The Importance of a Fifth Element in Transcription

Transcriptional Discrimination in *Escherichia coli*

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Cover picture:
Large filamentous cells and minicells
of *Escherichia coli* Δ*minB* ppGpp^0 strain.

To My Loved Ones

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ABSTRACT

Upon growth arrest of the bacterium *Escherichia coli*, RNA polymerase ($E\sigma^{70}$) is redirected from transcribing genes encoding the protein synthesizing system (PSS) to those involved in maintenance and stress resistance. The small nucleotide ppGpp, which is directly targeting $E\sigma^{70}$, is a key regulatory molecule required for this response. In addition, the protein DksA has been hypothesized to be required for the regulatory function of ppGpp. Cells defective in the synthesis of either ppGpp or DksA do not undergo a shift in gene expression upon growth arrest and are deficient in maintenance-related activities. The underlying mechanism of how regulation of gene expression is exerted by ppGpp and DksA is the main focus of this thesis.

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On top of a direct role of ppGpp in regulating gene expression, ppGpp can potentially affect gene regulation passively by modulating the levels of free $E\sigma^{70}$ in the cell. Using a mini-cell approach, I demonstrate an inverse correlation between the levels of ppGpp and free $E\sigma^{70}$. Thus, I hypothesize that ppGpp contribute to the redistribution of $E\sigma^{70}$ at promoters during growth arrest also by decreasing free $E\sigma^{70}$ levels, which would negatively affect promoters requiring high levels of $E\sigma^{70}$ for efficient transcription, such as those of the PSS.

List of Papers

This thesis is based on the following papers, which will be referred to in the text by their roman numerals;

I. **Identical, Independent, and Opposing Roles of ppGpp and DksA in *Escherichia coli*.**
Magnusson, L. U. [1)], B. Gummesson [1)], P. Joksimović, A. Farewell and T. Nyström
J. Bacteriol. (2007) **189**(14): 5193-5202.
[1)] contributed equally

II. **Increased RNA polymerase availability directs resources towards growth at the expense of maintenance.**
Gummesson, B. [1)], L. U. Magnusson [1)], M. Lovmar, K. Kvint, Ö. Persson, M. Ballesteros, A. Farewell and T. Nyström
EMBO J (2009) **28**(15): 2209-2219.
[1)] contributed equally

III. **-Text removed from public version-**
Gummesson, B., M. Lovmar and T. Nyström
Manuscript (2011)

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1. AIMS OF THIS THESIS

The main aim of this thesis was to elucidate what promoter elements are required for positive regulation by ppGpp/DksA and whether the same elements are required for responding to alterations in the levels of free RNA polymerase, using the *uspA* promoter of *E. coli* as a model promoter.

Aims in detail;

- Elucidate the proposed co-regulatory role of ppGpp and DksA in the activation of promoters during stringency *in vivo* (Paper I).

- Investigate similarities and differences between the relaxed phenotype ($ppGpp^0$) and the phenotype of a strain lacking DksA (Paper I).

- Test models for "passive" control of gene expression by assessing how growth-related promoters and stress response/maintenance promoters respond to artificial alterations in the levels of $E\sigma^{70}$ (Paper II).

- Elucidate whether ppGpp might modulate the levels of free $E\sigma^{70}$ (Paper II).

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2. INTRODUCTION

2.1 – *Escherichia coli* - the model organism

Escherichia coli belong to the *Enterobacteriaceae*, a family of rod-shaped and gram-negative bacteria. It is a large and diverse family that includes many genera, including known pathogens, such as *Salmonella*, *Shigella*, and *Yersinia*. Although most strains of *E. coli* are not classified as pathogens, some cause diarrheal disease while others colonize the urinary tract of humans and cause cystitis, or more severely, pyelonephritis (Johnson 1991; Mead and Griffin 1998). However, in their normal habitat, in the intestines of mammals and birds, they provide plentiful benefits for their host, including the production of vitamin K_2 (Bentley and Meganathan 1982), and protection against pathogenic microbes by producing gene-encoded antimicrobial peptides (Pons *et al.* 2002). In the generally anaerobic environment of the intestine, where the facultative *E. coli* is outnumbered 1.000-fold by anaerobic microbes, they manage to successfully compete and colonize a special niche (Slonczewski and Foster 2009). Colonization is aided by its ability to rapidly adapt to changing environmental conditions and its inherent potential of rapid growth and division. *E. coli cells* proliferate exponentially as long as essential nutrients are present (feast) or accumulation of growth-inhibiting products do not reach toxic levels. When the nutrients are depleted (famine), growth ceases and the bacteria will enter what is called stationary phase. Stationary phase cells of *E. coli* are metabolically active and, like many other bacterial species, *E. coli* cells express genes in this phase that increase the chances of survival throughout periods of non-growth.

Because of their inability to control their environment, *E. coli* cells need to change their gene expression upon changing environmental conditions. Thus, *E. coli* has evolved the capacity to regulate vast sets of genes quickly, accurately, and coordinately. Strategies for such coordinated gene regulation display many levels of organization. A milestone in the studies of synchronized gene control was the discovery of the operon by Jacob and Monod (Jacob *et al.* 1960). In this paper, the authors introduce the concept of an operon, in which several genes are co-transcribed as single transcriptional units controlled by a single "operator" – a site receiving regulatory signals. Ever since this seminal paper, the concept of coordinated gene expression has been increasingly expanded into the organization

of genes and operons into regulons (coordinated control of several genes/operons by a common regulator), modulons (coordinated control of several regulons), and stimulons (genes/operons/regulons/modulons responding to the same stimulus) (Neidhardt 1996). The *E. coli* cell harbors an ample variety of regulators, including proteinaceous transcription factors acting as activators or repressors, sigma factors and small non-proteinaceous modifiers such as nucleotides (ppGpp) to transduce the stimuli from the environment to genes and direct their expression pattern to appropriate cellular responses. Many of these regulators control the activity of genes primarily at the level of transcription and especially at the level of transcription initiation. This thesis focuses on two such regulators; DksA and ppGpp, two molecules that are the effectors of the stringent response, a response adapting the bacteria for periods of famine.

2.2 – Transcription

In *E. coli* the genetic material is arranged as a single circular molecule of DNA - a haploid chromosome. The DNA molecule of *E. coli* is approximately 1 mm long while the average size of an *E. coli* cell is 0.5 μm in width and 2 μm in length. To fit into this tiny space, the DNA is extraordinarily condensed but folded in such way that the appropriate DNA sequences are accessible for replication and transcription (Neidhardt *et al.* 1990). The condensation of the bacterial chromosome into a nucleoid is due to the action of supercoiling and nucleoid-associated proteins, NAPs, e.g. H-NS, IHF and Fis (Browning *et al.* 2010); see section 2.3.

The over 4 million base pairs that constitute the *E. coli* chromosomal genome were completely sequenced in 1997 (Blattner *et al.* 1997). The sequence contains 4464 annotated genes (Riley *et al.* 2006) each flanked by two distinct regulatory regions; the promoter and the transcriptional terminator. The promoter dictates where transcription is initiated whereas the transcriptional terminator determines where it ends. The sequence in between are transcribed into either messenger RNA (mRNA) or non-coding RNAs (rRNA, tRNA). The mRNAs are then further translated into proteins (Crick 1970). Transcription of DNA into mRNA, rRNA, and tRNA is accomplished by the enzyme called RNA polymerase.

2.2.1 – RNA polymerase and σ factors

In eukaryotes there are three different RNA polymerases that catalyze DNA-dependent RNA synthesis with specialized functions. Moreover, eukaryotes utilize different compartments for transcription and translation. In contrast, there is only one RNA polymerase responsible for all transcription in prokaryotes.

The bacterial RNA polymerase exists in two principal forms: core (E) and holoenzyme (Eσ). The core enzyme consists of five subunits: two copies of α in a dimer, one copy each of β, β' and ω ($\alpha_2\beta\beta'\omega$, with a molecular mass of about 400 kDa). The core enzyme harbors the catalytic activity required for transcribing the DNA template. The crystal structure of the bacterial RNA polymerase (Zhang *et al.* 1999; Vassylyev *et al.* 2002), suggests that the structure and function of the core enzyme has been evolutionarily conserved from bacteria to humans (Ebright 2000). The overall structure of the bacterial core RNA polymerase resembles a "crab claw", where the β and β' subunits form the two pincers of the crab claw and encompasses the active site of the enzyme. The α-dimer (specifically, the amino terminal domain [α-ATD]) assists in the assembly of β and β' (Zhang *et al.* 1999) and the carboxyl terminal domain (α-CTD) facilitate additional promoter contacts (Gourse *et al.* 2000; Ross *et al.* 2001), see figure 1. The role of the ω subunit is not fully understood since the enzymatic activity of the enzyme lacking ω is unaffected *in vivo* and the gene encoding ω is not essential (Mathew and Chatterji 2006). Nevertheless, it has been hypothesized that ω is required in RNA polymerase assembly (Mukherjee *et al.* 1999; Minakhin *et al.* 2001) and β' maintenance (Mathew *et al.* 2005), as well as in sensitizing RNA polymerase to small regulatory molecules (Vrentas *et al.* 2005).

