPTG -inducible promoter under various nutrient limitation conditions during which ppGpp is required for regulation of $\sigma^E$. Overexpression of dksA was sufficient to increase $\sigma^E$ activity in the absence of ppGpp during entry into stationary phase in LB and EZ-Rich and during phosphate starvation (Figure 2-15). However, the increase in $\sigma^E$ activity during amino acid starvation required ppGpp regardless of dksA overexpression (Figure 2-15). The data suggest that the mechanism by which DksA and ppGpp regulate transcription may vary depending on whether their roles are compensatory (phosphate starvation) or complementary (amino acid starvation).
Figure 2-15: Overexpression of dksA can compensate for the lack of ppGpp during most starvations. σE activity in the wild type (SEA7121, diamonds) and ppGpp0 (SEA7123, crosses) strains during various nutrient limitation conditions at 30°C is shown. IPTG was added to both strains at an OD600 of 0.2 (indicated by arrow) to overexpress dksA from the pIN plasmid. Samples were assayed for σE activity by monitoring β-galactosidase activity produced from the rpoH P3-lacZ reporter. The differential rate plot showing σE activity as a function of the optical density of the culture (left) is shown along with the corresponding growth curve (right) for each starvation condition tested. A representative data set from at least two independent data sets is shown.
When the X-ray crystal structure of DksA was solved, Perederina et al., showed that a DksA variant with two mutated residues at the coiled coil tip, D71 and D74, failed to augment ppGpp-dependent inhibition of transcription at the T7A1 promoter [45]. Recently, Lee et al., also demonstrated the importance of the D74 residue in the negative regulation of $\sigma^{70}$-dependent $rmb$ P1 transcription [101]. We analyzed the requirement of the D71/D74 residues in regulation of $\sigma^E$ activity following amino acid starvation during which the increase in $\sigma^E$ activity requires both DksA and ppGpp. $\sigma^E$ activity did not increase in a $\Delta$dksA strain complemented with a $p$dk$sA_{mut}$ plasmid (D71N/D74N $dksA$) during amino acid starvation, indicating that the acidic residues are functionally important in regulation of $\sigma^E$ (Figure 2-16).

**Figure 2-16:** The acidic residues at the coiled-coil tip of DksA are important for regulation of $\sigma^E$ activity during amino acid starvation. $\sigma^E$ activity during amino acid starvation in the wild-type (SEA7124), $\Delta$dksA strain (SEA7125) and ppGpp$^o$ (SEA7126) strains overexpressing $p$dk$sA_{mut}$ is shown. All strains were grown in limiting amino acid media at 30°C. Samples were assayed for $\sigma^E$ activity by monitoring $\beta$-galactosidase activity produced from the $rpoH$ P3-$lacZ$ reporter. The differential rate plot showing $\sigma^E$ activity as a function of the optical density of the culture (A) is shown along with the corresponding growth curve (B). A representative data set from two independent data sets is shown.
Discussion

Regulation of $\sigma^E$ by RseA in response to cell envelope-related stress is a well-characterized process that has been studied for many years. The proteolytic cascade that releases $\sigma^E$ from RseA, the genes transcribed by $\sigma^E$ (the $\sigma^E$ regulon) and a structural insight into the $\sigma^E$-RseA complex have been determined, giving us a detailed understanding of this process (reviewed in [102]). In contrast, we are still at the early stages of understanding regulation of $\sigma^E$ activity during nutrient limitation-induced stress. Previous work showed that when cells enter stationary phase, $\sigma^E$ activity increases in a ppGpp- and DksA-dependent manner [24, 56]. Before in vitro transcription experiments showed that $E\sigma^E$ activity could be directly enhanced by ppGpp and DksA [56]; the indirect model was the only proposed mechanism of regulation of alternative sigma factors by ppGpp and DksA [103]. I demonstrate in this chapter, that while ppGpp and DksA can have a complementary effect on $\sigma^E$ activity (amino acid starvation and entry into stationary phase in LB), ppGpp can also regulate $\sigma^E$ activity in the absence of DksA (phosphate starvation and entry into stationary phase in EZ-Rich) (Figure 2-1). During the course of my studies, I also found that ppGpp levels increase in strains carrying non-functional SpoT hydrolase suggesting that additional mechanisms exist to maintain ppGpp levels in the cell.

Finally, we note that $\sigma^{70}$-dependent transcription at ribosomal operons, specifically at $rrnB$ P1 and at $rrnE$, is not ppGpp-dependent during phosphate starvation. To the best of our knowledge, an increase in ppGpp levels, without a concomitant decrease in $rrn$ transcription has not been observed before. The data suggests that if ppGpp does not play a role in regulating transcription at $rrn$ promoters, then models based on the inhibitory action of ppGpp at $rrn$ promoters cannot sufficiently explain the increase in transcription by alternative sigma factors $\sigma^E$ and $\sigma^S$ during...
phosphate starvation since there would be no enhanced competition for the newly available core RNAP. Moreover, since ppGpp is essential for regulating $\sigma^E$ and DksA is essential for regulating $\sigma^{70}$ during phosphate starvation, it seems that ppGpp and DksA (alone or with additional unknown coregulators) regulate different targets in parallel during phosphate starvation. Such segregation of function may be an effective way to rapidly mount a response to limiting phosphate by using different regulators to simultaneously target transcription by various sigma factors.

**Regulation of $\sigma^{70}$ activity at ribosomal genes during phosphate starvation**

In addition to ppGpp and DksA, levels of iNTP can strongly influence transcription at $rrn$ P1 promoters. During rapid growth in rich media such as LB, high iNTP concentrations bind and stabilize open promoter complexes at $rrn$ P1 promoters allowing transcription to proceed. During starvation, iNTP availability is low resulting in lower rates of successful transcription initiation [71]. Along with iNTP levels; to some extent, the elongating NTP level can also affect $rrnB$ P1 transcription [71]. Our data show that ATP (iNTP at 6 of the 7 $rrn$ P1 promoters including $rrnB$ P1), GTP (iNTP at the 7th $rrn$ P1 promoter) and CTP levels (elongating nucleotide at $rrnB$ P1 promoter) decrease when cells enter phosphate starvation (Figure 2-11C, 2-11E, 2-11F) [48]. We hypothesize that the low NTP levels lower the rate of successful transcription initiation at the inherently unstable $rrn$ promoters during phosphate starvation. In addition, DksA can by itself inhibit transcription initiation at the short-lived unstable $rrn$ open promoter complexes [48]. Therefore, we propose that the combination of low iNTP levels and presence of DksA is sufficient to overcome the requirement of ppGpp to regulate $\sigma^{70}$ activity at $rrnB$ P1 and $rrnE$ during phosphate starvation.
**Regulation of σ^{S} during phosphate starvation**

Expression and activity of σ^{S} is regulated by many factors. ppGpp and DksA stimulate expression of rpoS (the gene encoding σ^{S}) during stationary phase [59,60]. In addition, the indirect model which predicts that ppGpp modulates the increase in core RNAP availability has been proposed to lead to an increase in σ^{S} activity during entry into various types of starvation. However, since ppGpp does not regulate transcription at rrn promoters during phosphate starvation (Figure 2-5, 2-6, 2-7), the increase in σ^{S} activity cannot be attributed to the indirect model. Yet, σ^{S} activity is ppGpp- and DksA- dependent. Recent work from the Gottesman lab has shown that ppGpp is a positive regulator of the anti-adaptor protein IraP, which interferes with the delivery of σ^{S} to the ClpXP protease by binding to the adaptor RssB [104]. Thus IraP mediates stabilization of σ^{S} during phosphate starvation in a ppGpp- and DksA -dependent manner. Hence, we propose that a more probable explanation for the increase in σ^{S} activity during phosphate starvation is the role of ppGpp and DksA in expression of iraP and rpoS and not necessarily enhanced competition for or availability of core RNAP.

**Regulation of σ^{E} activity during phosphate starvation**

ppGpp levels increase by ~2.5-fold during phosphate starvation in a ΔdksA strain compared to a wild-type strain (Figure 2-11D). We do not know whether subtle increases in ppGpp levels during phosphate starvation compensate for the lack of DksA to regulate σ^{E} activity in vivo. Since high ppGpp levels alone have only shown a slight effect on transcription initiation in vitro and DksA is required to potentiate the effect [48,56], it is likely that the DksA -independent regulation of σ^{E} activity in vivo is mediated by additional factors. None of the known homologs of DksA compensate for the lack of DksA to regulate σ^{E} activity during phosphate starvation.
(Figure 2-4). DksA is also required to complement ppGpp -dependent regulation of $\sigma^E$ under non-stress conditions (Figure 2-2). One intriguing possibility involves polyphosphate whose levels increase during phosphate starvation and which is hypothesized to regulate gene expression during starvation conditions.

**Role of SpoT during phosphate starvation**

The *E. coli* RelA has two domains; a ppGpp synthase domain that synthesizes large amounts of ppGpp in response to amino acid starvation and an inactive hydrolase domain. SpoT shares sequence similarity with RelA and also has two functional domains - a weak synthase domain and a strong hydrolase domain. Accumulation of ppGpp during various starvations is thought to result from lower degradation by SpoT hydrolase [105]. In *E. coli*, deleting *spoT* in the presence of a functional RelA is lethal, hence until now it has been difficult to understand the differential role of RelA and SpoT during various types of starvations (except amino acid starvation). We demonstrated in this chapter that ppGpp levels increase even if SpoT synthase is inactive suggesting that SpoT synthase plays a minor role (if any) in accumulation of ppGpp during phosphate starvation. Secondly, the observation that ppGpp levels were regulated even if SpoT hydrolase is non-functional during phosphate starvation suggests that inhibition of SpoT hydrolase is the not the only means of ppGpp accumulation. We suggest that an increase in ppGpp levels may not only occur via inhibition of ppGpp degradation (either by SpoT hydrolase or other compensatory phosphatases) but also by regulation of the ppGpp synthase (RelA) following specific starvations.
Existing models fail to explain regulation of transcription during phosphate starvation

The observation that $\sigma^E$ activity increases in a DksA-independent manner and $\sigma^{70}$ activity at $rnnB$ P1 and $rnnE$ decreases in a DksA-dependent manner during phosphate starvation is inconsistent with the direct and the indirect model which are based on the concerted action of ppGpp and DksA to modulate transcription. Additionally, the indirect model predicts that an increase in $\sigma^E$ activity is a consequence of the decrease in $\sigma^{70}$ activity at ribosomal promoters mediated by ppGpp, which is not applicable during phosphate starvation. The indirect model further suggests that all alternative sigma factors are regulated in the same manner by ppGpp and DksA following inhibition of transcription at $rnn$ operons. Our data clearly shows that two alternative sigma factors, $\sigma^E$ and $\sigma^S$, are regulated differently by ppGpp and/or DksA. In addition, our data does not support the general assumption that an increase in ppGpp levels necessarily correlates with a decrease in $rnn$ promoter activity, since transcription at $rnnB$ P1 and $rnnE$ during phosphate starvation is ppGpp-independent. We conclude that while ppGpp serves as a general starvation signal and can modulate activity of most sigmas, the contribution and independent effects of additional regulators such as DksA, iNTP and other starvation-specific regulators such as polyphosphate should be considered while proposing new models.

Specificity within a coordinate regulation system

Bacteria such as *E. coli* are found in a wide variety of environments in nature. Their survival during unfavorable circumstances depends on their ability to utilize various regulatory molecules quickly and efficiently to modulate gene expression. Regulation is a complex process and each type of starvation differs in its intensity and its effect on bacterial physiology. Some of the key players remain the same (for example, RseA functions as a direct regulator to control $\sigma^E$
availability under all conditions) but there are others that are either not required (such as DksA) or are replaced by other regulators (such as iNTP or additional unknown factors) depending on the type of nutrient limitation condition.

Each sigma factor is regulated by a dedicated signaling pathway(s) that determines the amount and activity of available sigma factor. The general stress response factor, ppGpp, acts in addition to these pathways; thus ppGpp fine-tunes the activity of the sigma factor. Secondly, ppGpp does not regulate all transcription in the same manner; the requirement for DksA and the overall effect (stimulatory or inhibitory) varies with the type of promoter, the type of starvation and the type of sigma factor. Thus, ppGpp exhibits specificity within a coordinate regulation system (Figure 2-17).

**Figure 2-17:** The global starvation signal, ppGpp, has specific effects on various sigma factors. The dedicated signaling pathway for each sigma factor (in green font) regulates sigma factor amount and/or availability and/or activity during particular stresses. Global stresses such as entry into nutrient limitation leads to ppGpp accumulation. ppGpp modulates global gene expression changes and poises the cell for worsening situations by lowering energy-consuming processes and pre-loads the cell with stress-response factors.
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**Plasmids**

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Materials and Methods

Media, strains and plasmids

Strains used in this chapter are listed in Table 2-1. Strains were grown in various media as described: LB, EZ-Rich with normal amounts of phosphate (1.3 mM phosphate), EZ-Rich with low phosphate (0.13 mM phosphate) or EZ-Rich with low isoleucine (0.06 mM instead of 0.4 mM). EZ-Rich, purchased from Teknova is a commercial preparation of rich defined media containing specific amounts of glucose, amino acids and vitamins, phosphate, and MOPS buffer which are added separately, thus allowing manipulation of specific components. For amino acid starvation experiments, MOPS buffer and glucose were obtained from commercial preparations of EZ-Rich. Amino acid mixture were prepared using the protocol for EZ-Rich on http://www.genome.wisc.edu/resources/protocols/ezmedium.htm and added separately. All amino acids except isoleucine were added at the prescribed amounts to trigger amino acid starvation.

Mutant alleles were moved into appropriate strains using P1 transductions according to standard techniques [107]. All P1 transductions were performed with P1 vir and transductants were isolated by selection on medium containing the appropriate antibiotic. Antibiotics were used at the following concentrations: ampicillin (*amp*) 100 µg/ml, kanamycin (*kan*) 15 µg/ml, tetracycline (*tet*) 10 µg/ml and chloramphenicol (*chlor*) 20 µg/ml. Experiments with ppGpp⁻ strain and *spoT* hydrolase- mutation were performed with at least three independent transductants to ensure that the results were not affected by spontaneous suppressor mutations. All ppGpp⁻ strains were verified by as being unable to grow on minimal media lacking amino
acids. All experiments were performed at 30°C with aeration except when noted otherwise. TLC data was collected by growing strains at 37°C and σE activity was monitored to ensure that similar regulation patterns were seen at both 37°C and 30°C. SEA7062 was made by targeted disruption of the ybiI gene in SEA001. The deletion was made according to the procedure of Datsenko and Wanner, 2000 [108]. The rnk::kan, phoB::kan and crl::kan alleles were obtained from the Keio collection [109] and moved by P1 transduction into SEA001.

