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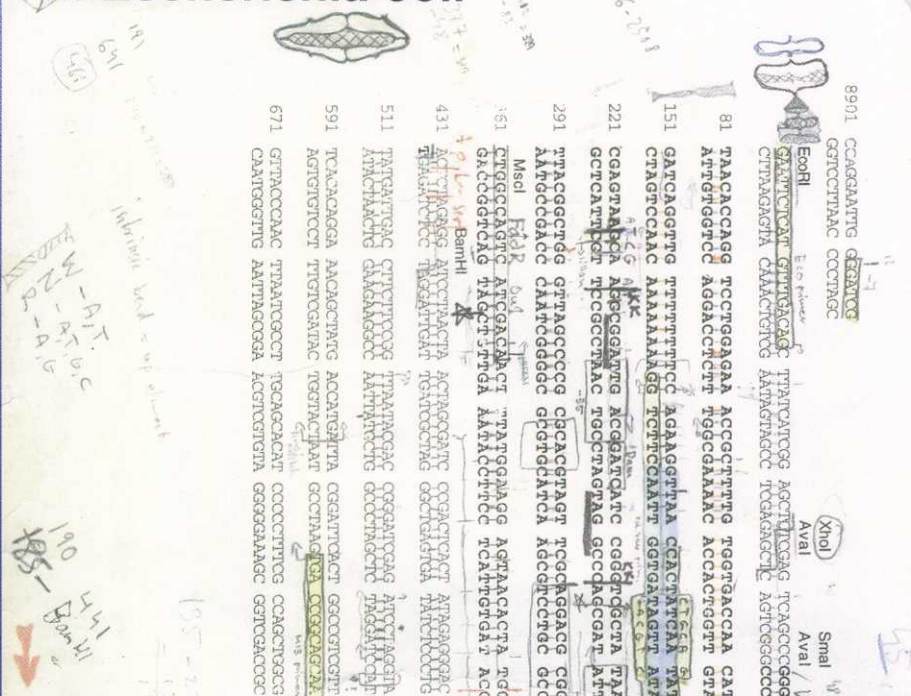
On the role of sigma factor competition and the alarmone, ppGpp, in global control of gene expression in *Escherichia coli*



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AKADEMISK AVHANDLING

För filosofie doktorsexamen i mikrobiologi (examinator Professor Thomas Nyström), som enligt sektionsstyrelsens beslut kommer att offentligt försvaras fredag den 7 Juni 2002, Kl 14.00 i föreläsningssal Inge Schiöler (F1405). Medicinaregatan 9B, Göteborg.

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Abstract

The *uspA* promoter, driving production of the universal stress protein A in response to diverse stresses, is demonstrated to be under dual control. One regulatory pathway involves activation of the promoter by the alarmone guanosine 3',5'-bisphosphate, via the β-subunit of RNA polymerase while the other consists of negative control by the FadR repressor. In contrast to canonical dual control by activation and repression circuits which depends on concomitant activation and derepression for induction to occur, the ppGpp-dependent activation of the *uspA* promoter overrides repression by an active FadR during starvation. The ability of RNA polymerase during stringency to overcome repression depends, in part, on the strength of the FadR operator. This emergency derepression is operative on other FadR regulated genes induced by starvation and is argued to be an essential regulatory mechanism operating during severe stress.

The open reading frame immediately upstream *uspA* was demonstrated to encode a protein (UspB) involved in stationary phase induced resistance to ethanol. *uspB* is dependent of the stationary phase sigma factor, σ^S. A mutation in the gene encoding σ^S (*rpoS*) not only abolishes transcription of some genes (e.g. *uspB*) in stationary phase, but also causes "superinduction" of other stationary phase-induced genes, such as *uspA*. The data suggest that the superinduction of *uspA* is caused by an increased amount of σ^{70} bound to RNA polymerase in the absence of the competing σ^S. Increasing the ability of σ^{70} to compete against σ^S by overproducing σ^{70} mimics the effect of an *rpoS* mutation by causing superinduction of σ^{70}-dependent stationary phase-inducible genes (*uspA* and *fadD*) and silencing of σ^S-dependent genes (*uspB*, *bolAp1* and *fadL*). Similarly, overproduction of σ^S markedly reduce stationary phase induction of *uspA* (σ^{70}-dependent), Thus, it seems that sigma factors compete for limiting amounts of core RNA polymerase during stationary phase.