The core enzyme cannot initiate promoter dependent transcription on its own. To initiate transcription, the core enzyme must bind a specificity factor, σ, that recognizes specific promoter sequences. Thus, the holoenzyme (Eσ) contains the core subunits and one σ factor (see figure 1). The σ subunit provides specificity for the correct initiation of transcription at different regulons and of different sets of genes needed at different conditions. In *E. coli,* there are seven different species of σ subunits; σ^{70}, σ^{S} (also called σ^{38}), σ^{H} (σ^{32}), σ^{E} (σ^{24}), σ^{N} (σ^{54}), σ^{F} (σ^{28}), and σ^{FecI} (Burgess *et al.* 1969; Ishihama 2000; Gruber and Gross 2003). Most of the genes needed for growth and proliferation (housekeeping genes) are transcribed by the

holoenzyme containing σ^{70} (Gross *et al.* 1998), while the stationary phase specific σ factor σ^{S} are responsible for transcribing genes in growth-arrested cells (reviewed in Hengge-Aronis 2002). σ^{S} activation is triggered by a complete stop of growth or partial reduction in growth rate generated by a variety of stress signals (Loewen *et al.* 1998). The other five alternative σ factors respond to specific stresses to control specialized regulons. The set of genes that are induced upon conditions causing the accumulation of unfolded proteins in the cytoplasm are dependent on the heat shock σ factor σ^{H} (Jenkins *et al.* 1991; Gross 1996). Stresses that disrupt the folding of outer membrane and periplasmic proteins results in the activation of σ^{E} (Ades 2008) while the holoenzyme containing σ^{N} is primarily involved in the transcription of genes in nitrogen scavenging (Merrick 1993) and some stress responses (Shingler 1996). σ^{F} is needed for the expression of chemotaxis genes and genes expressed late during the development of flagella (Chilcott and Hughes 2000). The σ^{FecI} sigma factor is required for the transcription of genes involved in ferric citrate transport (Braun *et al.* 2003). There is an apparent correlation between the number of alternative σ factors a bacterial species possesses and how varied the environments are that the species typically inhabit. For example, *Mycoplasma genitalium* that live in a relatively constant host environment contains only one σ factor, while the soil-dwelling *Streptomyces coelicolor* has 63 σ factors (Mittenhuber 2002; Gruber and Gross 2003; Ghosh *et al.* 2010). For organisms containing several sigma factors, an important means of altering the transcription pattern is to switch σ species associated to the core enzyme and the mechanism to do so is highly regulated.

The pool size of active σ factors in the cell is partly regulated by anti-σ factors that form a complex with its cognate σ subunit and thereby titrate it away from the transcription apparatus (reviewed in Helmann 1999). The anti-σ factor for σ^{70}, Rsd (regulator of sigma D), was first described by Jishage and Ishihama (1999). Rsd levels are increasingly elevated with declining growth rates and are suggested to aid alternative σ factors in binding RNA polymerase core under conditions of limited growth (Jishage and Ishihama 1999).

Another example of an anti-σ factor is FlgM, which inactivates σ^F until the hook and basal body of the flagella is assembled. Once the flagellum is fully assembled, FlgM is secreted by the flagellar export system, making σ^F available for the late genes in flagella assembly (Chilcott and Hughes 2000).

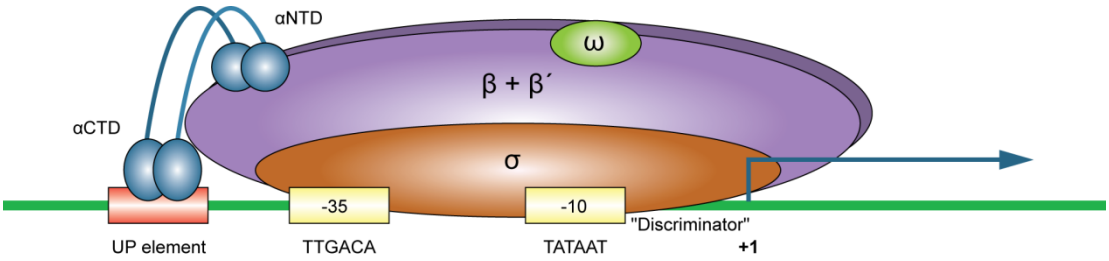

Figure 1. The different subunits of RNA polymerase (α_2, β, β', ω and σ) and the interactions with different promoter elements. The consensus sequence for σ^{70} is shown. Adapted from (Browning and Busby 2004).

2.2.2 – The promoter

The σ factor makes direct promoter contacts and the sequence of a promoter therefore dictates how efficient the promoter is in recruiting the RNA polymerase holoenzyme. There are three DNA sequence elements that define the bacterial core promoter (see figure 1). The two major determinants for RNA polymerase holoenzyme recognition of the promoter are the -10 hexamer, also called the Pribnow box (Pribnow 1975), and the -35 hexamer, located approximately 10 and 35 base pairs upstream from transcriptional initiation site (+1), respectively (Siebenlist 1979; Murakami *et al.* 2002). The consensus sequence of these two elements confers transcriptional dependency on their cognate σ factor. However, some σ factors are somewhat promiscuous and exhibit overlapping promoter specificities (Gaal *et al.* 2001). The stretch of nucleotides between the -10 hexamer and -35 hexamer is the third core promoter element. In *E. coli*, it has a characteristic length of 17 ± 1 base pairs (Siebenlist *et al.* 1980) but sequence-wise display a low conservation among promoters (O'Neill 1989). Nevertheless, bases at position -14 and -15 (denoted the extended -10 element) in the spacer region have been shown to be important for regulation of some promoters and interact with the RNA polymerase holoenzyme (Burr *et al.* 2000; Murakami *et al.* 2002).

Some promoters contains AT-rich regions immediately upstream the -35 element that has been considered a fourth promoter element. Such upstream sequence regions (USR), also called UP elements, can interact with the α-CTD of RNA polymerase (Estrem *et al.* 1998; Ross *et al.* 2001). Thus, the UP-element provide some promoters with a third region for RNA polymerase interactions and such an extra interaction is argued to make the promoter more efficient in polymerase recruitment (Hirvonen *et al.* 2001; Ross and Gourse 2005).

A fifth additional promoter element is called the discriminator, which is a G+C-rich motif of around 8 bases located immediately downstream the -10 hexamer, preceding the transcriptional initiation site (Travers 1980). The G+C rich discriminators are found in promoters driving rRNA expression and act as targets for negative control by the alarmone ppGpp during a stringent response (Zacharias *et al.* 1989; Zacharias *et al.* 1991) -Text removed from public version-

2.2.3 – The transcription cycle

Transcription is a cyclic process that can be divided into four major steps – RNA polymerase recognition of the promoter and binding (I), initiation (II), elongation (III), and termination (IV).

(I) As pointed out above, the first step of transcription is dependent on the recognition of the promoter sequence by the RNA polymerase holoenzyme (Eσ). The enzyme is believed to find the target promoter by a facilitated diffusion-like mechanism; a combination of the enzyme sliding along the DNA molecule and intersegment transfer between adjacent DNA segments (Berg *et al.* 1981; Elf *et al.* 2007). The binding of RNA polymerase holoenzyme to the promoter includes the σ factor interaction with the core promoter elements as well as interactions between the β, β' and α-dimer to any important flanking regions of the specific promoter (Record Jr *et al.* 1996). In this initial stage of enzyme-promoter complex formation, the promoter DNA base pairs remain undisrupted.

(II) When RNA polymerase (R) finds the promoter (P), it binds (facilitated by hydrogen bonds) and forms a RNA polymerase-DNA closed complex (RP_C). At this step, it has been shown that RNA polymerase protects the promoter DNA approximately from position -54 to -6 relative to the transcriptional start site (Mecsas *et al.* 1991; Murakami and Darst 2003). The complex undergoes several isomerization steps to form a strand-separated open complex (RP_O). The DNA melts around the -10 hexamer and extends past the transcription start site (+1) where the initiating nucleotide (iNTP) can pair, forming the initiation complex (RP_{init}). From most promoters, RNA polymerase synthesizes and releases short RNAs (2-8 nucleotides); a general phenomenon described as abortive transcription. During abortive initiation the σ subunit remain attached to the enzyme-promoter complex (Wagner 2000). Finally, when an initial productive transcript has been synthesized (>8 nucleotides), the RNA polymerase loses contact with the promoter and the transcribing RNA polymerase moves away from the promoter (promoter clearance) and forms an elongating complex (EC + P) (Record Jr *et al.* 1996) (see figure 2). The σ subunit is most often released upon promoter clearance, but the release can occur as late as after 100 nucleotides of transcription (Reppas *et al.* 2006). Additionally, a recent study suggest that σ^{70} can remain attached to the transcription apparatus, up to 700 nucleotides away from the transcriptional start site, aided by promoter-proximal σ^{70}-dependent pause elements (Deighan *et al.* 2011). Once RNA polymerase is cleared from the promoter, the core promoter elements are exposed and available for another round of the initiation phase.

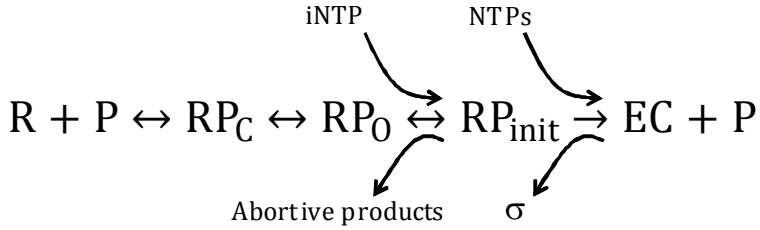

Figure 2. General view of the different steps in transcription initiation. RNA polymerase holoenzyme (R) binds to the promoter (P) and forms a closed complex (RP_C). After several isomerization steps, the DNA opens up and forms a promoter-enzyme open complex (RP_O). Addition of iNTP drives the reaction forward to the initiating complex (RP_{init}) and subsequently the elongating complex (EC) clear the promoter and the σ factor dissociates from R. Adapted from (Record Jr *et al.* 1996).