**β-galactosidase assays**

Overnight cultures were diluted to an OD<sub>600</sub> of 0.025 and grown with shaking in a gyratory water bath at 30°C or 37°C as indicated in figure legends. For overexpression of plasmid-borne relA or dksA, IPTG was added in early exponential phase at OD<sub>600</sub> of 0.2. Samples (0.5ml, 0.2ml or 0.1ml as indicated in the Figures) were collected throughout the growth curve. The β-galactosidase activity of each sample was measured by the standard assay [107] and is expressed as the OD<sub>420</sub> of reaction mixture divided by the reaction time (o-nitrophenol min<sup>-1</sup>). In all figures except 2-11B, the data are presented as differential rate plots in which β-galactosidase activity in a given sample volume is plotted versus the optical density (OD<sub>600</sub>) of the sample; therefore the slope of the curve indicates the change in β-galactosidase activity with increased cell number. The plots illustrate how sigma factor activity changes throughout the growth curve, in recovery from stationary phase, exponential phase and re-entry into stationary phase. A complete explanation of differential rate plots is presented in [24]. In Figure 2-11B, σE activity is plotted as Miller units (o-nitrophenol min<sup>-1</sup> OD<sub>600</sub><sup>-1</sup>) to emphasize that there is no increase in β-galactosidase accumulation in the ppGpp<sup>o</sup> strain compared to wild-type and ΔdksA strains. For measurements of σE activity as a function of growth rate (Figure 2-4), sigma factor activity was determined by the slope of the line on a differential rate plot after entry into specific nutrient
limitation condition. The slope value on the differential rate plot is equivalent to the Miller unit (β-galactosidase activity in a fixed volume of cells divided by the increase in cell density = β-galactosidase activity per cell).

Alkaline phosphatase assay

Alkaline phosphatase activity was assayed as described in [86]. The assay is similar to the β-galactosidase assay with the exception that p-nitrophenyl phosphate (PNPP) was used as a chromogenic substrate for alkaline phosphatase. AP converts PNPP to p-nitrophenol, which is deprotonated at alkaline pH to produce p-nitrophenolate, a yellow colored compound that is measured at OD_{420}. Alkaline phosphatase activity is plotted as (p-nitrophenolate min^{-1} OD_{600}^{-1}).

Primer Extension

Primer extension was performed as described in [110]. Briefly, monolysogens containing the rrnB P1-lacZ fusion were grown in the EZ-Rich for 3-4 generations to an OD of 0.25-0.3. Amino acid starvation was triggered by addition of 1 mg/ml serine hydroxamate and phosphate starvation by growing cells in low phosphate media (0.13 mM phosphate). At the time points indicated in the figures, RNA was extracted using boiling lysis method [110]. The γ-32P-ATP labeled primer was hybridized to the RNA template in M-MLV buffer (Promega). The primer annealed to the transcript at 112 nucleotides downstream of the 5’ terminus of the promoter in the rrnB P1-lacZ fusion. Reverse transcription of the unstable mRNA made from rrnB P1-lacZ fusion was performed as described in [111] using M-MLV (Promega). Extension was carried out at 42°C and reactions were stopped by the addition of formamide loading buffer. Samples were run on 6% acrylamide gel and visualized by phosphorimaging.
Nucleotide Extraction and Quantification

Wild type, \( \Delta dksA \) and ppGpp\textsuperscript{o} strains were grown in EZ-Rich and then switched to low phosphate media at an OD of 0.2. This culture was divided, and one set of cells immediately received 20 \( \mu \)Ci of \(^{32}\)P-orthophosphoric acid ml\(^{-1}\) (Specific activity 8500Ci/mMole, Perkin-Elmer). Samples were extracted for measurement of promoter activities (via \( \beta \)-galactosidase assay) from the non-radioactive culture and NTPs were extracted from the radioactive culture in parallel. NTPs were extracted with formic acid as described in [110] and resolved by PEI-cellular thin layer chromatography (TLC). For most experiments, 0.85 M phosphate buffer, pH 3.4 was used as running buffer but for Figure 2-12B, 1.5 M buffer was used to separate ppGpp from pppGpp. TLC plates were exposed to phosphorimager screens and scanned on Typhoon 8600 Imager. As described in [112], blank corrected GTP, ATP, CTP and (p)ppGpp values were quantified via ImageQuant 5.2 (Molecular Dynamics). Total ppGpp was expressed as a fraction of (ppGpp)/(ppGpp+GTP). Reported values represent the average of quantification from three independent data sets.
Chapter 3

The *Escherichia coli* transcriptional factor, LeuO, regulates $\sigma^E$ activity by modulating outer membrane porin expression and also plays a novel role in cell division

Abstract

In *Escherichia coli*, the extracytoplasmic function sigma factor, $\sigma^E$, is essential for its role in maintaining cell envelope homeostasis. $\sigma^E$ activity increases in a growth phase dependent manner and is subject to two regulatory mechanisms. The anti-sigma factor, RseA physically interacts with and prevents $\sigma^E$ from associating with RNA polymerase and DNA. Disruptions in the proper folding of outer membrane proteins results in a proteolytic cascade that degrades RseA, releasing $\sigma^E$, thereby promoting E$\sigma^E$-dependent transcription. The increase in $\sigma^E$ activity during starvation is mediated by the alarmone guanosine tetraphosphate (ppGpp) and co-regulator protein, DksA. We report that in addition to these regulatory mechanisms, the DNA-binding transcriptional regulator, LeuO, indirectly regulates $\sigma^E$. In a genetic screen for identifying additional regulators of $\sigma^E$ activity, we found that a transposon insertion upstream of *leuO* decreased $\sigma^E$ activity. Overexpression of *leuO* resulted in lower levels of outer membrane porins OmpC, OmpX and OmpL that, via RseA, decrease $\sigma^E$ activity. We also identified a putative role for LeuO in inhibiting cell division. Overexpression of *dksA* or inactivation of *damX* negates the inhibitory effect of *leuO* overexpression on cell division and $\sigma^E$ activity. DamX is a septal murein-binding protein that inhibits cell division and our work suggests that it may have additional roles. The cell division defect and the decrease in $\sigma^E$ activity appear to be unrelated events mediated by LeuO. The data suggest that LeuO may function as an accessory regulator to control various cellular processes during growth and survival.
Introduction

Bacterial RNA polymerase (RNAP) consists of five subunits (α₂ββ’ω) which comprise the core enzyme. RNAP is responsible for transcription of DNA templates; however, it cannot recognize specific promoters by itself. Promoter specificity is conferred by the sigma (σ) subunit, which recognizes specific DNA elements, assists in DNA melting and subsequently in transcription initiation. Apart from the housekeeping sigma factor, σ⁷⁰, which conducts the majority of transcription during exponential phase, *Escherichia coli* is equipped with six alternative sigma factors which can also associate with core RNAP to form specific holoenzymes (Eσ). σ^E was initially identified for its role in transcribing the heat shock response sigma factor, σ^H [113]. Both σ^H and σ^E transcribe specific genes encoding chaperones and proteases, which process misfolded or unwanted proteins. While σ^H responds to signals originating from within the cytoplasm, σ^E responds to stresses in the extracytoplasmic compartment [5,102,114]. The cell envelope stresses that trigger σ^E activity include chromosomal mutations in genes encoding periplasmic folding catalysts, heat shock, misfolded and overproduction of outer membrane porins[5,27,82,115,116].

The σ^E -specific anti-sigma factor RseA, is an inner membrane protein with a cytoplasmic domain that binds to the DNA and RNAP binding domain of σ^E [18]. When porin folding is disrupted, the C-terminal domain of the porin is exposed. The exposed peptides at the C-terminal tail interact with the DegS protease which initiates a proteolytic cascade mediated further by proteases DegP and ClpXP. The proteases cleave RseA, releasing σ^E, which binds to RNAP and directs transcription of genes whose products alleviate the membrane stress [115-119]. During non-stress conditions, continual proteolysis of RseA; a rather unstable protein, provides the cell
with sufficient free $\sigma^E$ to ensure viability [115,116]. Extracytoplasmic stress increases the degradation rate of RseA, leading to more available $\sigma^E$ and consequently higher activity [120].

A major determinant of $\sigma^E$ activity is the level of outer membrane porins (OMPs) which serve as a sensitive monitor of the health of the bacterial envelope. Multiple regulators exist to maintain precise levels of OMPs during all stages of bacterial growth. OmpC is one of the most abundant porins in *E. coli* and perturbations in its levels have a strong impact on $\sigma^E$ activity [5]. *ompC* expression increases by the action of EnvZ, OmpR and by CpxR [121-123], whereas its expression decreases by the action of Lrp, IHF and small RNAs RybB and MicC [124-128]. $\sigma^E$ in turn, controls *ompC* expression by transcribing *rybB* [129]. Overexpression of OMPs increases the degradation rate of RseA, elevating $\sigma^E$ activity, whereas underexpression or deletion of OMPs lowers the degradation rate of RseA, leading to lower $\sigma^E$ activity [5].

$\sigma^E$ activity also increases following nutrient limitation in the absence of obvious cell envelope damage. The increase in $\sigma^E$ activity following entry into stationary phase is mediated by the RNAP-binding factors ppGpp and DksA [24]. In addition, we discovered that ppGpp leads to an increase in $\sigma^E$ activity in a DksA-independent manner during phosphate starvation, suggesting that another factor may substitute for DksA. In a screen designed to identify additional regulators of $\sigma^E$ activity which may substitute for DksA, we identified the transcriptional effector, LeuO, as a potential regulator of $\sigma^E$ activity.

In the *E. coli* genome, *leuO* is located adjacent to the leucine biosynthesis operons (*leuABCD*) and is part of a complex *cis*-acting promoter relay system that connects the *leuABCD* and *ilvH*
operons [130-132]. In *E. coli*, LeuO has additionally been implicated in the bacterial response to starvation-induced stress [133,134]. In *Salmonella*, LeuO positively regulates outer membrane porins OmpS1 and OmpS2, which are known virulence factors in *Salmonella* [135-137]. These observations indicate that LeuO is not a dedicated regulator of the *leu* operons but a global regulator of various cellular functions.

In this chapter, we report that overexpression of *leuO* lowers $\sigma^E$ activity and lowers expression of the outer membrane porins, OmpC, OmpX and OmpL. We propose that the LeuO-mediated underexpression of OMPs stabilizes RseA, which results in low $\sigma^E$ activity. Deletion or overexpression of *leuO* did not compensate for the lack of DksA to regulate $\sigma^E$ activity; hence we conclude that LeuO is not part of the ppGpp-dependent, DksA-independent regulation of $\sigma^E$ activity. Additionally, we found that overexpression of *leuO* led to cellular filamentation, suggesting a novel role of LeuO in mediating cell division. The negative effect of LeuO on $\sigma^E$ activity and cell division were overcome by overexpression of *dksA* or inactivation of *damX*. The LeuO-mediated decrease in $\sigma^E$ activity and the cell division defects are unrelated. The data suggests that LeuO regulates various aspects of cellular physiology including maintenance of envelope integrity and cell division.
Results

Strategy for isolation of mutants affected in $\sigma^E$ activity

We recently identified a novel ppGpp-dependent and DksA-independent mechanism for regulation of $\sigma^E$ activity during entry into stationary phase in EZ-Rich (MOPS-based rich defined media, see \textit{Materials and Methods}) and during phosphate starvation. Since DksA is required to potentiate the \textit{in vitro} effect of ppGpp on $\sigma^E$-dependent transcription and is also required for the increase in $\sigma^E$ activity during entry into stationary phase in LB and amino acid starvation \textit{in vivo}, we hypothesized that an additional regulator compensated for DksA when regulation occurred in a DksA-independent manner.

A genetic screen was employed to identify additional regulators of $\sigma^E$ activity. Mutants were generated by transducing a kanamycin-resistant transposon library into recipient cells carrying a single-copy $\sigma^E$-dependent \textit{rpoH P3-lacZ} transcriptional fusion reporter gene (See \textit{Materials and Methods} for details on transposon library construction). The host cells were deleted of \textit{dksA} to mimic conditions during which a DksA-compensatory factor might be active. Transductants were analyzed based on their color on plates containing EZ-Rich, kanamycin and X-gal. The parental $\Delta dksA$ strain appeared blue on X-Gal plates; hence we screened for pale blue colonies which may be indicative of a disruption in a gene whose function normally activates $\sigma^E$ in the absence of DksA. We screened 40,000 colonies and identified 250 mutants that appeared white or pale blue in color. These mutants were divided into two categories based on the effect of the transposon insertion. The first category consisted of colonies that were completely white on X-Gal plates (8 out of 250). These mutants could indicate either very low $\sigma^E$ activity or disruption.
of the reporter gene. β-galactosidase activity in overnight cultures of each of these mutants was used to determine levels of σE activity in stationary phase in EZ-Rich. If there was no change in color after addition of ONPG, the β-galactosidase substrate, the transposon insertion was presumed to have disrupted the reporter fusion and was not pursued further. All 8 white mutants fell in this category. The second category of mutants (242 out of 250) consisted of mutants that exhibited a pale blue color on X-Gal plates. β-galactosidase activity in overnight cultures of each of these mutants indicated that in 240 of the 242 mutants analyzed, σE activity was at a level comparable to the wild-type strain in stationary phase. The pale blue color was attributed to slower growth as observed by small colony phenotypes on plates. The remaining two mutants which had low σE activity were analyzed further by measuring β-galactosidase activity at various time points throughout growth. σE activity in these mutants was low both in exponential and stationary phase (data not shown).

Identification and localization of the transposon insertion that lowers σE activity

The transposon insertion in these mutants was mapped as described in Materials and Methods. In the first mutant, the transposon was inserted within ompR, which codes for a transcriptional activator. OmpR is required for the expression of outer membrane porins OmpC and OmpF. Deletion of ompR has been characterized to result in low σE activity as RseA does not get degraded as readily [5]. In the second mutant, the transposon was inserted 27 bp upstream of the leuO open reading frame (Tn-leuO) (Figure 3-1). LeuO is a member of the LysR-type transcriptional regulator (LTTR) family of proteins which consist of an N-terminal DNA-binding helix-turn-helix motif and a C-terminal coinducer-binding domain [138]. LeuO has been implicated in a variety of cellular functions such as leucine biosynthesis, stringent response and
multidrug resistance [134,139,140]. The transposon that mapped to *leuO* was moved to a fresh wild type background and its effect on $\sigma^E$ activity verified (Figure 3-2).