σ^S requires ppGpp for its own accumulation and it was suggested that the similar phenotypes found between ppGpp^0 and Δ*rpoS* mutants was due to this fact. However, we found that no activity from the σ^S-dependent promoters tested (P*uspB*, *bolA*P1, P*cfa* and P*fadL*) was detectable in the ppGpp^0 strain even when σ^S levels were ectopically produced to levels corresponding to wild type levels. The results suggested that ppGpp confers dual control on the RpoS regulon by i) being essential for efficient expression and accumulation of σ^S and, ii) required for σ^S function *per se*. Interestingly, the *rpoB* allele *rpoB*3449 (A532Δ) that is epistatic to defects exhibited by a ppGpp^0 strain (i.e. growth in minimal media) suppressed the lack of induction of the σ^S-dependent promoters in the Δ*relA* Δ*spoT* strain. Thus, the *rpoB*3449 allele restores both accumulation of σ^S and the function of Eσ^S. This requirement of ppGpp can be explained, in part, by the fact that alternative sigma factors (σ^S and σ^{32}) compete better against σ^{70} for core RNA polymerase in the presence of ppGpp. Underproduction of σ^{70}, specific mutations in *rpoD (rpoD40* and *rpoD35)*, or overproduction of Rsd (anti-σ^{70}) restored expression from σ^S-dependent promoters *in vivo* in the absence of ppGpp accumulation. An *in vitro* transcription/competition assay with reconstituted RNA polymerase demonstrated that addition of ppGpp reduces the ability of wild type σ^{70} to compete with σ^{32} for core binding and the mutant σ^{70} proteins, encoded by *rpoD40* and *rpoD35*, compete less efficiently than wild type σ^{70}. Similarly, an *in vivo* competition assay demonstrated that the ability of both σ^{32} and σ^S to compete with σ^{70} is diminished in cells lacking ppGpp. Consistently, the fraction of σ^S and σ^{32} bound to core was drastically reduced in ppGpp deficient cells. Thus, the stringent response encompasses a mechanism that alters the relative competitiveness of sigma factors in accordance with cellular demands during physiological stress.

Keywords: Transcription, *Escherichia coli*, *uspA*, *uspB*, sigma factors, stationary phase, stress, *rpoS*, *rpoD*, *rpoB*, FadR, ppGpp, stringent response.

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To Hanna, Julia and Liza

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This thesis is based on the following papers, which are referred to by their Roman numerals:

I. **Kvint, K.**, Hosbond, C., Farewell, A., Nybroe, O. and Nyström, T. 2000. Emergency derepression: stringency allows RNA polymerase to override negative control by an active repressor. *Mol Microbiol* **35:** 435-443.

II. Farewell, A., **Kvint, K.** and Nyström, T. 1998. *uspB*, a new sigmaS-regulated gene in *Escherichia coli* which is required for stationary-phase resistance to ethanol. *J Bacteriol* **180:** 6140-6147.

III. Farewell, A., **Kvint, K.** and Nyström, T. 1998. Negative regulation by RpoS: a case of sigma factor competition. *Mol Microbiol* **29:** 1039-1051.

IV. **Kvint, K.**, Farewell, A. and Nyström, T. 2000. RpoS-dependent promoters require guanosine tetraphosphate for induction even in the presence of high levels of sigmaS. *J Biol Chem* **275:** 14795-14798.

V. Jishage, M., **Kvint, K.**, Shingler, V. and Nyström T. 2002. Regulation of sigma factor competition by the alarmone ppGpp. *Genes Dev* Accepted.

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1 Introduction

Bacteria are highly specialized species and are found in almost all environments on earth. For example, *Pyrolobus fumarii* can thrive at temperatures around 110°C (Blochl et al., 1997) and some species of *Halobacteriaceae* require at least 12% but can live in 25% NaCl (e.g. Kamekura, 1998). In addition, bacteria must be equipped with strategies to meet rapid changes in their near surroundings, such as depletion of carbon and nitrogen, exposure to H_2O_2, near-UV irradiation, temperature shifts, changing osmolarity, and exposure to antibiotics. Prokaryotes grow and proliferate exponentially as long as a supply of essential nutrients are present but enters the so-called stationary phase when one or more of these nutrients are exhausted. Many gram-positive bacteria respond to starvation by differentiating into dormant spores e.g. *Bacillus subtilis*. In contrast, most gram-negative bacterial species, such as *Escherichia coli*, respond to starvation by developing increased resistance to a large number of stresses without becoming dormant. The morphology of *E. coli* cells during starvation is recognized by its small and round shape, a very condensed cytoplasm, a highly cross-linked cell wall, and an increased periplasmic space (Huisman et al., 1996).

In order to adapt to environmental changes bacteria have to change their pattern of gene expression and to do this quickly the bacterial cells have evolved clever chromosomal structures and regulatory circuits. As bacteria have been around for some 3000 million years (compared to the much younger eukaryote: only 1000 million) it is reasonable to believe that basic molecular mechanisms that support life has first passed severe tests in prokaryotes.