(III) The growing RNA is synthesized in the elongating complex (EC) by incorporation of successive nucleotides, complementary to the template strand. This complex is much more stable than any preceding complexes, thus ensuring long transcripts. The step time (transcription rate) in *E. coli* varies from, roughly about 50 nt/s for mRNAs and up to 100 nt/s for RNAs that are not translated (Richardson and Greenblatt 1996). These step times represent an average step time and the elongating complex can experience sites on the template that pause transcription. Even though no consensus pause sites have been identified so far, it has been shown that RNA polymerase sometimes is inhibited in elongation by two distinct classes of pause signals; hairpin-dependent pausing and pausing by backtracking (Artsimovitch and Landick 2000).

(IV) Termination of transcription occurs when the elongating complex encounters a termination signal in the DNA template (factor-independent) or a protein factor, i.e. ρ. The factor-independent terminal signal consist of two distinct sequences; a stretch of G and C residues in an inverted repeat followed by a stretch of A residues. When the termination signal is transcribed, the RNA forms a hairpin loop that destabilizes the interaction with the elongating complex whereby the complex spontaneously dissociates. The ρ-dependent termination requires the ρ factor to bind to the growing RNA. Such binding causes the RNA-DNA interaction to unwind and the RNA polymerase to be released from the DNA. The ρ dependent termination site has no consensus sequence. However, there are sequences proximal the 3'-ends of terminated transcripts that has higher than normal proportion of C residues called *rut* (Rho utilisation sites), which is the site where ρ is belived to be bind (Richardson and Greenblatt 1996; Henkin 2000).

Regulatory control of the transcription cycle is exerted at all these four steps but the most cost effective way to regulate transcription in bacteria is in the very beginning of transcription, avoiding unnecessary synthesis of precursors that are not utilized. Thus, a large number of factors affecting gene expression act on transcription initiation.

2.3 – Transcriptional regulation

The ability of *E. coli* to regulate vast sets of genes quickly and accurately with changing growth conditions and/or environmental stimuli can be accounted for by a large number of *trans*-acting factors, i.e. transcription factors that act by binding to DNA and activate or repress transcription. In *E. coli* there are a total of 314 known and predicted genes that encode transcription factors (Perez-Rueda and Collado-Vides 2000).

The mechanism by which transcription can be increased by activators is diverse but they often improve the performance of the promoter by either improving its affinity for RNA polymerase or helping the polymerase to escape the promoter. In many cases, the activator binds to a sequence upstream the -10 element and interacts with the α-CTD of RNA polymerase. In addition, the activator can bind near the -35 hexamer and improve the promoter's interaction with the σ factor. Some activators induce conformational change in the promoter, positioning the promoter core elements for optimal RNA polymerase holoenzyme binding.

Repressors, on the other hand, usually act by hindering the RNA polymerase to bind the promoter. The simplest way is to sterically block binding of the polymerase by utilizing overlapping DNA sequences or blocking a required activator. Another mechanism includes making the promoter inaccessible by looping the DNA. In addition, transcriptional activators and repressors can integrate their signals for a specific gene, thus increasing the regulatory specificity (Browning and Busby 2004).

Other factors in the cell, besides activators and repressors, affecting transcription are the nucleoid-associated proteins (NAPs), such as Fis (factor for inversion stimulation), IHF (integration host factor) and H-NS (histone-like nucleoid-structuring protein). The NAPs bind to the DNA and condense the chromosome (Browning *et al.* 2010). By doing so, they influence the distribution of RNA polymerase between promoters, but a more specific effect by the NAPs has been shown for the *rrn* operons where Fis activates transcription (Hirvonen *et al.* 2001). Also, NAPs, such as IHF, can bend DNA such that activators binding relatively far upstream a promoter can interact with the RNA polymerase.

Many transcription factors regulate more than one gene or operon and some act as "global" regulators in the sense that a large number of genes are affected by the same regulator. An example of a global regulator is LexA of the SOS response. The SOS response is normally shut-down by the LexA repressor by its binding to specific operator sites (SOS boxes) upstream the SOS genes, encoding products required for DNA damage repair. The RecA protein is the transducer of the regulon, which, in response to DNA damage (the sensor), binds to single-stranded DNA (the signal). RecA together with single-stranded DNA interacts with LexA and triggers its autoproteolysis to relieve repression (Butala *et al.* 2009).

Another global regulatory system in *E. coli* is the stringent response elicited by amino acid starvation; a response regulated by the nucleotide guanosine-3',5'-bispyrophosphate (ppGpp), which is of special interest in this thesis (see next section for details). ppGpp radically differ from the transcription factors described above, instead of interacting with DNA, ppGpp binds directly to RNA polymerase to accomplish its regulatory role.

2.4 – ppGpp and the stringent response

The hallmark of the stringent response in *E. coli* is the sudden accumulation of the small nucleotide ppGpp (Cashel and Gallant 1969; Lazzarini *et al.* 1971; Ryals *et al.* 1982), followed by a rapid down-regulation of stable RNA synthesis (rRNA and tRNA) and ribosome production upon amino acid starvation (Sands and Roberts 1952; Stent and Brenner 1961; Cashel and Gallant 1969; Travers 1976). ppGpp, together with DksA (see below) mediate this global switch in transcription from highly expressed genes required for growth to amino acid biosynthetic operons to other genes required for homeostasis during slow/non-growth. Global transcriptome profiling of the stringent response during amino acid and carbon starvation shows that ppGpp (and DksA) are truly *bona fide* global regulators, affecting a vast set of genes, both positively and negatively (Traxler *et al.* 2006; Durfee *et al.* 2008; Traxler *et al.* 2008; Aberg *et al.* 2009; Traxler *et al.* 2011). Figure 3 summarizes the major effects on global transcription as a consequence of ppGpp synthesis.

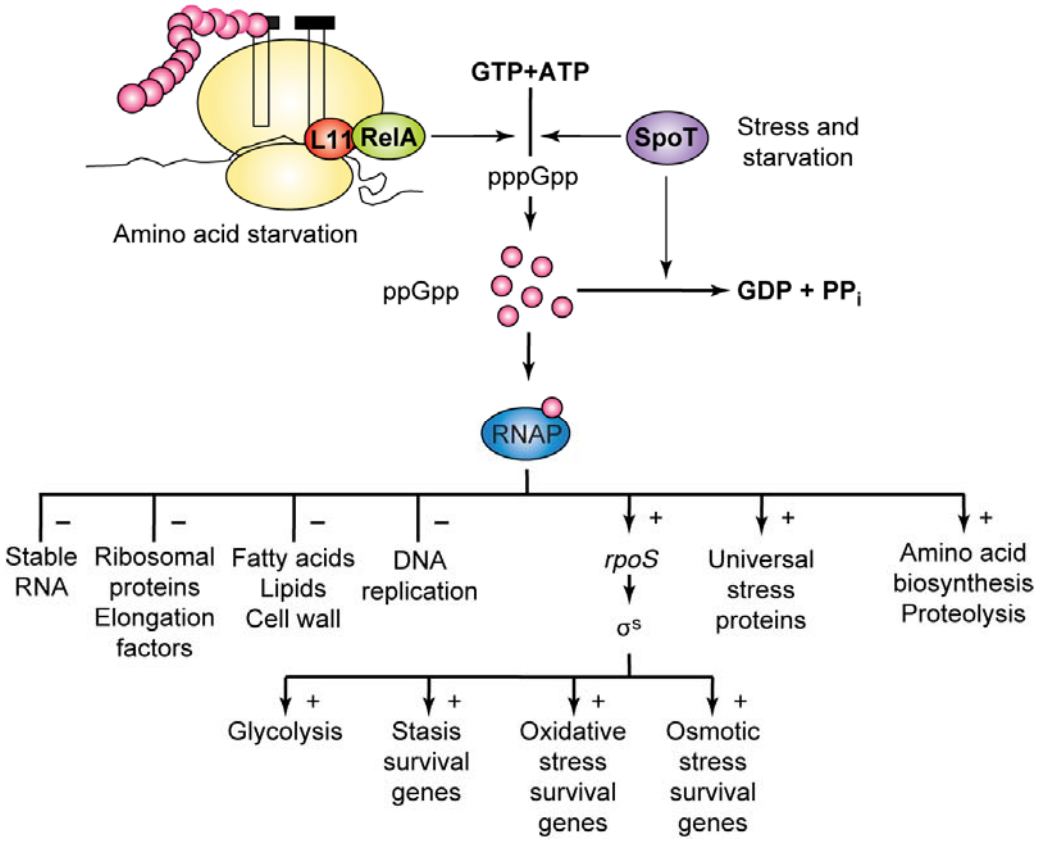

Figure 3. Summary of ppGpp synthesis and the effects on global gene transcription. ppGpp binds RNA polymerase (RNAP) and redirects transcription from growth-related genes to genes involved in stress resistance and starvation survival. Previously published in (Magnusson *et al.* 2005) Copyright © 2005, Elsevier.

Cashel and Gallant (1969) first noticed the existence of ppGpp by performing two-dimensional thin-layer chromatography of radiolabeled nucleotides from amino acid-starved *E. coli* cells (Cashel and Gallant 1969). They found two unusual nucleotides, or "magic spots", pppGpp (guanosine 3'-diphosphate,5'-triphosphate) and ppGpp (guanosine-3',5'-bispyrophosphate), collectively called ppGpp herein. These "magic spots" are synthesized by phosphorylation of GTP or GDP, using ATP as a phosphate donor (Cashel *et al.* 1996). In *E. coli*, the synthesis of ppGpp is mediated by two related proteins, RelA and SpoT; each requiring different signals for activation. The RelA protein is associated with the ribosome and the synthesis of ppGpp is triggered when the ribosome encounters an uncharged tRNA at the A-site (Haseltine and Block 1973). While RelA only possesses synthesizing activity, the related SpoT protein is bifunctional. Besides synthesizing ppGpp, SpoT can

hydrolyze the same to GDP and pyrophosphate (PP_i) (Xiao *et al.* 1991; Murray and Bremer 1996), thus allowing a way out of stringency when conditions permit. In addition, the accumulation of ppGpp induced by stress conditions, including carbon, phosphorus, iron, or fatty acids deficiency, is SpoT dependent (Xiao *et al.* 1991; Spira *et al.* 1995; Cashel *et al.* 1996; Vinella *et al.* 2005; Battesti and Bouveret 2006; Potrykus and Cashel 2008).