**Figure 3-1:** Identification of the mutation. Tn10-kanamycin insertion 27 bases upstream of the start of *leuO* lowers $\sigma^E$ activity (Image modified from [http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).

**Figure 3-2:** Tn-*leuO* leads to decrease in $\sigma^E$ activity. The wild type strain (SEA001, diamonds) and the Tn-*leuO* strain (SEA7089, crosses) were grown in EZ-Rich at 30°C. $\beta$-galactosidase activity of the $\sigma^E$-dependent *rpoH* P3-*lacZ* reporter was measured throughout the growth of the culture. The differential rate plot showing $\sigma^E$ activity as a function of the optical density of the culture in exponential phase (B) and following entry into stationary phase (C) is shown along with the corresponding growth curve (A). Data compiled from three independent experiments are shown.
**Overexpression of leuO lowers σ^E activity**

The transposon used in this experiment carries a promoter that can constitutively direct transcription from the transposon into the adjacent chromosomal DNA (See Materials and Methods for details). In the Tn-leuO strain with lower σ^E activity, the transposon was oriented with the promoter upstream of the leuO ORF. Therefore, leuO expression could be disrupted or constitutively activated. To distinguish between these possibilities, we measured σ^E activity in a ΔleuO strain. σ^E activity in the ΔleuO strain was indistinguishable from the parental wild type strain indicating that elimination of LeuO did not affect σ^E activity (Figure 3-4).

To determine if increased expression of leuO led to decreased σ^E activity, wild type cells were transformed with a plasmid carrying leuO gene under the control of an IPTG-inducible promoter (pLeuO strain). Overexpression of leuO with 1mM IPTG resulted in strong repression of σ^E activity both in exponential phase (~6.5-fold) and in stationary phase (~125-fold) compared to the wild-type strain carrying a control plasmid (Figure 3-3). The Tn-leuO strain probably only leads to moderate overexpression of leuO compared to the pLeuO system. Consistent with this interpretation, σ^E activity in the Tn-leuO strain was ~1.6 fold lower in exponential phase and ~5.2-fold lower in stationary phase compared to the wild-type (Figure 3-3). We further confirmed that the decrease in σ^E activity following overexpression of leuO was not unique to the rpoH P3 promoter since a comparable decrease in σ^E activity was observed in a strain carrying the σ^E-dependent rybB-lacZ reporter fusion (data not shown).
Figure 3-3: Overexpression of leuO lowers $\sigma^E$ activity. $\sigma^E$ activity in the wild type strain (SEA001), Tn-leuO strain (SEA7089), wild type with control plasmid (SEA7146) and pLeuO strain (SEA7074) grown in EZ-Rich at 30°C is shown. Samples were assayed for $\sigma^E$ activity throughout the growth of the culture by monitoring $\beta$-galactosidase activity produced from a single-copy $rpoH P3-lacZ$ reporter. Average $\sigma^E$ activity from three independent experiments is shown. Error bars represent the standard deviation between the individual data sets. $\sigma^E$ activity in the wild type strain was normalized to 1 both during exponential and stationary phase. $\sigma^E$ activity in the mutant strains is shown relative to that in the wild-type strain at the particular growth phase.

LeuO does not compensate for the lack of DksA to regulate $\sigma^E$ activity

Since LeuO was identified in a screen designed to identify regulators of $\sigma^E$ that compensate for the lack of DksA, we measured $\sigma^E$ activity in a $\Delta$dksA $\Delta$leuO strain. We found that $\sigma^E$ activity was regulated in the double deletion strain, indicating that LeuO does not compensate for the lack of DksA (Figure 3-4A, B). When leuO was overexpressed in a $\Delta$dksA strain, $\sigma^E$ activity was repressed to the same extent as when leuO was overexpressed in an otherwise wild-type background indicating that LeuO lowers $\sigma^E$ activity regardless of the absence or presence of DksA (data not shown). The data suggests that LeuO and DksA regulate $\sigma^E$ activity via distinct pathways. Consistent with this interpretation, $\sigma^E$ activity partially decreased in a strain that
overexpressed both LeuO and DksA, suggesting that the negative regulation by LeuO counters the positive regulation by DksA (Figure 3-4C, D, E).

**Figure 3-4:** LeuO does not compensate for the lack of DksA. All strains were grown in EZ-Rich at 30°C. β-galactosidase activity of the σE-dependent rpoH P3-lacZ reporter was measured throughout the growth of the culture. (A, B) σE activity in wild type (SEA001), ΔdksA (SEA6020), ΔleuO (SEA7103) and ΔdksA ΔleuO (SEA7148) strains is shown along with the corresponding growth curve. A representative data set from three independent experiments is shown. (C, D, E) σE activity during exponential phase (D) and during stationary phase (E) in wild type with control plasmid (SEA7146), pDksA (SEA7121), pLeuO (SEA7074) and pLeuO pDksA (SEA7132) strains is shown along with the corresponding growth curve (C). IPTG was added at OD_{600} of 0.2 (indicated by arrow). A representative data set from three independent experiments is shown.
Overexpression of \( \text{leuO} \) does not affect \( \sigma^E \) levels

To determine whether LeuO lowered \( \sigma^E \) activity by modulating \( \sigma^E \) levels by Western blotting in the pLeuO strain compared to the wild-type strain. \( \sigma^E \) levels during exponential phase, transition to stationary phase and in stationary phase were comparable to those in the wild-type strain; suggesting that \( \text{leuO} \) overexpression does not affect synthesis of \( \sigma^E \) (Figure 3-5).

**Figure 3-5:** Overexpression of \( \text{leuO} \) does not affect steady-state levels of \( \sigma^E \).
(A) Wild-type (SEA7146, circles) and pLeuO strain (SEA7074, crosses) was grown in EZ-Rich at 30°C. Protein samples were extracted at time points indicated by arrows during exponential phase, transition to stationary phase and stationary phase. IPTG was added at OD\(_{600}\) of 0.2 to induce \( \text{leuO} \) overexpression.
(B) Western blots probed with anti-\( \sigma^E \) and anti-RpoB antibody are displayed. Equal amounts of protein extracts as determined by BCA assay were loaded in each lane. The \( \sigma^E \) band (bottom panel) and the RpoB band (top panel) are indicated. As a control, total protein from a \( \Delta \text{rpoE} \) strain (SEA45113) was used. A representative blot is shown and similar results were obtained in three separate experiments.

Overexpression of \( \text{leuO} \) has a different effect depending on the sigma factor

Overexpression of \( \text{leuO} \) has been shown to repress the expression of the small RNA, \( \text{dsrA} \), which positively regulates \( \text{rpoS} \) translation (gene encoding the stationary phase sigma factor, \( \sigma^S \)). Thus, overexpression of \( \text{leuO} \) decreases \( \sigma^S \) activity [141]. Since overexpression of \( \text{leuO} \) lowers \( \sigma^E \)
activity as well, we examined whether LeuO has a negative effect on activity of other sigmas. The plasmid carrying \textit{leuO} was transformed into strains carrying transcriptional reporter fusions for either the \(\sigma^S\) (\(\text{bolA-lacZ}\) reporter), \(\sigma^H\) (\(\text{htpG-lacZ}\) reporter) or \(\sigma^{70}\) (\(\text{rrnBp1-lacZ}\) reporter). \(\beta\)-galactosidase activity assays indicated that overexpression of \textit{leuO} led to a decrease in \(\sigma^S\) activity by \(\sim 3\)-fold in exponential phase and \(\sim 50\)-fold in stationary phase. However, \(\sigma^H\) activity increased by \(\sim 7\)-fold during exponential phase and by \(\sim 3\)-fold during stationary phase. \(\sigma^{70}\) activity was only modestly reduced by LeuO, indicating that the effect of LeuO is different on each sigma factor (Figure 3-6).

Figure 3-6: LeuO has a different effect depending on the sigma factor. Activity in the wild type strain (black bars) is normalized to 1 both during exponential and stationary phase. Sigma factor activity in the pLeuO strain (gray bars) is shown relative to that in the corresponding wild type at the particular growth phase. All strains were grown in EZ-Rich at 30°C. Average data compiled from three independent experiments is shown. Error bars represent the standard error between the individual data sets. 
(A) \(\sigma^S\) activity in the pLeuO strain (SEA7078) is shown relative to the corresponding wild-type strain with control plasmid (SEA6325). Samples were assayed for \(\sigma^S\) activity throughout the growth of the culture by monitoring \(\beta\)-galactosidase activity produced from a single-copy \(\sigma^S\)-dependent \(\text{bolA-lacZ}\) reporter. 
(B) \(\sigma^H\) activity in the pLeuO strain (SEA7087) is shown relative to the corresponding wild-type strain with control plasmid (SEA7086). Samples were assayed for \(\sigma^H\) activity throughout the growth of the culture by monitoring \(\beta\)-galactosidase activity produced from a single-copy \(\sigma^H\)-dependent \(\text{htpG-lacZ}\) reporter. 
(C) \(\sigma^{70}\) activity in the pLeuO strain (SEA7083) is shown relative to the corresponding wild-type strain with control plasmid (SEA6583). Samples were assayed for \(\sigma^{70}\) activity throughout the
growth of the culture by monitoring β-galactosidase activity produced from a single-copy σ\(^{70}\)-dependent \(rrnB\) P1-\(lacZ\) reporter.

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**LeuO does not regulate σ\(^E\) activity via H-NS**

A majority of the LeuO regulated genes are also regulated by the silencer protein H-NS.

LeuO and H-NS have overlapping binding sites in the genome and LeuO is thought to antagonize H-NS action [139,135,142]. To determine whether a similar LeuO-mediated anti-silencing effect might explain the decrease in σ\(^E\) activity by LeuO, we first analyzed the effect of \(hns\) overexpression and deletion on σ\(^E\) activity. While overexpression of \(hns\) (\(phns\)) had no discernible effect on σ\(^E\) activity; deletion of \(hns\) led to a ~15-fold increase in σ\(^E\) activity during exponential phase and a ~2-fold increase during stationary phase, supporting a role for H-NS as a silencer of σ\(^E\) activity (Figure 3-7). If LeuO regulates σ\(^E\) activity by antagonizing the silencing effect of H-NS, we would expect overexpression of \(leuO\) to lead to increased σ\(^E\) activity. Our data does not support the anti-silencing model; hence we conclude that LeuO does not regulate σ\(^E\) activity via H-NS.

![Figure 3-7: LeuO does not regulate σ\(^E\) activity via H-NS.](image)

Wild type (SEA001), \(Δhns\) (SEA7098) and \(phns\) (SEA7104) strains were grown in EZ-Rich at 30°C, and β-galactosidase activity of the
σE-dependent *rpoH* P3-*lacZ* reporter was measured throughout the growth of the culture. The differential rate plot showing σE activity as a function of the optical density of the culture (B) is shown along with the corresponding growth curve (A). The inset in (B) shows σE activity during exponential phase in the wild type and Δ*hns* strain. A representative data set from three independent experiments is shown.

**LeuO regulates σE activity via RseA**

The anti-sigma factor RseA is the primary regulator of σE activity under both stress and non-stress conditions. To determine if LeuO lowers σE activity via RseA, σE activity was measured in a Δ*rseA* pLeuO strain during exponential phase. We found that σE activity was no longer repressed by pLeuO in the Δ*rseA* pLeuO strain, indicating that regulation of σE activity by LeuO is RseA-dependent (Figure 3-8).

![Figure 3-8: LeuO regulates σE activity via RseA.](image)

(A) σE activity during exponential phase in the Δ*rseA* strain with control plasmid (SEA7175, diamonds) and Δ*rseA* pLeuO strain (SEA7085, crosses) is shown along with the corresponding growth curve. Strains were grown in EZ-Rich at 30°C. IPTG was added at OD600 of 0.2 (indicated by arrow). Samples were assayed for σE activity by monitoring β-galactosidase activity produced from the *rpoH* P3-*lacZ* reporter. One representative data set from three independent repeats is shown.

(B) Average σE activity was determined using compiled data from three independent experiments. Error bars represent the standard deviation between the individual data sets. σE activity in the Δ*rseA* pLeuO strain is shown relative to that in the parental Δ*rseA* strain which is normalized to 1.

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**LeuO represses outer membrane porin expression**

Our data suggests that LeuO regulates $\sigma^E$ activity via RseA. Since RseA regulates $\sigma^E$ in response to outer membrane stresses such as porin overexpression, we determined whether LeuO was involved in regulation of porins. Overexpression of ompC alone strongly stimulates $\sigma^E$ activity (Figure 3-9; [5]). When leuO and ompC were both overexpressed, LeuO was no longer able to repress $\sigma^E$ activity (Figure 3-9). These results were verified using a plasmid variant that upon arabinose induction, overexpresses a gene carrying only the terminal peptide region of ompC (pYYF, [82]). The effect of overexpressing pYYF is not as lethal as overexpressing full-length ompC but is sufficient to initiate the RseA proteolytic cascade (Figure 3-10).

**Figure 3-9:** Overexpression of full-length ompC counters LeuO-mediated inhibition of $\sigma^E$ activity. $\sigma^E$ activity (B) in wild type with control plasmid (SEA7159, diamonds), pLeuO (SEA7158, squares), pOmpC (SEA7160, triangles) and pLeuO pOmpC (SEA7161, crosses) strains is shown along with the corresponding growth curve (A). Strains were grown in EZ-Rich at 30°C and 1mM IPTG added at OD$_{600}$ of 0.1 (indicated by arrow in A). Samples were assayed for $\sigma^E$ activity throughout the growth of the culture by monitoring $\beta$-galactosidase activity produced from the rpoH P3-lacZ reporter. A representative data set from three independent experiments is shown.
Overexpression of \( \text{ompC} \) terminal peptide pYYF counters LeuO-mediated inhibition of \( \sigma^E \) activity. \( \sigma^E \) activity (B) in wild type strain (SEA001), Tn-leuO (SEA7089), Tn-leuO + pYYF (SEA7093) and wild type + pYYF (SEA7096) is shown along with the corresponding growth curve (A). Strains were grown in EZ-Rich at 30°C and 0.2% arabinose added at OD\(_{600}\) of 0.1 (indicated by arrow in A). Samples were assayed for \( \sigma^E \) activity throughout the growth of the culture by monitoring \( \beta \)-galactosidase activity produced from the \( \text{rpoH P3-lacZ} \) reporter. Data compiled from two independent experiments are shown.