A strain lacking both RelA and SpoT proteins are incapable of producing any ppGpp and is referred to as $ppGpp^0$. Such mutants are auxotrophic for multiple amino acids and unable to grow in minimal media (Xiao *et al.* 1991). This phenotype is most likely due to the fact that the expression from amino acid biosynthesis operons are dependent on ppGpp (Cashel *et al.* 1996). As mentioned above, a $ppGpp^0$ strain also continues to accumulate stable RNA and ribosomal proteins during starvation conditions; a response referred to as a relaxed response (Neidhardt 1963).

The stringent response have mainly been studied in *E. coli* but several other bacterial species has RelA and SpoT homologues or bifunctional RelA-SpoT homologues with both synthetase and hydrolase activity (Mittenhuber 2001). Even though ppGpp acts as a general "alarmone" in most bacterial species, it influences diverse, species-specific, cellular processes. In the opportunistic pathogen *Legionella pneumophila*, when nutrients becomes limited, ppGpp levels increase and the bacteria differentiate to a motile, coccoid, virulent form that exhibits increased resistance to stress and has the ability to transmit from one host cell to another (Molofsky and Swanson 2004). Similarly, accumulation of ppGpp increases host persistence of *Mycobacterium tuberculosis* in mice (Dahl *et al.* 2003). The development of starvation-induced fruiting bodies (myxospores) in *Myxococcus xanthus*, is also dependent on ppGpp (Singer and Kaiser 1995). While the presence of genes responsible for ppGpp synthesis and hydrolysis is widespread among different species, some obligate intracellular bacteria lack any homologues for RelA-SpoT, e.g. *Chlamydia* species (Mittenhuber 2001). The presence of ppGpp is not restricted to prokaryotes, RelA-SpoT homologues have been found in plants (van der Biezen *et al.* 2000; Givens *et al.* 2004; Takahashi *et al.* 2004). ppGpp is produced in the chloroplast and an increase level of ppGpp could be seen in plant extracts after wounding or applying physical stress (Takahashi *et al.* 2004).

2.4.1 – DksA and ppGpp, brothers in arms?

DksA is a small protein (17 kDa; 151 aa) originally described as a multi-copy suppressor of the temperature sensitive growth and filamentation of a *dnaK* mutant (Kang and Craig 1990). Later studies showed that DksA is involved in a variety of cellular processes including quorum sensing in *Pseudomonas aeruginosa* (Jude *et al.* 2003), virulence in *Salmonella, Shigella flexneri* and *Legionella pneumophila* (Turner *et al.* 1998; Sharma and Payne 2006; Dalebroux *et al.* 2010) and in increased resistance to reactive oxygen species in *Salmonella* (Henard *et al.* 2010). In addition, DksA has also been implicated in the maintenance of DNA replication by resolving the conflicts between arrested DNA replication forks and RNA polymerase engaged in transcriptional elongation (Tehranchi *et al.* 2010).

While specific roles have been assigned to DksA, several studies suggest DksA to be a co-factor of ppGpp. For example, DksA was shown to augment regulation by ppGpp of the general stress σ factor σ^S in transcription as well as in translation (Brown *et al.* 2002; Hirsch and Elliott 2002). In addition, the inhibition of ribosomal RNA synthesis by ppGpp was shown to be dependent on the presence of DksA *in vivo* (Paul *et al.* 2004; Perron *et al.* 2005). Moreover, DksA has been shown to be an important player in the stringent response despite the fact that the levels of DksA are not changed with growth rate or growth arrest (Brown *et al.* 2002; Paul *et al.* 2004). From such observations it may be hypothesized that ppGpp acts through DksA during stringency by controlling its activity.

Numerous studies have confirmed that DksA and ppGpp act together in transcription. For example, the regulation of the transcription factor *fis* is negatively regulated by the combined action of ppGpp and DksA (Mallik *et al.* 2006). Moreover, it has been shown both *in vivo* and *in vitro* that the negative and the positive effects of ppGpp are amplified by DksA for the *rrn* promoter and amino acid promoters, respectively (Paul *et al.* 2004; Paul *et al.* 2005). In addition, as reported in this thesis, DksA and ppGpp together stimulate transcription from the universal stress protein promoter of *uspA* both *in vivo* and *in vitro*. Furthermore, a DksA mutation that bypasses the need of ppGpp for positive and negative control *in vivo* has been identified, providing further support for DksA as a possible partner to ppGpp (Blankschien *et al.* 2009).

On the other hand, some phenotypes of the *dksA* and ppGpp^0 mutants are not overlapping. For example, the amino acid requirements of a ppGpp^0 and a *dksA* mutant are not exactly the same (Brown *et al.* 2002). Moreover, ectopic DksA overproduction can completely compensate for the lack of ppGpp in the regulation of cell-cell aggregation, motility, filamentation, and σ^S-dependent morphology (Paper I). In addition to such independent functions, DksA and ppGpp have opposing roles in cellular adhesion (Paper I) and biofilm formation (Aberg *et al.* 2008). It has also been shown that DksA can exert ppGpp-independent effects on transcription both *in vivo* and *in vitro* (Paper I; Aberg *et al.* 2008; Lyzen *et al.* 2009).

2.4.2 – RNA polymerase, the target of DksA and ppGpp

Genetic suppressor screens have indicated that ppGpp might interact with the β and β' subunits of RNA polymerase. Specifically, mutations in the RNA polymerase, most of which mapped to the genes encoding β and β', have been identified that confer a stringent behavior in the absence of ppGpp (Oostra *et al.* 1981; Little *et al.* 1983; Zhou and Jin 1998). Similarly, mutations causing resistance to high levels of ppGpp was isolated and mapped to the RNA polymerase β subunit (Tedin and Bremer 1992). A biochemical cross-linking approach demonstrated that ppGpp analogs interact with the β and β' subunits of RNA polymerase (Chatterji *et al.* 1998; Toulokhonov *et al.* 2001).

The details of ppGpp interactions with RNA polymerase were believed to be clarified further when the X-ray structure of RNA polymerase-ppGpp co-crystal was resolved in *Thermus thermophilus* (Artsimovitch *et al.* 2004). The co-crystal positioned ppGpp in the interface between β and β', proximate to the active center of RNA polymerase. Based on these results, it was argued that ppGpp provides the means to modulate the catalytic Mg^{2+}-ions (Artsimovitch *et al.* 2004). However, a subsequent study called for reevaluating the data of Artsimovitch *et al.*, pointing out that substituting the interacting amino acids responsible for positioning ppGpp in the crystal, did not affect ppGpp-mediated regulatory effects (Vrentas *et al.* 2008). Thus, the binding site for ppGpp on RNA polymerase is still an unresolved issue.

DksA has been found to be structurally positioned close to ppGpp (Perederina *et al.* 2004) and a model of its interaction with RNA polymerase may be deduced or at least hypothesized from studies of the transcription factors GreA and GreB. These factors ensure continuous RNA polymerase transcription by suppressing

arrested transcriptional elongation complexes caused by backtracked RNA (reviewed in Fish and Kane 2002). As in the case for ppGpp, the Gre-factors bind directly to RNA polymerase rather than DNA (Stebbins *et al.* 1995; Opalka *et al.* 2003). The structure of GreA/B consists of a C-terminal globular domain and an anti-parallel coiled coil N-terminal domain. The coiled coil domain protrudes into the narrow substrate entry channel (secondary channel) towards the active site of RNA polymerase, where the factors facilitate RNA polymerase dependent hydrolysis of backtracked RNA (Laptenko *et al.* 2003; Opalka *et al.* 2003). The crystal structure of DksA reveals an anti-parallel coiled coil domain structurally similar to GreA/B (Perederina *et al.* 2004; Vassylyeva *et al.* 2004). Even though no co-crystal of DksA and RNA polymerase has been reported, the fact that DksA co-purifies with RNA polymerase and is structurally similar to the Gre-factors strongly indicates that DksA might associate with the secondary channel of RNA polymerase (Perederina *et al.* 2004). Notably, in *Mycobacterium tuberculosis*, which lacks DksA, CarD has been identified as a functional homolog of DksA. CarD is required for eliciting the mycobacterial stringent response to starvation and control of rRNA but CarD interacts with a different site on RNA polymerase than DksA (Stallings *et al.* 2009).

An additional functional interaction between RNA polymerase and DksA is indicated by the fact that DksA suppresses the inability of an RNA polymerase lacking the ω-subunit to respond to ppGpp (Vrentas *et al.* 2005). The physiological significance of this effect *in vivo* is obscure, however, since the ω-subunit of RNA polymerase is not required for the stringent response (Mukherjee *et al.* 1999; Ghosh *et al.* 2001).

The fact that GreA/B are structurally similar to DksA raised the possibility that they have similar roles in transcription. Over-expressing GreA antagonizes the negative effect of DksA, independently of ppGpp, on *rrn* expression *in vivo* (Potrykus *et al.* 2006). In contrast, GreB mimics the negative effect of DksA on *rrn* expression *in vitro* but not the positive effects of ppGpp on amino acid biosynthetic promoters (Rutherford *et al.* 2007). Thus, GreA/B might affect transcriptional output from stringently controlled promoters but are doing so in a rather unpredictable fashion, so far. In addition, it is not clear to what extent the GreA/B proteins contribute to stringency in wild type cells harboring both ppGpp and DksA.

2.5 – Mechanism of regulation by DksA and ppGpp

By reviewing the suggested mechanisms of action of ppGpp and DksA, two principal modes of regulation is emerging; direct and indirect control.

2.5.1 – Direct negative and positive regulation of transcription by DksA and ppGpp

The repression of the *rrn* promoters by DksA and/or ppGpp has been studied extensively *in vitro* (Kajitani and Ishihama 1984; Gourse 1988; Barker *et al.* 2001b; Paul *et al.* 2004). These studies show that the *rrn* promoters form intrinsically instable open complexes during transcription initiation and are very sensitive to further destabilization. A model derived from these data suggest that only intrinsically unstable promoters can be inhibited by DksA and ppGpp since they promote collapse of the open complex before NTP incorporation can occur. Both DksA and ppGpp have been implicated in contributing to open complex collapse, in concert and independent of each other (Barker *et al.* 2001b; Paul *et al.* 2004; Rutherford *et al.* 2009), but a conflicting report shows that DksA does not contribute to open complex collapse at the *rrn* promoter (Potrykus *et al.* 2006). The *rrn* core promoter has features that could contribute to the instability of the open complex; a suboptimal -35 hexamer, a suboptimal (extended) -10 hexamer, a suboptimal spacer length (16 bp) and a G and C rich discriminator region (Haugen *et al.* 2006). However, mutations in the -35 hexamer, spacer and discriminator regions that increase the stability of the *rrn* open complex did not totally abrogate DksA and ppGpp dependent inhibition (Haugen *et al.* 2006) suggesting that an unstable open complex is not an absolute requirement for negative regulation by ppGpp. In line with this notion, the λpR promoter forms intrinsically stable open complexes but is nonetheless inhibited by ppGpp (Potrykus *et al.* 2002).

Besides reducing the stability of the open complex, a second model suggest that ppGpp competes with NTPs in the active center of the RNA polymerase (Jores and Wagner 2003), leading to a general inhibition of transcription initiation. It has been argued that a possible consequence of ppGpp and NTP competition is an enhanced transcriptional pausing (Jores and Wagner 2003) and a decreased rate of transcriptional elongation (Kingston *et al.* 1981).

A third model for the direct inhibition of transcription by ppGpp relies on the interpretation of the co-crystal of RNA polymerase and ppGpp, showing that ppGpp is positioned close to the transcription bubble. In this model, ppGpp is suggested to pair with cytosine residues at the non-template strand, at position -1 and -2 in the promoter, destabilizing the open complex, possibly by slowing down RNA polymerase translocation (Artsimovitch *et al.* 2004).

A fourth mechanistic model of ppGpp-dependent inhibition of transcription is contrasting the open complex stability model outlined above. This model is based on experiments with stringent RNA polymerase mutants (i.e. mutants with an RNA polymerase behaving as if ppGpp is always present), which demonstrated that RNA polymerase of such mutants was often trapped in the closed promoter complexes and unable to initiate transcription (Maitra *et al.* 2005). Interestingly, DksA seems to be important in trapping RNA polymerase (Potrykus *et al.* 2006). If the negative effect by DksA is exerted at the closed complex, the negative effect by both ppGpp and DksA on the subsequent step in transcription initiation, the open complex, might not make a big difference in total transcriptional output. However, the different mechanisms presented above are not mutually exclusive and might work in concert in fine-tuning negative regulation by DksA and ppGpp.

Direct positive regulation by DksA and ppGpp has not been studied as extensively as negative regulation. Promoters requiring DksA and/or ppGpp for direct positive effects are found among genes needed for stationary phase survival and stress adaptation. Among these promoters, some are dependent on alternative σ factors; σ^S (Gentry *et al.* 1993; Lange *et al.* 1995; Kvint *et al.* 2000a), σ^N (Carmona *et al.* 2000), and σ^E (Costanzo and Ades 2006). In addition, the major housekeeping σ factor, σ^{70}, recognizes many promoters positively regulated by ppGpp/DksA. Specifically, the expression of the universal stress proteins and genes required for amino acid biosynthesis and uptake, are σ^{70}-dependent genes requiring ppGpp for their induction (Cashel *et al.* 1996; Kvint *et al.* 2003).

It is commonly stated that promoters activated by DksA and ppGpp display stable open complexes in transcriptional initiation (Bartlett *et al.* 1998; Barker *et al.* 2001a; Paul *et al.* 2005). Nevertheless, although the λ*paQ* promoter has intrinsically unstable open complexes, it is activated by ppGpp (Potrykus *et al.* 2004). An alternative model for activation suggests that DksA and ppGpp stimulate the rate of an isomerization step on the pathway to open complex formation, thus promoting the forward reaction (Paul *et al.* 2005).

2.5.2 – Indirect effects on regulation of transcription by ppGpp

It has been suggested that ppGpp can alter gene expression indirectly by influencing the availability of free RNA polymerase in the cell. This implicates ppGpp as a factor involved in "passive control" of gene expression, a phenomenon I will now review before considering the potential role of ppGpp in this type of control.

2.5.2.1 – Passive regulation of transcription

When considering gene regulation *E. coli* it is important to take into account that RNA polymerase is in short supply (Shepherd *et al.* 2001; Bremer *et al.* 2003) and that promoters compete with each other. Ole Maaløe introduced the concept of "passive" regulation, in which promoters are differentially regulated by changes in the concentration of free and available RNA polymerase (Maaløe 1979).

What regulates the distribution of RNA polymerase between promoters in passive control of gene regulation? If one disregards specific factors, the intrinsic property of the promoter itself must partly account for such passive regulation. During steady-state growth, over 50% of all transcripts that can be found in *E. coli* originate from the 14 promoters of the *rrn* operons (coding for rRNA), while there are over 2000 mRNA promoters (Wagner 2000). Thus, the promoters of *rrn* genes appear to harbor promoter elements that contribute to an exceptional high activity.

The activity of the promoter can be compared to the activity of an enzyme, thus the Michaelis-Menten parameters can be used to characterize the properties of different promoters. The Micaelis-Menten equation relates the turn-over rate of the promoter, V, to the concentration of free RNA polymerase holoenzyme [$E\sigma^{70}$] using the promoter specific parameters V_{max} and K_m;

$$V = \frac{V_{max} * [E\sigma^{70}]}{[E\sigma^{70}] + K_m}$$

V_{max} represent the maximal initiation rate of transcription (or maximal promoter regeneration (turn-over) time) at saturating concentration of RNA polymerase, while K_m is the concentration of free RNA polymerase that keeps the promoter occupied half of the time or, in other words, the concentration [$E\sigma^{70}$] that results in half the maximal initiation rate (V_{max}). The factor $[E\sigma^{70}]/([E\sigma^{70}] + K_m)$, represents the probability that the promoter is occupied by an RNA polymerase (Dennis *et al.*

2004). Thus, when$[E\sigma^{70}] \rightarrow \infty$, this factor approaches 1, so that V approaches V_{max} (saturation). Accordingly, when $[E\sigma^{70}] \rightarrow 0$, the equation can be expressed as: $V = (V_{max}/K_m) * [E\sigma^{70}]$, where the factor V_{max}/K_m represents the promoter strength (Dennis *et al.* 2004). Thus, the three dependent parameters, V_{max}, K_m and V_{max}/K_m are sufficient to compare promoter strengths and the sensitivity of the promoter to changes in $[E\sigma^{70}]$.

A model for passive control of gene regulation suggested by Jensen and Pedersen (1990) is illustrated in figure 4. The figure illustrates two types of promoters with intrinsically different Michaels-Menten parameters where the initiation rate V_{max} (in arbitrary time units (tu^{-1})) is plotted as a function of $[E\sigma^{70}]$ (in arbitrary concentration units (cu)). Promoters that are highly expressed during growth, such as those producing rRNA and ribosomal proteins in the protein synthesis system (PSS), exhibit a very high turn-over rate which gives them very high capacity, high V_{max}, but also makes them hard to saturate (high K_m) (blue curve). Stress- and amino acid biosynthetic promoters on the other hand, mainly expressed during slow growth or growth arrest, are argued to have both low V_{max} and K_m (red curve).

If we, based on the assumptions made, now test the effects of increasing the concentration of available RNA polymerase ($E\sigma^{70}$) in the cell, from 0.4 cu (grey line 1) to 0.8 cu (grey line 2), we notice that the initiation rates from the PSS promoters (with high V_{max} and high K_m) increase while stress- and amino acid promoters remain unaltered (with low V_{max} and low K_m). If we instead lower the concentration of available RNA polymerase from 0.4 cu (grey line 1) to 0.05 cu (grey line 3), the stress- and amino acid promoters will now show higher initiation rates relative to PSS promoters. This can be accounted by the fact that promoters with low V_{max} and low K_m show increased promoter strength (high V_{max}/K_m) with decreasing concentration of RNA polymerase.