To further determine if LeuO was involved in regulating OMPs, \( E. \text{coli} \) outer membrane proteins were extracted by a differential solubility assay (See \textit{Materials and Methods}) from a wild type strain and compared with the outer membrane proteins from the Tn-leuO strain and the pLeuO strain (Figure 3-11). Analysis of the outer membrane profiles revealed that strains that overexpress LeuO had lower levels of certain porins. Comparisons with the molecular weight markers and single deletion strains of particular OMPs revealed that LeuO specifically lowered expression of OmpC (~75% decrease), OmpX (~80% decrease), OmpL (~65% decrease) and to a lesser extent, OmpF (~20% decrease) (Figure 3-11).
**Figure 3-11:** Overexpression of *leuO* lowers OMP levels.

(A) Outer membrane was extracted from equal volume of wild type (SEA001), pLeuO (SEA7074) and Tn- *leuO* (SEA7089) cells at OD<sub>600</sub> of 0.5 and 1. Equal amounts of sample were loaded in each lane and run on 6M urea 12% polyacrylamide SDS gels. OMPs affected by *leuO* overexpression include OmpC (40.3 kDa), OmpL (27.2 kDa), OmpX (18.6 kDa) and to a lesser extent OmpF (39.3 kDa). One representative gel from three independent repeats is shown.

(B) OMP levels in the wild type strain (SEA001, black bars), Tn-*leuO* strain (SEA7089, light gray bars) and pLeuO strain (SEA7074, dark gray bars) is shown. Data represents the average from three independent experiments. Error bars represent the standard deviation between the individual data sets.

To determine the level of gene expression at which LeuO modulated OMP expression, we examined the effect of *leuO* overexpression on expression of *ompC* and *ompF* -transcriptional fusion reporters. β-galactosidase activity assays indicated that overexpression of *leuO* led to
significantly less activity from the *ompC-lacZ* reporter fusion (~25-fold, Figure 3-12A), and to a lesser extent from the *ompF-lacZ* reporter (~4-fold, Figure 3-12B), suggesting that LeuO likely regulates OMPs at the level of transcription (or mRNA stability).

**Figure 3-12**: LeuO lowers expression of *ompC* and *ompF*. Wild type and pLeuO strains were grown in LB at 37°C. IPTG was added at OD$_{600}$ of 0.2 (indicated by arrow). A representative data set from three independent experiments is shown.

(A) *ompC* expression in wild type with control plasmid (SEA7235, diamonds) and pLeuO (SEA7236, squares) strains is shown along with the corresponding growth curve (inset). β-galactosidase activity produced from the single-copy *ompC-lacZ* reporter was monitored throughout the growth of the culture.

(B) *ompF* expression in wild type with control plasmid (SEA7237, circles) and pLeuO (SEA7238, triangles) strains is shown along with the corresponding growth curve (inset). β-galactosidase activity produced from the single-copy *ompF-lacZ* reporter was monitored throughout the growth of the culture.

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**Overexpression of leuO leads to filamentation**

In the course of investigating phenotypes associated with elevated levels of LeuO, we found that cells overexpressing LeuO started to become filamentous in late exponential phase for the Tn-leuO strain and after one doubling following addition of IPTG for the pLeuO strain (Figure 3-13). The filamentation phenotype exaggerated with time since ~10% of Tn-leuO cells and ~80%
of pLeuO cells were filamentous in stationary phase. We noticed individual cells that were elongated as well as centrally septated cells that did not separate and continued to elongate over time. The observation suggests that LeuO may be involved in mediating cell division.

**Figure 3-13:** Overexpression of *leuO* from Tn-*leuO* strain and from pLeuO strain induces filamentation.

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**Overexpression of *leuO* does not affect overall cell growth**

The filamentation defect caused by *leuO* overexpression prompted us to examine whether LeuO affected cell growth along with cell division. We determined the effect of *leuO* overexpression on total protein amounts and total protein profiles. Quantification of total protein via BCA assay indicated that the Tn-*leuO* and pLeuO strains accumulated as much protein as the wild-type strain throughout growth (data not shown). The overall protein profiles were also comparable between the two strains; suggesting that cells that overexpress *leuO* continue to grow despite the filamentation defect (Figure 3-14).
Figure 3-14: Total protein profiles in the wild type and pLeuO strain. Total protein was extracted from equal volume of wild type strain carrying control plasmid (SEA7146, WT) and pLeuO strain (SEA7074) at various time points in the growth curve. Lane 1 = marker (M). The arrowhead indicates a band that corresponds by molecular weight to LeuO (35.7 kDa). Increase in total protein concentration over time is equivalent for both strains as determined by BCA assay, hence equal amount of sample was loaded in each lane. Samples were run on 12% polyacrylamide SDS gels. One representative gel from two independent repeats is shown.

Overexpression of dksA antagonizes the filamentation defect caused by overexpression of leuO

Overexpression of dksA (pDksA) has been shown to alleviate the filamentation defects in dnaKJ, grpE280, prc, yhhP and ppGpp null mutants [40,41,100,143]. To determine whether pDksA could also overcome the filamentation defects caused by pLeuO, we observed wild type, pLeuO and pLeuO pDksA strains under the microscope at various stages of the growth curve following IPTG induction. In contrast to the pLeuO strain, cells that overexpress both dksA and leuO did not filament and looked phenotypically like a wild-type strain suggesting that DksA assists in overcoming cell division defects in strains that overexpress leuO (Figure 3-15).
**Figure 3-15:** Overexpression of dksA antagonizes the filamentation defects caused by overexpression of leuO. Wild type (SEA001), pLeuO (SEA7074) and pDksA pLeuO (SEA7132) were grown in EZ-Rich at 30°C and treated with IPTG at OD$_{600}$ of 0.2. The image was taken in stationary phase at OD$_{600}$ of 2.5.

**DamX may be involved in the LeuO -mediated regulation pathway**

To identify additional proteins that may be directly or indirectly involved in the LeuO -mediated regulation of σ$^E$ activity, pLeuO host cells were transduced with the transposon library described earlier with the goal of obtaining mutants that suppress the effect of leuO overexpression. The Tn-leuO strain cannot be transduced with the transposon library since they are both kanamycin resistant so we used the chloramphenicol resistant pLeuO strain as the transposon recipient. leuO was induced with 5 μM IPTG final concentration which has an effect on σ$^E$ activity that is similar to the Tn-leuO strain (Figure 3-16). Transductants were selected on plates containing chloramphenicol, kanamycin and IPTG. In the presence of 5 μM IPTG, pLeuO cells appeared pale blue on X-Gal plates, hence after transduction, mutants that produced blue colonies (similar to wild-type) were considered as mutants of interest as they may be indicative of a mutation that suppresses the effect of leuO overexpression on σ$^E$ activity. Transduction efficiency was generally low, possibly because in this experimental setup the host cells are forced to retain the
plasmid as well as accept a transposon insertion in the chromosome for survival. We screened ~150 colonies and obtained one blue transductant using this approach. PCR amplification of the region adjacent to the inserted transposon indicated that the transposon was inserted within the gene *damX* (Tn-*damX* pLeuO), which codes for a septal murein-binding protein [144].

**Figure 3-16:** Comparison of $\sigma^E$ activity in the wild type, Tn-*leuO* strain and pLeuO strain. $\sigma^E$ activity and corresponding growth in the wild type strain (SEA001) and strains overexpressing *leuO* is shown. Tn-*leuO* (SEA7089) and pLeuO strains (SEA7074) were grown in LB at 30°C. Overexpression of *leuO* was induced in parallel SEA7074 cultures with varying amounts of IPTG at OD$_{600}$ of 0.2 (indicated by arrow): 1mM IPTG, 0.1 mM IPTG, 0.01 mM IPTG, 0.005 mM IPTG. Samples were assayed for $\sigma^E$ activity throughout the growth of the culture by monitoring $\beta$-galactosidase activity produced from the *rpoH* P3-*lacZ* reporter. Data compiled from two independent repeats are shown.

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**Loss of damX antagonizes the effect of leuO overexpression**

Unlike the parental pLeuO strain, the mutant Tn-*damX* pLeuO strain did not filament when *leuO* was overexpressed using 5 μM IPTG. Additionally, we deleted *damX* from a wild-type strain and moved Tn-*leuO* to this background by P1 transduction. No abnormal cell morphology was
observed in the independently constructed ΔdamX Tn-leuO strain (data not shown). Since overexpression of damX has been previously been shown to induce filamentation [145], the data suggests that absence of DamX can overcome the effect of leuO overexpression or that LeuO may enhance damX expression and thereby cause filamentation.

To determine if loss of DamX could also rescue the decrease in σE activity caused by leuO overexpression, we measured σE activity in a ΔdamX Tn-leuO strain. Our data indicates that σE activity was completely restored in this strain (Figure 3-17). In the pLeuO strain, ΔdamX could restore σE activity when leuO was overexpressed with 5 μM IPTG, but not with 1mM IPTG (data not shown), suggesting that overexpression of leuO from the plasmid overwhelms the system or that inactivation of damX can only overcome the effects of a certain threshold level of LeuO.

**Figure 3-17:** Loss of DamX antagonizes the effect of leuO overexpression. σE activity in the wild type strain (SEA001), ΔdamX strain (SEA7110), Tn-leuO strain (SEA7089) and ΔdamX pLeuO strain (SEA7115) grown in EZ-Rich at 30°C is shown. Samples were assayed for σE activity throughout the growth of the culture by monitoring β-galactosidase activity produced from the rpoH P3-lacZ reporter. Data represents the average σE activity compiled from three independent experiments. Error bars represent the standard deviation between the individual data sets. σE activity in the mutant strains is shown relative to that in the wild-type strain which is normalized to 1.
**LeuO-mediated filamentation is unconnected to regulation of \( \sigma^E \) activity**

Overexpression of *leuO* led to both a decrease in \( \sigma^E \) activity and filamentation. To determine whether these effects were related, we analyzed whether filamentation alone could result in low \( \sigma^E \) activity. Since *damX* overexpression can independently lead to filamentation (data not shown, [145]), we measured \( \sigma^E \) activity in strains that overexpress *damX* (pDamX) in an otherwise wild-type background. Our data indicates that \( \sigma^E \) activity is high, not low, in pDamX strains suggesting that filamentation in general does not lead to low \( \sigma^E \) activity (Figure 3-18). We conclude that the effect of LeuO on cell division is unrelated to the effect of LeuO on \( \sigma^E \) activity.

**Figure 3-18:** Filamentation does not lead to low \( \sigma^E \) activity. \( \sigma^E \) activity (B) in the wild type strain with control plasmid (SEA7146), pDamX strain (SEA7127) and pLeuO strain (SEA7074) is shown along with the corresponding growth curve (A). Strains were grown in EZ-Rich at 30°C and treated with IPTG at OD\(_{600}\) of 0.2 (indicated by arrow). Data compiled from two independent experiments is shown.
Discussion

Our analysis of an *E. coli* transposon insertion mutant that exhibited reduced $\sigma^E$ activity led to the identification of LeuO as a regulator of OMPs in *E. coli*. I demonstrated in this chapter that LeuO lowers expression of major OMPs such as OmpC, as well as other minor OMPs like OmpL and OmpX. We also discovered a putative role of LeuO in the cell division process. Overexpression of *dksA* or inactivation of *damX* is sufficient to counter the effects of *leuO* overexpression. The LeuO-mediated repression of $\sigma^E$ activity appears to be independent from the LeuO-mediated cell division defects. The exact mechanism by which LeuO regulates these processes was not determined.

**The effect of *leuO* overexpression varies with the type of sigma factor**

Our data suggests that LeuO regulates $\sigma^E$ activity indirectly via its effects on OMP expression. LeuO has been shown to lower $\sigma^S$ levels via its inhibitory effects on *dsrA* expression [141]. Since *leuO* overexpression does not affect $\sigma^E$ levels, the analysis suggests that the mechanism by which LeuO negatively regulates two alternative sigma factors is different. In contrast to the observed negative effects with $\sigma^E$ and $\sigma^S$, LeuO increases $\sigma^H$ activity, indicating that *leuO* overexpression follows a different regulatory pathway for each affected sigma factor.

**LeuO regulates OMP expression**

The outer membrane represents the first line of defense bacteria have against all external agents. OMPs regulate the passage of various ions, solutes and antibiotics through the cell membrane. Apart from these well-studied roles, some OMPs like OmpX have also been implicated for their
role in regulating cell surface contact [146]. Others like OmpL allow solutes to pass into and out of the periplasm but have not been studied in further detail [147]. Regulating levels of these porins is important not only during exponential growth, but also for survival during adverse conditions such as low nutrient availability or exposure to antibiotics. It makes sense therefore, that multiple factors are engaged in maintaining OMP levels. LeuO directly increases $ompS1$ and $ompS2$ expression and inhibits $ompX$ expression in *Salmonella typhi* [136,137]. Whether LeuO directly inhibits $ompX$ expression in *E. coli* is not known. In the case of OmpC and OmpL, LeuO may directly or indirectly regulate expression. For OmpC, it is possible that LeuO may increase expression of an inhibitor of $ompC$ expression and thereby lead to lower porin expression. A genomic SELEX screening study for identification of LeuO-binding sites in the *E. coli* genome identified the small RNA, $micC$, as a potential target of LeuO. LeuO possibly binds upstream of $micC$, which inhibits $ompC$ expression post-transcriptionally [142].

**LeuO and DksA regulate cell division**

Cell division is an energy-consuming process and as such, must be regulated for efficient use of resources. In this chapter, I showed that overexpression of *leuO* results in filamentation; a defect that can be overcome by overexpression of *dksA*. Overexpression of *dksA* can, by an unknown mechanism, alleviate filamentation defects caused by a variety of mutations. One hypothesis suggests that overexpression of *dksA* enhances transcription of *rpoS* (the gene encoding $\sigma^5$) which transcribes the cell shape regulatory factor, BolA. BolA in turn, restores cell shape and division [100]. Overexpression of *leuO* inhibits *rpoS* translation, which may in turn lead to lower *bolA* expression. Overexpression of *bolA* results in round morphology in stationary phase [148] but whether *bolA* underexpression results in filamentation has not been determined. While the
BolA-mediated pathway is one possible mechanism to explain regulation of cell division by DksA and LeuO; it does not rule out other possible mechanisms by which these regulators may affect cell division.

**Role of DamX**

DamX accumulates at the septal ring when overexpressed and possibly leads to filamentation by inhibiting FtsQ and/or by excluding FtsN from the septal ring [144,149]. Since inactivation of damX negates the effect of leuO overexpression; it is possible that LeuO increases damX expression which leads to the observed inhibition of cell division. Alternately, loss of damX may overcome the inhibitory effects of LeuO via a bypass mechanism. DamX is present even in non-dividing cells, suggesting that it plays a role in addition to regulating cell division [150]. Our work has shown that ΔdamX by itself does not affect σE activity, but can restore σE activity in a leuO overexpression background (Figure 3-16). Additionally, overexpression of damX leads to high σE activity (Figure 3-18), suggesting that DamX regulates σE activity via an unknown mechanism.

**Physiological relevance of the role of LeuO**

Levels of OMPs are regulated during exponential phase by a variety of regulators that act at all levels of gene expression. Levels of LeuO increase when cells are starved for phosphate or enter stationary phase [133,134,140,151]. While loss of leuO does not lead to noticeable phenotypes in otherwise wild type cells growing in rich media; LeuO is required for amino acid starved cells to resume growth following addition of amino acid in the media [134]. This suggests that even if LeuO is generally non-essential, it plays a special role during specific starvation scenarios. The
LeuO-mediated inhibition of OMP expression may be part of a proactive measure by the cells to quickly overcome problems in the cell membrane and to promote survival. By regulating OMP levels, LeuO can also fine-tune $\sigma^E$ activity, which is essential; since very high $\sigma^E$ activity can be detrimental to cell survival [162,163]. Finally, LeuO may act as an accessory regulator of cell division to ensure that cellular resources are conserved during unfavorable conditions.
Materials and Methods

Media, strains and plasmids

Strains used in this work are listed in Table 3-1. Cultures were grown in Luria-Bertani (LB) broth or in EZ-Rich at 30°C with shaking unless otherwise noted. EZ-Rich purchased from Teknova is a commercial preparation of rich defined media containing glucose, amino acids, vitamins, phosphate and MOPS buffer. The ΔleuO::kan and ΔdamX::kan alleles were obtained from the Keio collection ([109]) and moved by P1 transduction into SEA001 by standard P1 transduction ([107]). Antibiotics were used at the following concentrations: ampicillin (amp) 100μg/ml, kanamycin (kan) 15μg/ml, tetracycline (tet) 10μg/ml and chloramphenicol (chlor) 20μg/ml. For unmarked strains, antibiotic resistance genes were removed by FLP recombinase method ([108]). Plasmids carrying leuO, damX and hns under the control of an IPTG -inducible promoter were obtained from the ASKA collection ([152]) and transformed into host strains as indicated. For all overexpression studies, 1mM IPTG (final concentration) was used except for the genetic screen for isolating mutations that overcome the effect of leuO overexpression, where plates contained 5 μM IPTG.

Construction of Transposon library

Detailed description of transposon library construction is described in [153]. Mini-Tn10 derivative 108 carrying kanamycin resistance and containing the cis-ATS transposase was used to construct the library. The derivative consists of a plac-UV5 promoter which can direct transcription from the transposon into the downstream genes leading to higher expression of the adjacent chromosomal gene(s). The transposon delivery λ phage lysate was prepared in the C600
permissive bacterial host. Recipient cells were grown overnight in TBMM (Trypton BI broth with maltose and magnesium) and incubated with various quantities of phage for 15 minutes at 37°C to give an MOI of between 0.1 to 1 phage/cell. The infected culture was then grown for 1 hour at 37°C to allow expression of antibiotic resistance and then plated on selective media. To identify particular genes of interest within a specific pathway, we pooled ~10,000 successful transductants and prepared P1lysate on the pooled colonies to allow a modest level of redundancy. The P1 lysate carrying a mixture of insertions now represents the ‘transposon library’ that was used for transduction into a secondary host (MG1655) to identify mutants of interest.

**Mapping the site of the transposon insertion**

To map the transposon insertion site, a two-step PCR method was used as described in *Yao-Guang and Ning, 1998* as follows: A first PCR amplification was done in 20µL final volume using 0.25 mM dNTPs, 100ng of chromosomal DNA, 0.25µM of primer LacUV51 (5’-CACTCATTAGGCACCCCAGG-3’) or NK1327R (5’-ATCAGAGATTTTGAGACACAACG-3’) hybridizing on each end of the mini Tn10::kan, and 2µM of random primer RandomPrim1 (5’-GGCCACGCGTCGACTAGTACNNNNNNNNGCTGG-3’ where N=A, C, G or T). Six amplification cycles (94°C for 30sec, 59°C for 30”, 72°C for 2min 30sec) were performed to enrich in single stranded DNA template corresponding to the end to the Tn10::kan and the sequence adjacent to the mini transposon. Two amplification cycles (94°C for 30sec, 25°C for 2min ramping to 72°C in 2min, 72°C for 2min 30sec) were then performed to allow hybridization and amplification using the degenerate primer. Finally, 25 cycles (94°C 30sec, 59°C 30”, 72°C 2min 30sec) were performed to specifically amplify the fragments amplified...
during the previous two cycles. The PCR product was diluted 1:2.5 in sterile water and 1µl of this dilution was used as template for a second PCR in 25µl final volume using 0.25 mM dNTPs, 0.25µM of primer LacUV52 (5’-CCGGCTCGTATGATGTGTGG-3’) or NK1327R2 (5’-TTACAGGATCCGGGATCATATG-3’) hybridizing on each end of the mini Tn10::kan between the hybridization sites of LacUV51 or NK1327R and the end of the Tn10::kan, and 2µM of primer RevPrim2 (5’-GGCCACCGTCGACTAGTAC-3’) homologous to the non-degenerated part of RandomPrim1. After amplification for 35 cycles (94°C for 30sec, 59°C for 30sec, 72°C for 2min 30sec), the fragments were purified using QIAquick spin columns and the DNA concentration estimated by agarose gel electrophoresis and measure of the A260nm. The fragments were sequenced using primer Tn10-1 (5’-GACAAGATGTATCCACTTAATGA-3’) hybridizing in the inverted sequence of the mini Tn10::kan. Analysis of the sequence revealed the chromosomal location where the transposon was inserted.

**β-galactosidase assays**

Overnight cultures were diluted to an OD$_{600}$ of 0.025 and grown with shaking in a gyratory water bath at 30°C or 37°C as indicated. Samples (0.5ml or 0.2ml as indicated in the Figures) were collected throughout the growth curve. The β-galactosidase activity of each sample was measured by the standard assay [107] and is expressed as the OD$_{420}$ of reaction mixture divided by the reaction time (o-nitrophenol min$^{-1}$). The data are presented as differential rate plots in which β-galactosidase activity in the sample is plotted versus the optical density (OD$_{600}$) of the sample. β-galactosidase activity (o-nitrophenol min$^{-1}$) per reaction volume is plotted, rather than standard Miller units (o-nitrophenol min$^{-1}$ OD$_{600}$), therefore the slope of the curve at each time point indicates the change in β-galactosidase activity with increased cell number. The plots
illustrate how $\sigma^E$ activity changes throughout the growth curve, in recovery from stationary phase, exponential phase and re-entry into stationary phase. A complete explanation of differential rate plots is presented in [24]. For measurements of $\sigma^E$ activity as a function of growth rate, $\sigma^E$ activity was determined by the slope of the line on a differential rate plot either during exponential phase or stationary phase as indicated. Experiments were repeated a minimum of three times with independent cultures.

**Outer membrane preparations and quantification**

Outer membrane was separated from other membrane fractions by differential solubility in sarcosyl as described in Mecsas et al., 1993 [5]. Wild type and strains overexpressing leuO were grown in LB medium at 30°C and 10 ml culture volume harvested at OD$_{600}$ of 0.5 and 1. Cell pellets were resuspended in 500 μl of 100 mM Tris-HCL (pH 8), 10 mM EDTA and digested with 100 μg/ml lysozyme. After 10 minutes on ice, spheroplasts were treated with 10 mM MgCl$_2$, 50 μg/ml DNase I and subject to 3 freeze-thaw cycles. After 30 minutes centrifugation at 15,000 g, the supernatant was removed. The membrane-containing pellet was washed with 500 μl of 20 mM NaPO$_4$, pH 7. The inner membrane fraction was separated from the outer membrane fraction by treating with 100 μl of 0.5% sarcosyl in 20 mM NaPO$_4$ and incubating for 30 minutes at room temperature. After removing the soluble inner membrane by centrifugation at 15,000 g for 10 minutes, the outer membrane pellet was washed again with 100 μl of 0.5% sarcosyl in 20 mM NaPO$_4$. The final pellet was resuspended in SDS sample buffer and boiled for 5 minutes. Outer membrane proteins were analyzed on a 6M urea 12% polyacrylamide-SDS gel. Blank-corrected protein values were quantified via ImageQuant 5.2 (Molecular Dynamics). Reported values represent the average of extracts from three independent data sets.
**Western blotting**

Whole-cell extracts were prepared as described in Gentry *et al.*, 1993 [60]. Briefly, cells were lysed in protein sample buffer; proteins precipitated with acetone, and re-suspended in 2% SDS. Protein concentrations were determined using the BCA Protein Assay (Pierce). 10 μl from each sample was loaded onto 12% polyacrylamide-SDS gels and transferred to HybondTM-P, PVDF membrane (GE Healthcare). Bands containing σE were detected by probing the blots with rabbit polyclonal antibodies raised against σE (gift from CA Gross), then with alkaline phosphatase conjugated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). The secondary antibody was visualized with the ECF reagent from GE Healthcare according to the manufacturer’s instructions. Blots were scanned using Typhoon 8600 Imager in fluorescence mode.
<table>
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| **Plasmids** |
| **pLeuO** | *leuO* in pCA24N, *chlorR* | ASKA library collection |
| **pDamX** | *damX* in pCA24N, *chlorR* | ASKA library collection |
| **pH-NS** | *hns* in pCA24N, *chlorR* | ASKA library collection |
| **pACYC184** | plasmid control, *chlorR* | Ades lab collection |
| **pTrc99a** | vector in pBR322, *ampR* | Pharmacia |
| **pOmpC** | *ompC* in pBR322, *ampR* | SEA45122 |
| **pINdksA** | *lpp-lac* promoter fused to *dksA*, *ampR* | pRLG6333, [47] |
Regulation of $\sigma^E$ by RseA in response to cell envelope-related stress is a well-characterized process that has been studied for many years. A detailed description of this process is reviewed in [102] and [114]. In contrast, we are still at the early stages of understanding regulation of $\sigma^E$ activity during nutrient limitation-induced stress. Previous work showed that when cells enter stationary phase, $\sigma^E$ activity increases in a ppGpp and DksA-dependent manner [24, 56]. Before in vitro transcription experiments showed that ppGpp and DksA could directly increase $E\sigma^E$ activity [56], the indirect model was the only proposed mechanism of regulation of alternative sigma factors by ppGpp and DksA [103]. The model suggested that during starvation, ppGpp and DksA repress $\sigma^{70}$-dependent transcription at ribosomal $rrn$ operons, tipping the competition for available core RNAP in favor of alternative sigma factors. However, there is little evidence that ppGpp and DksA directly affect association/dissociation of sigma factors to/from core RNAP. The kinetic model instead offers a better alternative to how ppGpp and DksA stimulate transcription at some promoters and repress transcription at others. However, all known models are based on the concerted action of ppGpp and DksA to regulate transcription. I show in Chapter 2 that while ppGpp and DksA can have a complementary effect on $\sigma^E$ activity, ppGpp can also regulate $\sigma^E$ activity in the absence of DksA.

This observation led to the hypothesis that “Regulation of $\sigma^E$ activity by the global stress response factor, ppGpp, follows a different regulation pattern depending on the nutrient limitation condition and potentially involves cofactors other than DksA.”
In accordance with the predictions of the hypothesis, I found that whether DksA and ppGpp work together to regulate transcription by Eσ^E varies with the nutrient limitation condition. The increase in σ^E activity during amino acid starvation and entry into stationary phase in LB requires the concerted action of both ppGpp and DksA, whereas during phosphate starvation and entry into stationary phase in EZ-Rich, σ^E activity increases in a DksA-independent, ppGpp-dependent manner (Figure 2-1).

My data also suggests that regulation of σ^E during phosphate starvation is unconnected to the PhoB-dependent response to phosphate starvation and is also independent of RseA, the anti-sigma factor that directly regulates σ^E. Additionally, higher levels of ppGpp observed in vivo in the absence of DksA (Figure 2-11) may contribute to the observed DksA-independent regulation of σ^E during phosphate starvation. However, since high ppGpp levels alone have little effect on Eσ^E activity in vitro, I conclude that an unknown co-regulator assists ppGpp-dependent regulation of σ^E activity in vivo during phosphate starvation.

I also found that σ^70-dependent transcription at ribosomal operons, specifically at rrnB P1 and rrnE, is not ppGpp-dependent during phosphate starvation (Figure 2-5, 2-6, 2-7). To the best of our knowledge, an increase in ppGpp levels, without a concomitant decrease in transcription at rrn genes had not been observed. The data suggest that if ppGpp does not play a role in regulating transcription at rrn, then the simplistic models based on the inhibitory action of ppGpp at rrn promoters cannot sufficiently explain the increase in transcription by σ^E and σ^S during phosphate starvation. Since ppGpp is necessary for regulating σ^E, and DksA is necessary
for regulating $\sigma^{70}$ during phosphate starvation, it seems that ppGpp and DksA (alone or with additional unknown co-regulators) regulate different targets during phosphate starvation.

While determining the contribution of RelA and SpoT in regulation of $\sigma^E$ activity, I found that accumulation of ppGpp is not exclusively SpoT-mediated as has been suggested [86]. In Chapter 2, I show that ppGpp levels can be regulated even when SpoT hydrolase is inactive, suggesting that alternate mechanisms for maintaining ppGpp levels exist. I hypothesize that in addition to SpoT hydrolase, ppGpp levels may be determined by regulation of the ppGpp synthase (either RelA or SpoT) or that other phosphatases may compensate for SpoT hydrolase (Figure 2-14).