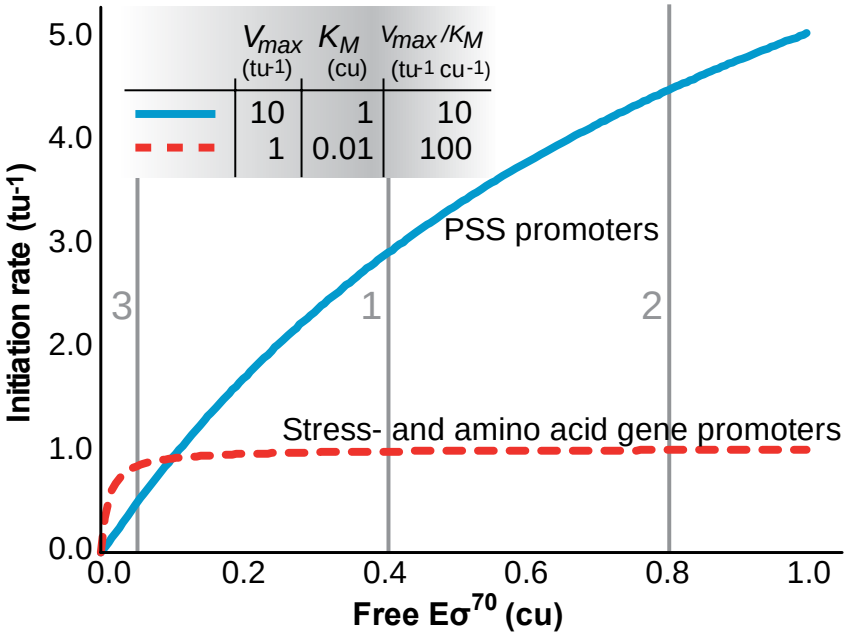

Figure 4. Initiation rates for PSS promoters (blue curve) and stress- and amino acid promoters (red curve) as a function of the free concentration of RNA polymerase holoenzyme. A promoter with a K_m value below the free RNA polymerase concentration is much less sensitive to changes in the concentration than a promoter with a K_m-value above the free RNAP concentration.

2.5.2.2 – ppGpp and passive regulation of transcription

The potential role of ppGpp in passive control is a controversial issue; some researchers argue that elevated levels of ppGpp cause an increase in the pool size of free RNA polymerase while others propose the exact opposite. Zhou and Jin (1998) originally suggested that RNA polymerase availability increases during stringency and that such an increase is a consequence of RNA polymerase falling off from rRNA promoters because of the specific inhibition by ppGpp on the promoter open complex stability (Zhou and Jin 1998; Barker *et al.* 2001b). The positively regulated promoters (e.g. stress-defense genes and amino acid biosynthetic operons) are, in this model, induced upon stringency because they have low affinity for RNA polymerase and are therefore benefited by an increased concentration of free RNA polymerase (Barker *et al.* 2001a). This model is referred to as the "affinity model" in this thesis (Paper II). The model also connects ppGpp-dependence of alternative σ factors to changes in RNA polymerase availability. It is hypothesized that the release of core RNA polymerase from rRNA promoters upon ppGpp accumulation, increases the levels of the polymerase available to bind alternative σ factors. Consequently, promoters requiring alternative σ factors will be expressed.

In contrast to the affinity model, Bremer and others have presented a model (referred to as the "saturation model" in this thesis) that argues that the levels of free, and transcription-available, $E\sigma^{70}$ should rise with the quality of the growth medium, associated with diminished levels of ppGpp (Jensen and Pedersen 1990; Bremer and Dennis 1996). Specifically, the free $E\sigma^{70}$ concentrations were calculated from the concentrations of total RNA polymerase and promoters in a model system with estimated Michaelis–Menten constants for transcription initiation and such calculations reveal that the concentration of free $E\sigma^{70}$ is about 0.4 and 1.2 μM at growth rates corresponding to 1.0 and 2.5 doublings/h, respectively, *i.e.* the free $E\sigma^{70}$ concentration increases with increasing growth rates (Bremer *et al.* 2003). In the saturation model, differential control by ppGpp relies heavily on the saturation kinetics of the promoter, as outlined above. Again, the model centers around the proposition that genes in high demand during rapid growth (e.g. rRNA genes) possess unsaturated promoters with high maximal initiation rates (figure 4, blue curve) while stress- and amino acid promoters,

induced during slow growth or growth arrest (figure 4, red curve), are often saturated with RNA polymerase with low initiation rates (Jensen and Pedersen 1990; Paper II).

In the saturation model, there is an inverse correlation between ppGpp levels and the levels of free RNA polymerase and several mechanisms by which ppGpp decrease RNA polymerase availability have been proposed. At some genes, ppGpp induce transcriptional pausing (Kingston *et al.* 1981; Bremer and Ehrenberg 1995; Krohn and Wagner 1996) and reduce the rate of elongation (Sorensen *et al.* 1994; Vogel and Jensen 1994), thus sequestering core RNA polymerase during slow growth and the stringent response. Moreover, it has been reported that σ^{70} can remain attached in the elongation cycle and such association between the σ factor and polymerase increases during stationary phase (Bar-Nahum and Nudler 2001; Mukhopadhyay *et al.* 2001; Deighan *et al.* 2011). Even though this association has not been confirmed to be dependent on ppGpp, it is possible that such a mechanism reduces available RNA polymerase.

An additional phenomenon that might reduce RNA polymerase programmed with σ^{70} in a ppGpp-dependent fashion is σ factor competition. A central feature of the σ factor competition model is that core RNA polymerase is limiting for transcription. Thus, a competition is brought about between alternative σ factors and the main σ factor σ^{70} during growth arrest (Farewell et al. 1998; Jishage et al. 2002; Laurie et al. 2003). Notably, the concentration of the main σ factor, σ^{70}, is predominant over alternative σ factors during growth and the concentration of σ^{70} does not change markedly with growth phase (Jishage *et al.* 1996). The other six σ factors accumulate in response to specific stresses and/or growth arrest, but do not reach the concentration of σ^{70} (Jishage *et al.* 1996). Moreover, σ^{70} has the highest affinity for RNA polymerase among all the σ factors (e.g. σ^{70} has 16 fold higher affinity for core RNA polymerase than σ^{S} (Maeda *et al.* 2000)). From these facts it appears feasible that additional factors are needed to aid the alternative sigma factors in their competition for core RNA polymerase and such factors include the anti σ^{70}-factor, Rsd, and ppGpp/DksA.

The anti-σ factor Rsd binds to σ^{70} (Jishage and Ishihama 1998; Jishage and Ishihama 1999) and allows alternative σ factors to compete more successfully for core RNA polymerase (Jishage *et al.* 2002; Laurie *et al.* 2003; Mitchell *et al.* 2007; Costanzo *et al.* 2008). Thus, besides its role in stimulating promoters dependent on alternative sigma factors, Rsd can be argued to indirectly influence the expression from promoters sensitive to the free levels of σ^{70} programmed RNA polymerase. Interestingly, ppGpp is a positive regulator of *rsd* expression (Jishage and Ishihama 1999) implicating ppGpp as a factor in σ factor competition through its effects on Rsd levels. In addition, σ^{S}, σ^{H} and σ^{N} compete better against σ^{70} *in vitro* in the presence of ppGpp (Jishage *et al.* 2002; Laurie *et al.* 2003) suggesting that ppGpp may have more direct effects on competition. In line with such results, it has been shown that less σ^{S} and σ^{H} and more σ^{70} are bound to RNA polymerase *in vivo* in cells lacking ppGpp (Hernandez and Cashel 1995; Jishage *et al.* 2002).

Recently, a small RNA, 6S RNA (*ssrS*), has been implicated in global regulation of gene expression (Neusser *et al.* 2010). 6S RNA is induced in stationary phase and it has been observed that 6S RNA can form a complex with σ^{70} programmed RNA polymerase, thus making the holoenzyme unavailable for transcription (Wassarman and Storz 2000; Wassarman 2007). This notion cannot explain induction of promoters dependent on competing alternative σ factors, since the levels of 6S RNA in stationary phase far exceeds the levels of RNA polymerase, thus in theory sequestering all of them. However, 6S RNA-mediated trapping of σ^{70} programmed RNA polymerase could contribute to reduce levels of σ^{70} programmed RNA polymerase, playing a part in passive regulation of gene expression. Interestingly, the expression of *relA* is negatively regulated by 6S RNA (Cavanagh *et al.* 2010; Neusser *et al.* 2010), thereby altering the levels of RelA available to respond to amino acid starvation and ppGpp synthesis, possibly providing means to fine-tune the level of the "alarmone".

2.6 – Growth rate regulation and ppGpp

During rapid growth, most resources in the *E. coli* cell are directed towards producing their protein synthesizing system (PSS) to match the high demand of protein synthesis (Neidhardt *et al.* 1990). Since the translation rate of the ribosomes in the cell changes relatively modestly at different growth rates, the number of ribosomes must be regulated in proportion to growth rate (growth medium allowing a certain growth rate) to sustain proper levels of protein synthesis (growth rate control). The intracellular concentration of ppGpp follows an inverse correlation with growth rate (Ryals *et al.* 1982; Bremer and Dennis 1996) and it is tempting to suggest that ppGpp might have a prominent role in growth rate control of stable RNA synthesis and the PSS similar to its role in shutting down expression of the PSS during a stringent response. Nevertheless, views on the importance of ppGpp for growth rate control vary significantly as elaborated below.

There are several models explaining growth rate dependent regulation of ribosomes and all include transcription initiation as the dominant site of control. One of the earliest models, the ribosome feedback model by Nomura (Jinks-Robertson *et al.* 1983; Cole *et al.* 1987), suggests that excess translating ribosomes feedback inhibit the transcription of new ones. The feedback signal to the ribosomal promoters of *rrn* was later suggested to be the pool size of ATP and GTP (Gaal *et al.* 1997). The reasoning is that excessive translating ribosomes drain the pool size of ATP and GTP and as a consequence, transcription from the *rrn* promoters is inhibited due to the promoters' requirement of high levels of these nucleotides (NTPs) to initiate transcription. However, studies have shown that the pool size of NTPs does not change with growth rate (Petersen and Moller 2000). Thus, the signal in the ribosome feedback model remains obscure. It should be noted also that this model excludes ppGpp as a controlling factor in growth rate regulation of the PSS.