My research has shown that existing models of regulation by ppGpp and DksA are based on an assumption, which when tested, failed to adequately explain the regulation of not only $\sigma^E$, but also $\sigma^S$ and $\sigma^{70}$ activity during phosphate starvation. This strongly suggests that even though ppGpp is central to long-term survival in *E. coli*, additional regulation players and mechanisms during various types of starvations need to be considered.

My data are consistent with the predictions of the main hypothesis noted earlier. I have shown that ppGpp does not regulate $\sigma^E$ and other sigma factors in the same way during all starvations, adding specificity to its regulation. My data suggests that additional pathways for regulation of $\sigma^E$ activity exist, leading to the following questions: What is the other factor(s) involved in regulation of $\sigma^E$ activity during phosphate starvation? How are ppGpp levels maintained in the cell in the absence of SpoT hydrolase? What signals RelA/SpoT to synthesize ppGpp during
phosphate starvation? How do ppGpp, DksA and iNTP affect the kinetics of \( \sigma^E \)-dependent transcription? I discuss in the proceeding sections the various strategies that can be employed to answer some of these questions. I also hypothesize possible contributions of iNTP and poly P in regulating \( \sigma^E \) activity.

To identify additional regulators of \( \sigma^E \) activity that may be part of the ppGpp -dependent, DksA - independent pathway, I examined the effect of known homologs of DksA. When none of the known homologs were found to compensate for DksA, a genetic screen based approach was employed. In Chapter 3, I describe how transduction using a transposon library that integrates randomly into \( E. coli \) genome allowed identification of the DNA -binding transcriptional regulator LeuO as a potential regulator of \( \sigma^E \) activity (Figure 3-1). LeuO did not compensate for DksA to regulate \( \sigma^E \) activity but overexpression of \( leuO \) did independently lower \( \sigma^E \) activity (Figure 3-2, 3-3, 3-4). I found that overexpression of \( leuO \) inhibited expression of outer membrane porins OmpC, OmpX and OmpL (Figure 3-10). Underexpression of OMPs stabilizes RseA; thus LeuO indirectly regulates \( \sigma^E \) activity. Independent of its role in regulating \( \sigma^E \) activity, overexpression of \( leuO \) led to filamentation (Figure 3-12). Inactivation of \( damX \) or overexpression of \( dksA \) was sufficient to overcome the effects of \( leuO \) overexpression (Figure 3-14, 3-16). I propose that LeuO regulates membrane porins and cell division during unfavorable conditions when survival takes precedence over continual growth.

**Role of DksA and ppGpp during phosphate starvation**

During amino acid starvation RelA synthesizes large amounts of ppGpp and co-regulation by DksA is essential for the increase in \( \sigma^E \) activity. In addition, ppGpp and DksA together directly
increase $E\sigma^E$ activity \emph{in vitro}. Contrary to the concerted mode of action, my data shows that during phosphate starvation, $\sigma^E$ activity is enhanced by ppGpp in the absence of DksA, while $\sigma^{70}$ activity at $rmbP$ is repressed by DksA in the absence of ppGpp. Both effectors modulate gene expression changes but they do not regulate the same target during phosphate starvation. Such segregation of function may be an effective way to rapidly mount a response to limiting phosphate by using different regulators to simultaneously target transcription by various sigma factors.

**Role of iNTP**

My data indicates that iNTP levels decrease when cells enter phosphate starvation (Figure 2-11). In addition to ppGpp and DksA, levels of iNTP can strongly influence transcription at $rmbP$ promoters. During rapid growth in rich media such as LB, high iNTP concentrations bind and stabilize open promoter complexes at $rmb$ promoters, allowing transcription to proceed. During starvation, iNTP availability is low, resulting in lower rates of successful transcription initiation. ppGpp and DksA further destabilize the open promoter complex following nutrient downshift. Consequently, transcription fails to initiate at the ribosomal promoters [71].

While regulation of $\sigma^{70}$ activity at ribosomal promoters has been studied for decades, regulation of $\sigma^E$ activity by ppGpp and DksA is still at a preliminary stage. It is theorized that $\sigma^E$-dependent promoters form stable open promoter complexes unlike those at $rmb$ promoters. If that is true, then it may further explain how less than optimal iNTP levels during starvation are sufficient to drive initiation at $\sigma^E$-dependent promoters forward. Previously, we have measured the stability of open complex formation at $rpoH$ and $rybB$ promoters in the presence of ppGpp after
adding heparin which competes with DNA for binding to RNAP [56]. Both promoters formed relatively stable complexes with $\sigma_E^E$. ppGpp destabilized open complex stability by $\sim 2.5$-fold.

Since ppGpp destabilizes open complexes on all promoters examined to date, and yet clearly enhance transcription at many promoters, it has been proposed that destabilizing open complex actually helps to move transcription forward at certain promoters by promoting promoter escape [69]. The decision to drive the reaction forward depends on properties of individual promoters [38,48,53,55,106]. The kinetics of transcription initiation for $\sigma_E^E$ have not yet been studied and this study mandates an in-depth analysis on the mechanics of transcription initiation and the influence of ppGpp and iNTP in regulation of $\sigma_E^E$-dependent promoters. We plan on performing transcription at three $\sigma_E^E$-dependent promoters: rpoH P3 (Promoter P3 of the heat shock sigma factor $\sigma^H$), rybB (promoter for the small RNA RybB) and fkpA (promoter for peptidyl-prolyl isomerase and chaperone). By only varying iNTP levels and keeping the concentration of other NTPs constant; we will be able to determine the influence of iNTP on promoting $\sigma_E^E$-dependent transcription. Performing this assay at different $\sigma_E^E$-dependent promoters might also assist in determining the promoter properties that influence transcription initiation.

**Identification of additional regulators of $\sigma_E^E$ activity**

The data from chapter 2 strongly suggests than in addition to ppGpp, an additional factor contributes to the increase in $\sigma_E^E$ activity during phosphate starvation. I discuss next, possible candidates as well as other approaches for finding the DksA-compensatory factor.
**Polyphosphate**

Apart from global regulatory molecules like ppGpp, bacteria are equipped with other specialized factors which can also influence transcription during specific stresses. A variety of starvations and environmental stresses lead to large accumulations of a phosphate polyanion known as polyphosphate (poly P) in the cell. Poly P is a chain of ten or many hundreds of phosphate residues linked by high-energy phosphoanhydride bonds. In the cell, poly P is synthesized by the polyphosphate kinase (PPK) enzyme through the transfer of a phosphoryl group from ATP to poly P polymer. Exopolyphosphatase (PPX) degrades poly P by removing orthophosphate processively [154]. (p)ppGpp can block PPX activity, thus resulting in poly P accumulation. Thus, (p)ppGpp and poly P metabolism are linked during phosphate starvation (Figure 4-1). In addition to serving as a reservoir of Pi, poly P is also theorized to have regulatory roles since its levels increase during amino acid starvation, phosphate starvation, nitrogen limitation and osmotic stresses [155-159]. Poly P readily interacts with basic proteins (such as histones) and with basic domains of proteins (such as RNAP). Thus, like ppGpp, Poly P may directly affect the activity of RNAP. Since a Δppk mutant, severely deficient in poly P, fails to express σS [160], it is possible that polyP may also influence other alternative sigma factors such as σE. In addition, mutants that fail to accumulate poly P do not survive more than a few days in stationary phase [158]. Whether this is because of a failure to induce the sigma factors σS and/or σE that adapt the cells to adjust to nutritional downshifts or both is as yet unknown. The possibility that poly P may regulate σE is intriguing and remains to be determined.
Figure 4-1: Accumulation of polyphosphate in *E. coli*. Synthesis of poly P by PPK or inhibition of PPX leads to accumulation of polyP. (p)ppGpp generated by RelA and/or SpoT in response to starvation inhibits PPX activity leading to further poly P accumulation. SpoT degrades ppGpp to GDP, which can be recycled to GTP. Image from [157].

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Alternate strategies

If polyP does not play a role in regulation of σ^E activity, then assuming that the factor binds to core RNAP like ppGpp and DksA, we will attempt to purify Eσ^E by immunoprecipitation experiments. An *E. coli* strain with his-tagged σ^E as the sole source of σ^E will be constructed. Eσ^E will be purified by affinity chromatography from cell extracts made during phosphate starvation. Co-purifying proteins present during phosphate starvation will be candidate regulators to be pursued further. Alternately, the genetic screen described in Chapter 3 can be employed to identify additional mutants that exhibit low σ^E activity. The kanamycin resistant transposon library that integrates at random sites within the *E. coli* genome has been used successfully to identify LeuO which indirect regulates σ^E activity. We can modify the screen to find genes that are involved in regulating σ^E activity during phosphate starvation by using a ΔphoBR strain that
would lead to phosphate starvation on plates. Mutants that are affected in the stress-signaling pathway will require RseA for modulating $\sigma^E$ activity and will be eliminated by examining their effect in a $\Delta rseA$ strain.

**Levels of DksA during phosphate starvation**

The data presented in Chapter 2 indicates that regulation of $\sigma^{70}$ at the $rrnB$ P1 promoter is DksA-dependent and ppGpp-independent during phosphate starvation. I suggest that the presence of DksA and low iNTP levels are sufficient to overcome the need for ppGpp to regulate transcription at $rrnB$ P1. Saturating amounts of ppGpp alone are unable to significantly inhibit transcription at $rrnB$ P1; however, in the presence of low amounts of DksA, greater inhibition is observed. More importantly, higher amounts of DksA alone can mimic the synergistic effect of ppGpp and DksA to inhibit transcription from $rrnB$ P1 *in vitro* [53]. One question that needs to be addressed is whether levels of DksA increase during phosphate starvation. This possibility can be easily determined by western blot with DksA-specific antibodies on cell lysates collected at various time points before and after phosphate starvation.

**Regulation of ppGpp levels**

*E. coli* RelA has two domains; a ppGpp synthase domain that synthesizes large amounts of ppGpp in response to amino acid starvation and an inactive hydrolase domain. SpoT shares sequence similarity with RelA but has two functional domains - a weak synthase domain and a strong hydrolase domain which is thought to regulate ppGpp levels in the cell [105]. Most starvation conditions, except amino acid starvation, are thought to lead to ppGpp accumulation via inhibition of SpoT hydrolase activity. I showed in Chapter 2 that ppGpp accumulated even if
SpoT synthase was inactive, suggesting that SpoT synthase plays a minor role (if any) in accumulation of ppGpp in the cell during phosphate starvation. Secondly, ppGpp levels were regulated even if SpoT hydrolase was inactive during phosphate starvation, which is in contrast to the model that inhibition of SpoT hydrolase leads to ppGpp accumulation. This observation leads to many questions. Most importantly, what is the signal that leads to ppGpp synthesis during phosphate starvation? To answer this question we need to determine whether accumulation of ppGpp depends on regulation of ppGpp synthase or by inhibition of ppGpp degradation (either by SpoT hydrolase or other compensatory phosphatases).

To determine if SpoT hydrolase is indeed involved in regulating ppGpp levels, we could induce active relA′ from plasmid pALS13 in a ΔrelA spoT synthase- hydrolase+ strain. This plasmid overexpresses a truncated variant of relA upon IPTG induction which synthesizes ppGpp without associating with ribosomes. We can measure ppGpp levels in this strain at specific time points following phosphate starvation and compare them with ppGpp levels in a ΔrelA ΔspoT (ppGpp0) strain that also synthesizes ppGpp from active relA′ from pALS13. A difference in ppGpp levels in the two strains may indicate that SpoT hydrolase is involved in regulating ppGpp levels.

To determine whether ppGpp synthase is being regulated, we would need to first determine the signal/regulator that leads to ppGpp synthesis during phosphate starvation. We could use the transposon library (described in Chapter 3) to set up a screen to find mutants that fail to induce ppGpp. The host strain would carry a σE-dependent reporter and mutants of interest would be chosen based on their color on X-Gal plates (pale blue color would indicate low σE activity compared to deeper blue mutants in which σE activity is higher). Alternately, if the signal is not a
regulatory molecule, but instead a conformation change within RelA or SpoT induced by high/low levels of phosphate in the cytoplasm, computational modeling studies may be used to determine whether phosphate can bind to RelA/SpoT. Determining the signal that leads to ppGpp synthesis during phosphate starvation, would allow us to hypothesize and determine whether the ppGpp synthases are regulated instead of the hydrolase.

**LeuO regulates outer membrane porins**

It is clear from Chapter 3 that low $\sigma^E$ activity is a downstream effect of LeuO -mediated repression of porin expression. I found that overexpression of *leuO* lowers levels of OmpC, OmpX and OmpL (Figure 3-10). Whether LeuO directly or indirectly regulates OMP expression in *E. coli* is not known. In *Salmonella*, LeuO binds directly to the 5’ intergenic region of *ompX* and inhibits its expression [137]. Whether the same mechanism of regulation exists for regulation of *E. coli ompX* is not known.

A recent genomic SELEX screen that identified binding sites for LeuO in the *E. coli* genome showed that LeuO may bind upstream of *micC* [142]. I propose that LeuO leads to an increase in *micC* expression. High levels of MicC have been shown to result in ~60% reduction in *ompC* expression [124]. MicC base pairs with a sequence located upstream of the ribosome binding site in the *ompC* mRNA, thereby repressing *ompC* translation by inhibiting binding of the 30S ribosomal subunit to *ompC* mRNA [124].

Whether LeuO directly controls expression of *micC* and *ompX* in *E. coli* has not been biochemically determined. DNA footprinting assays on the *ompX* and *micC* template with LeuO
will help to authenticate the DNA-protein interaction. Additionally, the effect of LeuO on expression of *micC* and *ompX* can be determined via *in vitro* transcription assays.

**LeuO affects stability of RseA**

Changes in RseA stability correlate with changes in $\sigma^E$ activity. In unstressed cells, the half-life of RseA is $\sim$8 minutes. Stresses such as heat shock reduce the half-life of RseA to $\sim$2 minutes, leading to increased $\sigma^E$ activity. Stress shutoff (return to optimum growth temperature) stabilizes RseA, with the half-life increasing by $>50$ minutes. The half-life of RseA then slowly returns to that observed in unstressed cells concurrent with the return of $\sigma^E$ activity to basal levels [120]. I hypothesize that LeuO-mediated underexpression of porins stabilizes RseA and thereby reduces $\sigma^E$ activity. Pulse-chase immunoprecipitation experiments on cells that overexpress *leuO* to determine RseA half-life would determine whether RseA is indeed more stable in the presence of high levels of LeuO.

**LeuO inhibits cell division**

The finding that cells that overexpress *leuO* are filamentous suggests a role of LeuO in mediating cell division. I found that deletion of *damX* restored normal cell division in cells that overexpress LeuO, suggesting that LeuO may be involved in enhancing *damX* expression. This is likely because DamX is an established cell division inhibitor and cells that overexpress *damX* look phenotypically like cells that overexpress *leuO*. DamX was not identified as a potential target of LeuO in a genome wide SELEX screen for LeuO-binding targets, hence whether LeuO actually targets *damX* or if DamX, via an unrelated pathway, counters LeuO-mediated inhibition is not known. Unraveling cell division regulation pathways can be a complicated process; however, we
can determine whether LeuO binds DamX (gel shifts) and increases *damX* expression (*in vitro* transcription) to either confirm or to eliminate the role of LeuO in modulating cell division via DamX.

**Conclusions**

Bacteria such as *E. coli* are found in a wide variety of environments in nature. Their survival during unfavorable circumstances depends on their ability to utilize various regulatory molecules quickly and efficiently to modulate gene expression. The aim of this study was to determine how the alternative sigma factor, σ^E^, is regulated during specific starvation regimes. I started my thesis work with the knowledge that the RNAP-binding factors, ppGpp and DksA, enhance σ^E^ activity during entry into stationary phase in LB. The finding that transcription at ribosomal operons is regulated by DksA in a ppGpp -independent manner during phosphate starvation indicated that the indirect model does not sufficiently explain the increase in σ^E^ activity during starvation. Focusing my work on phosphate starvation, I found that σ^E^ activity is regulated by ppGpp along with additional unknown co-regulators in the absence of DksA. Moreover, I assert that established models of transcription, such as the indirect model cannot be generalized to fit regulation of various sigma factors during all starvation conditions. Regulation is a complex process and each type of starvation differs in its intensity and its effect on bacterial physiology. Hence, some of the key players remain the same (for example, RseA functions as a direct regulator to control the availability of σ^E^ under all conditions), but there are other regulators that are either not required (such as DksA) or are replaced by other regulators (such as NTPs, polyP or other factors) depending on the starvation conditions.
Additionally, I discovered that the transcription factor LeuO, modulates porin expression and cell division in *E. coli*. Based on its chromosomal location, LeuO was originally thought to function as an accessory regulator of amino acid biosynthesis. Work from other labs has recently shown that LeuO regulates a variety of processes including recovery from amino acid starvation in *E. coli* and expression of virulence-related porin genes in *Salmonella*. I found that while loss of LeuO had no discernible effect on $\sigma^E$ activity, overexpression lowered $\sigma^E$ activity. The inhibition was found to be indirect; via the effect of LeuO in repressing expression of the porins OmpC, OmpX and OmpL. LeuO thus adds to the repertoire of porin modulators in *E. coli*. The data suggests that bacteria have evolved multiple pathways to control levels of membrane components. The integrity of the cell envelope is a crucial determinant of the health of the bacterium and can additionally affect $\sigma^E$ activity, a process that is fine-tuned by LeuO. Finally, the studies described here show that regulation of $\sigma^E$ is a complex process, and that while the key players RseA, ppGpp and DksA remain the same, their requirement and their involvement varies depending on the type of stress. Other accessory regulators such as LeuO indirectly affect $\sigma^E$ activity by modulating porin expression.
Appendix

Toxicity of high $\sigma^E$ during stationary phase

Introduction

High $\sigma^E$ activity has been proposed to lead to lysis of viable $E. coli$ cells in stationary phase cultures, suggesting that $\sigma^E$ may control a programmed cell death response, in addition to protecting the cell from envelope stress [162,163]. To examine this phenomenon in greater detail, a wild-type strain and a $\Delta rseA$ strain were grown for 10 days at 30°C in rich media with aeration. In addition to monitoring the optical density of these cultures, $\sigma^E$ activity from the chromosomally-encoded $\sigma^E$-dependent $rpoH$ P3-$lacZ$ fusion was regularly assayed. Aliquots of the cultures were plated on medium containing lactose and two indicators of LacZ activity, Terazolium chloride (TTC) and X-gal (LTX plates). Cells with high levels of $lacZ$ expression, such as the $\Delta rseA$ strain, form deep blue colonies in the presence of X-gal, white colonies in the presence of TTC, and blue colonies in the presence of TTC-Xgal. Cells with intermediate levels of $\sigma^E$ activity, such as the wild type strain, form medium blue colonies on X-gal, pink colonies on TTC, and purple colonies on TTC-Xgal. Cells with lower levels of $\sigma^E$ activity form pale blue or white colonies on X-gal, red colonies on TTC and red colonies on the combination. Using this method, we found that after 2 days in stationary phase, the population of $\Delta rseA$ cells with high $\sigma^E$ activity started to decrease and was replaced by colonies with lower $\sigma^E$ activity. Within 5 to 6 days in stationary phase, mutants with low $\sigma^E$ activity took over the entire culture. These data suggest that population of cells with lower $\sigma^E$ activity are more adept at long-term survival compared to the parental $\Delta rseA$ strain. Similar results were obtained with two different $\Delta rseA$ strains, each carrying a different tightly linked marker, $nadB::Tn10$ or $yfiC::kan$. Mutants with
low $\sigma^E$ activity were analyzed further. Most mutations mapped to the \textit{rpoE} locus and included point mutations in the \textit{rpoE} gene and IS5 insertions in the \textit{rpoE} promoter. Two mutations were found to map elsewhere in the genome, to the \textit{nsrR} gene and the \textit{rpoBC} region. Our work suggests that constant high $\sigma^E$ activity is lethal and precise regulation of $\sigma^E$ activity is essential for bacteria to survive in stationary phase.
Results

High $\sigma^E$ activity is lethal in stationary phase.

To measure the toxicity of high $\sigma^E$ activity, wild type strain and $\Delta rseA$ $nadB::Tn10$ mutant were cultured for a period of 10 days in rich media at 30°C (see Materials and Methods). After 2 to 3 days in stationary phase, the optical density (OD$_{600}$) of the $\Delta rseA$ culture dramatically decreased compared to that of the wild-type strain. Numerous translucent/lysed cells were visible under the microscope for the $\Delta rseA$ culture as well, but the total viable population remained similar to that of the wild type strain (Figure A-1). When the viable population was determined on LTX plates, we found that the population of cells with a high $\sigma^E$ activity (originally blue on LTX plates) started to decrease after 2 days and completely disappeared after 5 to 6 days in stationary phase. On days 7 and later, analysis of thousands of colonies isolated from several independent growths failed to detect any bacterial colonies with high $\sigma^E$ activity. Thus, bacteria with a high $\sigma^E$ activity could not survive more than few days in stationary phase. While the population with a high $\sigma^E$ activity decreased over time, we observed that it was efficiently replaced by a population of bacteria with lower $\sigma^E$ activity (deep red colonies on LTX). Similar observations were made with a variant $\Delta rseA$ $yfic::kan$ strain, revealing that the $nadB::Tn10$ and $yfic::kan$ markers were not involved in the observed phenotype.
Figure A-1. Survival of wild type (SEA6003) and ΔrseA (SEA6462) strain in stationary phase in LB. Wild type and ΔrseA were cultured for 10 days in LB at 30°C and monitored for changes in absorbance (A) and cell viability (B). The decrease in optical density (OD$_{600}$) and corresponding CFU/ml is shown for wild type (diamonds) and two independent cultures of ΔrseA (squares and crosses).

Isolation of spontaneous mutations that lower σ$^E$ activity

Parallel ΔrseA cultures were grown to stationary phase in rich media. Mutants were selected on LTX plates and reisolated which allowed identification of mutants with varying degrees of σ$^E$ activity (Figure A-2).
To understand the extent of the decrease in $\sigma^E$ activity needed for a $\Delta rseA$ strain to survive in the stationary phase, we analyzed and characterized all the mutations that lowered $\sigma^E$ activity. The analysis also allowed us to make a ‘mutant library’ which could potentially be used to further understand $\sigma^E$ regulation. The original study was carried out in the $\Delta rseA \ nadB::Tn10$ (SEA2000) strain. We obtained numerous suppressors with low $\sigma^E$ activity, of which, some mapped to point mutations in $rpoE$ (Table A-1). We isolated many others that lost tetracycline resistance which is part of the Tn10 insertion in $nadB$, the gene immediately upstream of $rpoE$ (data not shown). Attempts to amplify the $rpoE$ promoter region in these mutants failed, suggesting that imprecise excision of the Tn10 disrupted the promoter leading to low expression of the adjacent $rpoE$ operon. We repeated the experiment in a different parental background $\Delta rseA \ Delta yfiC::kan$ (SEA6462) to avoid problems associated with transposon excision. All mutants that arose from cultures of the $\Delta rseA \ Delta yfiC::kan$ (SEA6462) strain retained kanamycin resistance and did not have major disruptions of the $rpoE$ genomic region (Table A-1). The mutants with low $\sigma^E$ activity consisted of mutations that mapped to the $rpoE$ locus and included point mutations in the $rpoE$ gene and IS5 insertions in the $rpoE$ promoter. Two mutations were found to map elsewhere in the genome, to the $nsrR$ gene and the $rpoBC$ region (Table A-1). The precise mutation within $nsrR$ and $rpoBC$ has not been mapped. Several of the mutations were recovered multiple times from independent cultures.
Analysis of spontaneous mutants

To determine if the mutations alter the expression and/or activity of $\sigma^E$, the steady-state protein level and $\sigma^E$ activity were measured for each mutant. $\beta$-galactosidase assays determined that with the exception of two mutants, in the remaining 15 mutants, $\sigma^E$ activity was reduced to levels similar or below that found in wild type strain (Table A-1) Steady state $\sigma^E$ levels were assayed by probing western blots with a $\sigma^E$-specific polyclonal antibody. In all cases the steady state level of $\sigma^E$ was lower than that in the parental $\Delta rseA$ strain (Table A-1). Reductions in steady state level of $\sigma^E$ could have arisen from two major sources. First, the mutations could alter the structure of the $\sigma^E$ protein, making it more susceptible to proteolysis. Second, transcription of the rpoE gene could be affected, leading to lower rate of expression. Since rpoE has two promoters, one transcribed by $\sigma^70$ and the other by $\sigma^E$, decreases in the activity and stability of $\sigma^E$ will further reduce transcription from the $\sigma^E$-dependent promoter and consequently affect $\sigma^E$ levels.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mutation</th>
<th>$\sigma^E$ levels</th>
<th>$\sigma^E$ activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>SEA6003</td>
<td>wild type</td>
<td>1</td>
<td>1.00 ±0.04</td>
</tr>
<tr>
<td>SEA6462</td>
<td>$\Delta rseA yfic::kan$</td>
<td>1.81</td>
<td>13.24 ±0.17</td>
</tr>
<tr>
<td>SEA2000</td>
<td>$\Delta rseA nadB::Tn10$</td>
<td>1.79</td>
<td>13.03 ±0.12</td>
</tr>
<tr>
<td>SEA6526</td>
<td>$\Delta rseA L10P:rpoE$</td>
<td>Not detected</td>
<td>1.71 ±0.01</td>
</tr>
<tr>
<td>SEA7035</td>
<td>$\Delta rseA A60P:rpoE$</td>
<td>Not detected</td>
<td>0.58 ±0.02</td>
</tr>
<tr>
<td>SEA7008</td>
<td>$\Delta rseA E126K:rpoE$</td>
<td>0.64</td>
<td>0.68 ±0.02</td>
</tr>
<tr>
<td>SEA6542</td>
<td>$\Delta rseA L127Q:rpoE$</td>
<td>0.80</td>
<td>0.33 ±0.02</td>
</tr>
<tr>
<td>SEA6544</td>
<td>$\Delta rseA R149Q:rpoE$</td>
<td>0.23</td>
<td>0.67 ±0.01</td>
</tr>
<tr>
<td>SEA7036</td>
<td>$\Delta rseA S155I:rpoE$</td>
<td>0.12</td>
<td>1.15 ±0.04</td>
</tr>
<tr>
<td>SEA6543</td>
<td>$\Delta rseA V170M:rpoE$</td>
<td>0.18</td>
<td>0.82 ±0.01</td>
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<tr>
<td>SEA6525</td>
<td>$\Delta rseA R171C:rpoE$</td>
<td>0.24</td>
<td>0.33 ±0.01</td>
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<td>$\Delta rseA S172A:rpoE$</td>
<td>0.72</td>
<td>0.28 ±0.01</td>
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<tr>
<td>SEA6663</td>
<td>$\Delta rseA R173C:rpoE$</td>
<td>0.52</td>
<td>0.18 ±0.01</td>
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<tr>
<td>SEA7037</td>
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<td>0.73</td>
<td>0.68 ±0.01</td>
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<td>SEA7012</td>
<td>$\Delta rseA R176Q:rpoE$</td>
<td>0.84</td>
<td>0.32 ±0.01</td>
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<td>SEA6666</td>
<td>$\Delta rseA A177V:rpoE$</td>
<td>0.21</td>
<td>0.64 ±0.03</td>
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<tr>
<td>SEA7007</td>
<td>$\Delta rseA RNAP: \beta/\beta'$</td>
<td>0.76</td>
<td>0.72 ±0.01</td>
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<tr>
<td>SEA6657</td>
<td>$\Delta rseA nsrR$</td>
<td>0.58</td>
<td>6.56 ±0.17</td>
</tr>
</tbody>
</table>
Table A-1:
(a) Total protein extracted from the wild type, ΔrseA and various mutant strains was subject to western blotting analysis to determine the steady-state level of σE.
(b) σE activity in the wild type and mutant strains was determined by measuring β-galactosidase activity produced from the σE-dependent rpoH P3-lacZ reporter. σE activity is reported from the slope of the line which represents the accumulation of β-galactosidase as a function of the growth of the culture.
For (a) and (b), levels in the mutant strains are shown relative to that in the wild-type strain which is normalized to 1. Results are the average of two independent experiments.