In contrast to the ribosome feedback model, the "passive control" and the "RNA polymerase partitioning model" implicate ppGpp as an integral factor affecting growth rate dependent control. The model of passive control has been examined in the previous sections, where Jensen and Pedersen argue that the initiation rates from the *rrn* promoters are directly dependent on the concentration of free RNA polymerase (Jensen and Pedersen 1990) and that the levels of free RNA polymerase are inversely correlated with the levels of ppGpp, possibly by a

mechanism where ppGpp sequesters RNA polymerase in the elongation cycle (Sorensen *et al.* 1994; Krohn and Wagner 1996; Zhang *et al.* 2002, Paper II). In the RNA polymerase partitioning model, the accumulation of ppGpp (slow growth) is hypothesized to direct RNA polymerase away from *rrn* promoters. RNA polymerase is thought to exist in two forms, a ppGpp bound form and a free form. Only the free form of RNA polymerase is believed to be able to initiate transcription from *rrn* promoters. Consequently, only when the levels of ppGpp are low (fast growth), will RNA polymerase be apportioned correctly to the promoters needed during growth (Travers *et al.* 1980; Ryals *et al.* 1982).

As mentioned above, whether or not ppGpp is required for proper growth rate control has been a contentious topic, but a recent report (Potrykus *et* al. 2011) clearly demonstrates that slow growing cells lacking ppGpp show RNA/DNA ratios similar to fast growing cells (Potrykus *et al.* 2011) and that proper growth rate control of the PSS is completely abolished in cells lacking ppGpp or DksA. Interestingly, the data also show that DksA, when over-produced, is able to substitute for ppGpp in growth rate control (Potrykus *et al.* 2011). Thus, ppGpp and DksA emerge as the two primary factors involved in growth rate control by priming the cells protein synthesizing system according to the nutritional status of the cell.

3. RESULTS AND DISCUSSION

Global control of gene expression in *E. coli* has been hypothesized to be, in part, a consequence of changes in the levels of RNA polymerase available for transcription. However, there has been significant disagreement within the field as to whether the levels of free RNA polymerase core increase or decrease with growth arrest and what the outcome of such alteration in RNA polymerase availability is on the expression of growth-related genes versus maintenance genes. In addition, there have been conflicting reports concerning how and whether the "alarmone", ppGpp regulates the levels of free RNA polymerase core. The aim of this thesis was to elucidate what promoter elements are required for positive regulation by ppGpp/DksA and for responding to alterations in the levels of RNA polymerase, using the *uspA* promoter of *E. coli* as a model promoter.

3.1 – The universal stress protein A, UspA

The universal stress protein A, UspA, was isolated and cloned by Nyström and Neidhardt (1992). They demonstrated that the protein is becoming increasingly synthesized in growth-arrested cells, regardless of the condition inhibiting growth (Nystrom and Neidhardt 1992). It was hypothesized that UspA might have a general protective role in growth-arrested bacteria since the same pattern of UspA synthesis could be seen in cells exposed to diverse stressful conditions. Specifically, heat shock, heavy metal exposure, oxidative stress, osmotic shock, antibiotics, and DNA damaging agents induce UspA synthesis (Nystrom and Neidhardt 1992; 1994; Diez *et al.* 2000; Gustavsson *et al.* 2002). In line with this notion, mutants of *uspA* have an impaired ability to survive prolonged periods of different stresses and are more sensitive to carbon starvation (Nystrom and Neidhardt 1994), while ectopically overproducing UspA reduce the cells ability to exit starvation and grow when conditions are again becoming favorable (Nystrom and Neidhardt 1996).

UspA belongs to a superfamily of proteins that can be found in diverse bacterial species as well as in archaea, fungi, protozoa, and plants (Aravind *et al.* 2002; Kvint *et al.* 2003). *E. coli* has five additional *usp*s; *uspC*, *uspD*, *uspE*, *uspF* and *uspG*. They are all induced during growth arrest and stress conditions and their role in the cell is multifaceted. In general, these Usp proteins are, similar to UspA, involved in the cell's ability to withstand different stresses (Nachin *et al.* 2005). Moreover, all *usp* genes require the major σ factor, σ^{70}, for expression and are all positively regulated by ppGpp (Kvint *et al.* 2000b; Gustavsson *et al.* 2002).

3.1.1 – The *uspA* promoter

Nyström and Neidhardt (1992) determined the transcriptional start site of the *uspA* promoter and identified the -35 and -10 hexamers as characteristic for promoters dependent on σ^{70} for expression. Indeed, the -35 hexamer of the *uspA* promoter deviates only with one base from the consensus recognized by σ^{70} while the -10 hexamer has a perfect match with the three bases that shows the highest degree of conservation in the consensus sequence (Lisser and Margalit 1993; see Figure 5). In addition, the spacer region has the characteristic length of 17bp for σ^{70} preferred promoters. The wild type *uspA* promoter lacks an upstream sequence region (UP-element) that can aid promoter efficiency through interaction with α-CTD. It also lacks a GC-rich discriminator sequence preceding the transcriptional start site.

uspA harbors two FadR binding sites positioned downstream the promoter. The binding sites are found between the transcriptional and translational start sites and *uspA* can be classified to be a member of the FadR regulon (Farewell *et al.* 1996). FadR represses *fad* genes, which are induced in stationary phase and involved in fatty acid degradation. FadR is inactivated in the presence of long chain fatty acids, thus derepressing the *fad* genes (Raman and DiRusso 1995). In cells lacking *fadR*, the expression from the *uspA* promoter is somewhat derepressed, but only in log phase when ppGpp levels are low (Farewell *et al.* 1996). In stationary phase, in the presence of ppGpp, RNA polymerase can override repression by FadR (Kvint *et al.* 2000b). Thus, FadR regulation of the *uspA* promoter appears to be independent of regulation by ppGpp.

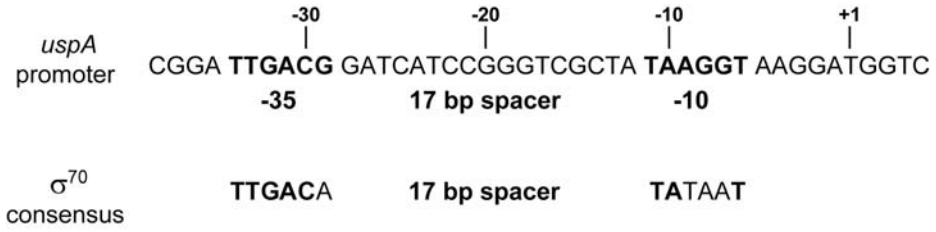

Figure 5. The *uspA* core promoter; spacer region, transcriptional start site (+1), -35 and -10 hexamers are indicated as well as consensus sequence recognized by σ^{70}

3.1.2 – The *uspA* promoter requires DksA and ppGpp for induction *in vivo*

The regulation of the *uspA* gene has been shown to be at the level of transcription (Nystrom and Neidhardt 1992). In addition, Kvint and co-workers (2000b) showed that ectopic production of ppGpp is sufficient to induce expression and the requirement of ppGpp can be bypassed by a mutant *rpoB* allele that mimics a constitutively stringent RNA polymerase. The data indicates that ppGpp might act directly to stimulate transcription on the *uspA* promoter via the RNA polymerase (Kvint *et al.* 2000b). Following such premises, I hypothesized that there might be a *uspA* promoter element that confers positive regulation by ppGpp and, possibly, by DksA. I further conjectured that the defining element would be found in the core promoter. Therefore, I constructed a minimal *uspA* promoter harboring only the four core elements: the -10 region, the -35 region, the spacer region, and the sequence immediately downstream the -10 element. This minimized the promoter to -38bp upstream and +5bp from transcriptional start site. The regulation of this promoter is identical to the wild type promoter previously assayed (-227bp +163bp), i.e. it displays a four-fold induction of expression upon growth arrest. Both the wild type promoter and the minimal promoter are positively controlled by ppGpp and DksA *in vivo*. While *relA spoT* mutations ($ppGpp^0$) totally abolish positive regulation of *uspA*, a *dksA* mutation did so to a somewhat lesser degree. -Text removed from public version-

3.1.3 – Text removed from public version

3.2 – ppGpp and DksA; more than just cofactors

It has been shown previously that DksA and ppGpp regulate many genes in a similar fashion, either positively or negatively (Paul *et al.* 2004; Paul *et al.* 2005). Indeed, we observed that the loss of DksA resulted in the same expression pattern as a loss of ppGpp *in vivo*, for the positively regulated promoters P*uspA* and P*livJ* as well as the negatively regulated promoter P*rrnB* P1 (Paper I). Such results have prompted the suggestion that DksA and ppGpp may be cofactors, in a sense similar to CRP and cAMP. The concentration of DksA in the cell is almost constant throughout growth and stationary phase (Rutherford *et al.* 2007) and ppGpp, therefore, does not appear to regulate DksA levels. Rather, ppGpp might contribute to DksA activity by, for example, increasing DksA affinity for $E\sigma^{70}$ under conditions of stringency. Overproducing of DksA in strains lacking ppGpp, stimulates and represses transcription from P*uspA* and P*rrnB* P1, respectively. Thus, DksA appears to be able to act alone, at least when overproduced (Paper I). In line with these findings, DksA have been shown to exert ppGpp-independent effects on transcription initiation and $E\sigma^{70}$-promoter complex formation (Aberg *et al.* 2008; Lyzen *et al.* 2009). Moreover, negative control by DksA on P*rrnB* P1 seems greater than that achieved by ppGpp in an *in vitro* transcription assay (Haugen *et al.* 2006). On the other hand, ppGpp appears to be the factor primarily responsible for the stimulation of P*uspA* and P*livJ* while positive regulation by DksA is only in operation together with ppGpp *in vivo* (Paper I). In conclusion, even though DksA and ppGpp in many cases appear to act together as cofactors in transcription, they might target different steps in transcription initiation that contribute to the independent effects of these factors seen *in vivo* and *in vitro*.