Classification of mutations
All of the residues mutated within rpoE were conserved and identical among σE homologues. In σ70 homologues, the equivalent residues were conserved and identical to that of σE (L127, R149 and R171), conserved and similar to that of σE (L10, A60, S155, V170 and S172), conserved but different than that of σE (R173, F175, R176 and A177) or not conserved (E126) [18].
Comparison of σE amount and activity in each mutant revealed three classes of mutations (Figure A-3).
**Figure A-3:** Nature and effect of the mutations in suppressors that survive prolonged stationary phase.

(A) Repartition of the mutations in suppressors selected from three independent growths of SEA6462 (ΔrseA yfiC::kan) (1, 2, 3) and in other suppressors selected from SEA2000 (ΔrseA nadB::Tn10) and SEA6462 in rich media. *rpoE* sequence was divided into four regions (regions 1 to 4) as described in Campbell et al., 2003.

(B) *σ*^E^ amount and activity in various spontaneous suppressors. Triangles represent *rpoE* mutations affecting residues binding the -35 DNA element. Squares represent *rpoE* mutations that potentially affect *σ*^E^ structure. Diamonds represent *rpoE* mutations that affect surface exposed residues. Circles represent mutations not in *rpoE*. Results are the average of two independent repeats and the standard deviation fell within the experimental standard deviation indicated by the shaded region “a”. The detection limit is indicated by the vertical line seen outside the X- and Y-axis. The amount of *σ*^E^ in the wild type corresponds to that of free *σ*^E^ and RseA-bound *σ*^E^, while amounts in the mutants (which are all ΔrseA) correspond to free *σ*^E^.

To roughly estimate the theoretical amount of RNA polymerase-available free *σ*^E^ in the wild type (theor. wt), the amount of free *σ*^E^ in the ΔrseA strain was divided by the difference between the *σ*^E^ activities in the ΔrseA strain and in the wild type strain. The theoretical amount obtained intersects the line representing the activity per amount of free *σ*^E^ in non-suppressor strains (diagonal line in shaded region “a” joining the point for ΔrseA strains to the origin of the X-axis and Y-axis). The horizontal line represents *σ*^E^ activity as measured in the wild type strain. The region “b” represents mutants affected in *σ*^E^ activity but not *σ*^E^ amount. The region “c” indicates mutants affected in both *σ*^E^ activity and *σ*^E^ amount. Mutants that are not in regions “b” or “c” are affected in *σ*^E^ amount rather than activity.
Class A mutations

Certain mutations were found to lower $\sigma^E$ activity without significantly lowering the amount of $\sigma^E$. These included the mutations E126K, L127Q, S172A, R173C, F175S, R176Q and rpoBC (Figure A-3, Table A-1). The slight decrease in the amount of $\sigma^E$ compared to that in the parental $\Delta rseA$ strain could be explained by lower rate of transcription of the $\sigma^E$-dependent P2 promoter of rpoE. This possibility has not been experimentally verified. Four of these mutations (S172A, R173C, F175S and R176Q) lowered $\sigma^E$ activity by targeting residues directly involved in $\sigma^E$ interaction with the DNA -35 element [164]. The L127 residue faces the last $\alpha$-helix of $\sigma^E$ and is involved in binding to the -35 DNA element while the E126 residue is surface-exposed [18]. The L127Q and E126K mutations therefore, probably destabilize the DNA binding domain of $\sigma^E$ without affecting the amount of $\sigma^E$. Not surprisingly, the mutation that mapped to the rpoBC genes encoding the $\beta$ and $\beta'$ subunits of the RNA polymerase involved in binding the sigma factors also is a Class A mutant [17]. $\sigma^E$ activity but not growth was affected in the rpoBC mutation, suggesting that the mutation might affect $\sigma^E$ binding to RNA polymerase. Alternately, the mutation may alter activity of E$\sigma^E$ with little or no effect on RNA polymerase bound or binding to other sigma factors such as $\sigma^{70}$.

Class B mutations

Class B mutations affected both the amount and activity of $\sigma^E$. This class includes the rpoE mutants R149Q, R171C and A177V. The R149Q and R171C mutations target residues that directly interact with the DNA -35 element [164]. The equivalent of R149 in $\sigma^{70}$ is also involved in the interaction with the RNA polymerase [17]. In addition to their effect on $\sigma^E$ activity, R149Q and R171C mutations also decreased $\sigma^E$ amount (Table A-1). These mutations may destabilize $\sigma^E$.
structure and increase its proteolytic degradation, or they could affect the \textit{rpoE} mRNA stability. The A177 residue is not located within a \(\sigma^E\) domain known for its role in binding to the DNA or the RNA polymerase. Mutation of this residue might affect \(\sigma^E\) structure and decrease both the activity and stability of the protein.

Class C mutations

Mutations from the class C affected \(\sigma^E\) amount but did not lower its activity (Table A-1). This class includes mutations L10P, A60P, S155I, V170M and \textit{nsrR}. L10 and V170 residues are not located within \(\sigma^E\) regions known for their role in binding to the DNA or the RNA polymerase, although insertion of a proline within the first \(\alpha\)-helix of \(\sigma^E\) (mutation L10P) likely affects \(\sigma^E\) structure. S155 makes strong interactions with E158 and thus could be important for correct orientation of residues Y156 and E157 which contact several nucleotides of the -35 DNA element [164]. Still, it is surprising that lower amounts of \(\sigma^E\) in L10P, S155I and V170M mutants do not lead to lower activity. We hypothesize that the mutated \(\sigma^E\) proteins might, for example, fold into two distinct structures, one being fully active and the other being actively degraded. Alternatively, the CTG to CCG (L10P), AGC to ATC (S155I) and GTG to ATG (V170M) mutations might not affect the protein but the \textit{rpoE} mRNA stability, causing lesser amounts of fully active \(\sigma^E\) to be produced. Mutation A60P (GCG to CCG) targets a surface-exposed residue and may affect \(\sigma^E\) at a post-transcriptional or translational level. Further investigation of these mutations is required to fully understand their effect on \(\sigma^E\). NsrR negatively regulates \(\sigma^E\) in an \textit{RseA} -independent manner [129]. While the exact mechanism of action is still unknown, we hypothesize that the \textit{nsrR} mutant we obtained in our study directly or indirectly affects \(\sigma^E\) at a post-transcriptional or translational level.
Summary of mutant analysis

Altogether, 6 of the 12 residues of $\sigma^E$ known to directly interact with the -35 element of the $\sigma^E$ target promoters (residues R149, R171, S172, R173, F175 and R176; [164] were found to be mutated in our collection of mutants that lower $\sigma^E$ activity. Only 1 of the 13 residues known to affect the $\sigma^{70}$-core RNA polymerase interaction was found to be mutated in our collection (residue R149 which interacts with the DNA -35 element [17], suggesting that most mutations were specific to $\sigma^E$.

Conclusion

Extracytoplasmic function (ECF) alternative sigma factors are key components of the bacterial response to extracellular stress, and their deletion or overproduction alters pathogenicity in numerous bacteria. However, little is known about the effects of high $\sigma^E$ activity on the cell. Here we show that constant high $\sigma^E$ activity in *E. coli* caused a lethal phenotype in stationary phase. After few days in stationary phase, the dying population of ΔrseA cells was efficiently replaced by a growing subpopulation with a GASP (Growth Advantage in Stationary Phase) phenotype caused by spontaneous mutations lowering $\sigma^E$ activity. This work suggests that precise regulation of $\sigma^E$ is essential for survival during unfavorable circumstances.
Materials and Methods

Strains, media and growth conditions

*E. coli* strains used in this work are described in table 1. All strains are derivatives of SEA6003 (wild-type MG1655 with a single-copy $\sigma^E$-dependent *rpoH* P3-*lacZ* reporter). LB (Beckton, Dickinson and Co.) or EZ-Rich (MOPS rich defined medium, Teknova) was used for culturing strains. P1 transductants were selected by addition of kanamycin (15µg/mL) or tetracycline (10µg/mL), both purchased from Calbiochem. Lactose TTC plates (2,3,5-triphenyltetrazolium chloride (TTC purchased from Research Organics, Cleveland, OH) supplemented with 50 µg/mL X-gal (Gold BioTechnology, St Louis, MO) were used to differentiate colonies with high (blue) or low (red) $\sigma^E$ activity. Because studies on $\sigma^E$ can involve mutants with a thermosensitivity at high temperatures, all studies were performed at 30°C.

*rpoE* PCR amplifications and sequencing

*rpoE* and its promoter were amplified with primers rpoEnadBmid (5’-CCGCTACCGATAATCAACACG-3’) and rseB1R (5’-GCTGATGAATGACAGCTCG-3’) using Taq polymerase (New England Biolabs, Inc, Ipswitch, MA) and a MJ Research PTC-100 thermocycler (Waltham, MA). Amplified fragment were purified using QIAquick spin columns (QIAGEN, Valencia, CA) and sequenced at the Nucleic Acid Facility (Huck Institute, The Pennsylvania State University, PA) using primers rpoE1 (5’-ACTGGTAGTGCCTATCAGC-3’) and rpoE1R (5’-TTTACAGCAATCCGATACAGCC-3’).
Mapping spontaneous mutations

The transposon library described in Chapter 3 was used to identify spontaneous mutants that did not map to \( rpoE \). The P1 library lysate was transduced into the strain carrying the unidentified spontaneous mutation. Kanamycin-resistant colonies reverting the phenotype of interest caused by the spontaneous mutation were selected. P1\(_{\text{vir}}\) lysate was prepared on a suppressor and used to transduce the kanamycin marker and the reverted mutation back into the original mutant. Percentage of co-transduction were calculated and the mini Tn10::kan insertion site determined using a protocol derived from Yao-Guang and Ning, 1998 as described in Chapter 3.

Stationary phase lethality studies

Strains were isolated on LTX for initial analysis of \( \sigma^E \) activity. One isolated colony from each strain with the correct \( \sigma^E \) activity (e.g. blue colony for the \( \Delta rseA \) strain SEA6462) was grown few hours at 30°C in 1mL LB until the absorbance reached ~1.0. Cells were then diluted to an OD\(_{600}\) of 0.1 (dilution -1) in fresh LB, and then further diluted 1:10 in fresh LB up to the dilution -5. Five microliters of the dilution -5 were used to inoculate 4 mL (in 15mL glass vials) of LB or EZ-Rich. Low cell count in the inoculates (<50 bacteria) lowered the probability that a suppressor with a low \( \sigma^E \) activity was already present at the beginning of the growth of strains with an initial high \( \sigma^E \) activity. Cultures were then incubated at 30°C for 10 days with constant aeration. Each day, the absorbance of the cultures was measured, and the colony forming units (CFU) determined on LTX plates. For the \( \Delta rseA \) strain, the proportion of bacteria which reverted the high \( \sigma^E \) activity was assessed by determining the proportion of red colonies growing on the LTX.
β-galactosidase assays

β-galactosidase assays were performed as described in Chapters 2 and 3.

Western blot hybridization

Cells were grown in LB up to an OD₆₀₀ of 0.8 and whole-cell extracts were prepared as described in Gentry et al., 1993 [60]. Proteins extracted from 0.16 O.D. units of cells were separated on SDS-PAGE 10% polyacrylamide gels. Samples were transferred to Hybond™-P, PVDF transfer membrane (Amersham Biosciences) using EBU-202 Mini-Electrophoretic Blotting System (C.B.S. Scientific Co.) and probed with anti-σ₇₀ rabbit polyclonal antibodies along with anti-RpoB antibody for control, then with ECL anti-rabbit IgG Horseradish peroxidase from donkey (Amersham Biosciences). The secondary antibody was visualized with the ECL reagent (Amersham Biosciences) according to the manufacturer's recommendations and exposed on HyBlot CL™ Autoradiography film (Denville Scientific Inc.). Films were scanned using an Epson Perfection 3170 Photo Scanner (Seiko Epson Corp) and the amount of proteins was quantified using the ImageQuant 5.2 software (Molecular Dynamics). The intensity of each band was normalized to that of the wild-type.


Vita
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Academic Training
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- Gopalkrishnan, S¹ and Ades, SE. The LysR-type regulator LeuO is a novel regulator of outer membrane porin expression and of cell division in *Escherichia coli* (manuscript in preparation)
- Gopalkrishnan, S¹, Nicoloff, H¹ and Ades, SE. Toxicity and Membrane defects caused by high σ^E activity in *Escherichia coli* (manuscript in preparation)

Presentations
- Gopalkrishnan, S¹ and Ades, SE. Regulation of the *Escherichia coli* alternative sigma factor σ^E, during nutrient limitation. *Gordon Research Conference on Microbial Stress Responses*, Mount Holyoke, MA. July 2012 (poster presentation)
- Gopalkrishnan, S¹ and Ades, SE. Regulation of the *Escherichia coli* alternative sigma factor σ^E, during nutrient limitation. *American Society for Microbiology General Meeting*, San Francisco, CA. June 2012 (poster presentation)
- Gopalkrishnan, S¹ and Ades, SE. Regulation of the *Escherichia coli* alternative sigma factor σ^E, during nutrient limitation. *Molecular Genetics of Bacteria and Phages Meeting*, Madison, WI. August 2011 (poster presentation)
- Gopalkrishnan, S¹ and Ades, SE. Regulation of the *Escherichia coli* alternative sigma factor σ^E, during starvation. *Pittsburgh Bacterial Meeting*, Duquesne University, Pittsburgh. March 2010 (oral presentation)
- Gopalkrishnan, S¹ and Ades, SE. Regulation of the *Escherichia coli* alternative sigma factor σ^E, during nutrient limitation. *American Society for Microbiology Allegheny Branch Meeting*, Juniata College, Huntingdon, PA. November 2009 (oral presentation)

Scholarships and Awards
- First place, Oral Presentation, *Pittsburgh Bacterial Meeting*, Pittsburgh, PA, March 2010
- Second place, Outstanding Teaching Assistant, BMB department, The Pennsylvania State University, Spring - Fall 2009
- Homer F. Braddock Fellowship, Eberly College of Science, The Pennsylvania State University, Fall 2007 - 2009

Teaching Experience
- Teaching Assistant, Elementary Microbiology Laboratory, Penn State, Fall 2010, Fall 2011
- Guest Lecturer, Molecular and Cell Biology II, Penn State, Spring 2011
- Teaching Assistant, Human Biology Lectures, Penn State, Spring 2009, Fall 2009, Spring 2010
- Teaching Assistant, Laboratory in Protein Purification and Enzymology, Penn State, Fall 2008