3.2.1 – The relaxed versus the Δ*dksA* phenotype

We found that overproduction of DksA could bypass the requirement of ppGpp to activate the promoter of *livJ* (a promoter that directs the transcription of a transporter of branched-chain amino acids). Therefore, we examined the effect of overproducing DksA in cells lacking ppGpp for their ability to grow on media without any amino acids. Since a hallmark of ppGpp^0 mutants is that they are auxotrophic for many amino acids (Xiao *et al.* 1991), and a *dksA* mutant has multiple amino acid requirements (Brown *et al.* 2002), we hypothesized that overproducing DksA in *trans* might rescue ppGpp^0 cells from amino acid auxotrophy. Indeed, it was possible to restore growth of the ppGpp^0 mutant on minimal media by overproducing DksA (Paper I). However, this phenotype was strain dependent occurring in MC4100 but not in MG1655. This is interesting since MC4100 has previously been shown to be more sensitive than MG1655 to high levels of ppGpp (Brown *et al.* 2002), and might also be more sensitive to high levels of DksA. Elucidating the reason for the different responses of these genetic backgrounds to elevated levels of ppGpp and DksA may reveal important aspects of ppGpp/DksA regulation.

Other phenotypes that can be observed in a relaxed cell (ppGpp^0), e.g. filamentous growth and a failure of becoming coccoid and self-aggregate in stationary phase, can be observed also in a Δ*dksA* mutant (Paper I). All these phenotypes in relaxed cells can be suppressed by DksA overproduction, supporting the idea that DksA can act alone, when overproduced. The failure to self-aggregate, as seen in ppGpp^0 and Δ*dksA* mutants, has been shown to be antagonized by the presence of type 1 fimbriae (Hasman *et al.* 1999). Therefore, we also tested Fim-dependent adhesion of the Δ*dksA* and ppGpp^0 mutants by scoring for their ability to agglutinate yeast cells, a fimbriae-dependent process. Interestingly, this phenotype of the relaxed cell did not match that of the *dksA* mutant. While ppGpp^0 cells were deficient in agglutination of yeast cells, Δ*dksA* cells showed increased agglutination efficiency compared to wild type cells (Paper I, Table 2). This is consistent with later findings demonstrating that ppGpp is a positive regulator and DksA a negative regulator of type 1 fimbriae production (Aberg *et al.* 2006; Aberg *et al.* 2008).

At the time of publication of Paper I, it was not known whether the reduced motility of a Δ*dksA* mutant was due to the lack of flagella or not. In contrast, the motility deficiency of a ppGpp^0 mutant was revealed by electron microscopy to be a consequence of cells being devoid of flagella (Paper I, Figure 5B). Using transcriptomics, it has been shown, however, that ppGpp^0 and DksA affect flagellum production differentially (Aberg *et al.* 2009). Surprisingly, ORFs coding for flagellum biosynthetic genes were induced in a DksA-deficient strain, contrasting the effect seen on low agar plates were a Δ*dksA* mutant shows reduced motility (Paper I, Figure 5A). The production of flagella was confirmed by microscopy and the detection of the major subunit of the flagella, FliC, by Western blot analysis (Aberg *et al.* 2009). Thus, the reduced motility of a Δ*dksA* mutant on low agar plates cannot be explained by the lack of flagella. Instead, reduced motility might be due to the fact that Δ*dksA* mutants are hyper-flagellated; thus, the flagella might be entangled or have reduced vigor. Alternatively the high expression of type 1 fimbriae seen in Δ*dksA* strains (Aberg *et al.* 2008) might impair movement due to increased adherence to surfaces. Moreover, the transcriptome study confirmed that the majority of ORFs coding for flagellum biosynthesis were downregulated in a ppGpp^0 mutant (Aberg *et al.* 2009), supporting the findings that ppGpp^0 cells are devoid of flagella and severely impaired in motility (Paper I).

The discrepancy in flagella regulation by DksA and ppGpp has been difficult to explain in relation to their co-regulatory role in the cell, but it seems that in the absence of DksA, other factors might have access to the secondary channel of RNA polymerase (i.e. GreA/B), exerting regulatory roles unrelated to DksA function in motility (Aberg *et al.* 2009). It appears that the positive effects seen on motility in Δ*dksA* are an effect of GreA through alleviation of competition for the secondary channel with DksA (Aberg *et al.* 2009).

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3.4 – Transcriptional regulation by alterations in the levels of RNA polymerase

Nutritionally induced alterations in the levels of σ^{70}-programmed RNA polymerase have been argued to be a regulatory mechanism controlling gene expression. This type of "passive control" is a result of promoters having different kinetic properties thus responding differently to changes in RNA polymerase concentration (see Figure 3). In this thesis (Paper II), I have investigated whether and how genes being positively and negatively regulated by ppGpp/DksA respond to altered levels of free $E\sigma^{70}$ and if alterations in RNA polymerase availability may be an integral part of the stringent response.

3.4.1 – Increased RNA polymerase availability creates a phenocopy of a relaxed cell

Using a system for artificial, ectopic, overproduction of the RNA polymerase, demonstrated that a 2-fold overproduction of $E\sigma^{70}$ holoenzyme increased the expression from the *rrnB* P1 promoter and increased the production of ribosomal proteins. In contrast, expression from stress defense genes (*uspA, uspF* and *uspD*) as well as amino acid biosynthesis genes (*livJ* and *thrABC*) decreased upon $E\sigma^{70}$ overproduction (Paper II). Moreover, cells overproducing $E\sigma^{70}$ displayed phenotypes similar to a $ppGpp^0$ strain; i.e. they were defective in motility and protein damage control (Paper I and II). Thus, increased availability of RNA polymerase directs expression towards growth-related genes at the expense of maintenance genes, a response typical of cells with diminished ability to make ppGpp (relaxed cells). This is also in line with the results of underproduction of RNA polymerase which has the opposite effect (Magnusson *et al.* 2003).

Repression of σ^{70}-dependent stress-defense genes and amino acid biosynthetic genes upon ectopic elevation of $E\sigma^{70}$ levels is in accord with the saturation model (Jensen and Pedersen 1990; Dennis *et al.* 2004), which argues that such genes are driven by promoters that are much more easily saturated than *rrn* promoters and would not, relative to *rrn* and ribosomal protein genes, benefit from increased concentrations of free $E\sigma^{70}$. We demonstrated that this is the case by scoring for promoter occupancy in a chromatin immunoprecipitation experiment (ChIP) using antibodies against β' and σ^{70}. This analysis demonstrated that $E\sigma^{70}$ overproduction

in vivo resulted in an 8- to 11-fold increase in the ratio of promoter occupancy at *rrn* relative to *uspA* promoters (Paper II).

As overproduction of $E\sigma^{70}$ mimics a relaxed response, we asked whether cells lacking ppGpp display an elevated concentration of free $E\sigma^{70}$ and whether such elevation is, in fact, an integral part of the relaxed response. We measured the levels of free RNA polymerase in purified chromosome-free mini-cell fractions and found that 60% of total $E\sigma^{70}$ was free in $ppGpp^0$ cells whereas wild type cells displayed 30% free $E\sigma^{70}$ (Paper II). These results are in accordance with calculations from the Bremer laboratory arguing that the concentration of free RNA polymerase is inversely correlated with ppGpp levels (Bremer *et al.* 2003).

The model of passive control by Jensen and Pedersen (1990) suggests that stringency is accomplished, in part, by a reduction in free $E\sigma^{70}$ levels. Therefore, we hypothesized that overproduction of $E\sigma^{70}$ should to, some extent, repress the stringent response. We approached this idea by using a mutant carrying a spoT allele (*spoT202*) resulting in constitutive elevated (2 to 3-fold) levels of ppGpp, which causes partial repression of the *rrnB* P1 promoter and elevates both *uspA* and *thrABC* expression, mimicking a stringent response. The repression of *rrnB* P1 and the increased expression of *uspA* and *thrABC* in the *spoT202* strain was counteracted by overproduction of $E\sigma^{70}$, suggesting that limitations in available free $E\sigma^{70}$ might be an integral regulatory component of a stringent response in *E. coli* (Paper II).

In summary, these results are in line with the saturation model presented by Jensen and Pedersen (Jensen and Pedersen 1990), which highlights that promoters of genes whose products are in high demand (e.g. ribosomes) need to exhibit a high maximal velocity of transcription, thus requiring high concentration of free $E\sigma^{70}$. Whereas promoters of, for example, stress-defense genes (*usps*) and amino acid biosynthetic gene are often saturated (or close to saturated) with $E\sigma^{70}$ and exhibit lower maximal initiation velocity (see Figure 4). Thus, passive control of gene expression through alterations in the levels of $E\sigma^{70}$ might play an integral part in regulating the trade-off between growth related genes and maintenance genes and this trade-off is, under some conditions, regulated by the "alarmone" ppGpp. However, a similar trade-off has been shown to occur in the absence of ppGpp accumulation by conditions reducing the availability of free $E\sigma^{70}$. Especially, Vogel *et al.* (1991) showed that partial pyrimidine starvation, reducing transcription elongation rates, created a phenocopy of the stringent response without elevated ppGpp levels.

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