Genes with related functions are often synchronically regulated. For instance, the genes required for lactose utilization are located in the *lac* operon (e.g. Jacob and Monod, 1961; Lederberg, 1948). An operon consists of two or more genes that are cotranscribed and can therefore be coordinately regulated. Because a response may require regulation above the operon level, some regulators have evolved to activate/repress several unlinked genes and/or operons and these will then form a regulon. In many cases, a stimulus will influence many different regulons, operons and cistrons simultaneously and these genetic networks are called stimulons (Smith and Neidhardt, 1983). For instance, all genes that respond to phosphate limitation

belong to the *psi* (phosphate starvation-inducible) stimulon. Several studies of gene regulation have shown that no regulon operates in isolation and that specific genes and operons can and do respond to more than one signal in a combinatorial fashion (Neidhardt and Savageau, 1996). For example, raising the temperature not only induces the heat-shock regulon but also represses the cold-shock response and causes a transient induction of the stringent response regulon (Jones and Inouye, 1994; Mackow and Chang, 1983). Estimations suggest that a bacterial cell has developed several hundred of such multigenic systems of which only a fraction has been discovered (Neidhardt et al., 1990b).

In order to carry out the responses required, the cell harbors a battery of "key-players" such as stimuli sensors, signal transducers, and regulators including activators, repressors, sigma factors and modifiers (e.g. cAMP and ppGpp), see figure 1. It should be pointed out that most of the regulation occurs at the level of transcription, but complementary RNA's and proteases play important regulatory roles at the post-transcriptional levels.

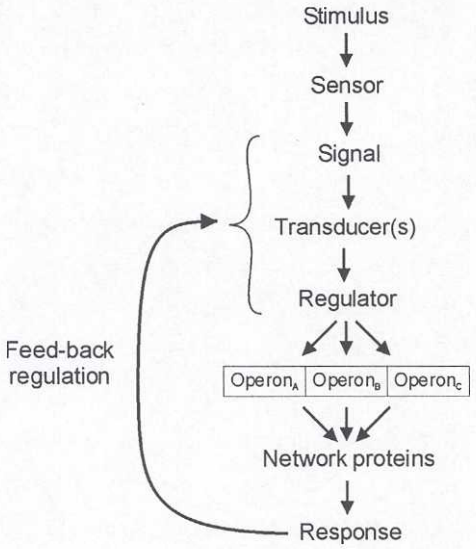

Fig. 1. Gene network depicted as a stimilus-response pathway (Adapted from Neidhardt et al. 1990).

2 Transcriptional control: some important players

2.1 DNA

In *E. coli*, the genome is a single circular, covalently closed double stranded DNA molecule. The sequencing of the *E. coli* K-12 chromosome was completed in 1997 and it turned out that it contains 4 639 221 base pairs (bp) that comprise 4288 open reading frames (ORFs) of which 1853 were previously described genes (Blattner et al., 1997). When fully extended the length of the chromosome is about 1 mm (Neidhardt et al., 1990a) and this has to be packed within 0.75 μm X 0.75 μm X 2 μm (the average size of a bacterial cell; Schmid, 1990). To do this a series of DNA binding proteins (e.g. HU, H-NS, IHF, Dps and Fis) has evolved that bend and coil the DNA into a structure called the nucleoid. Many, if not all, of these proteins are involved in gene regulation as well. For instance, IHF, which accumulates in stationary phase, bends DNA such that regulators that bind far away from promoters can come in contact with and help RNA polymerase to initiate transcription (Ditto et al., 1994; Nash, 1996). In contrast to IHF, Fis production is maximal during growth but is shut off in stationary phase (Ball et al., 1992; Nilsson et al., 1992). In addition, Fis has been shown to be important as an activator of *rrn* P1 (ribosomal RNA promoter) and several tRNA promoters (e.g. Gourse and Ross, 1996).

2.2 RNA polymerase

The multi-subunit, DNA-dependent RNA polymerase (RNAP) governs the process of all RNA synthesis in bacteria. In eubacteria, the 400 kDa RNAP core (E) consists of five different subunits, two α and one each of β and β', and ω ($\alpha_2\beta\beta'\omega$). RNAP core recognizes DNA unspecifically and is capable of elongation and termination of transcription. However, before RNAP can initiate transcription an additional subunit, sigma (σ), must associate to E, which then forms the holoenzyme ($E\sigma$) that has the ability to bind DNA in a promoter-specific manner (Burgess et al., 1969; Losick and Pero, 1981; Travers and Burgess, 1969). A promoter is the DNA sequence upstream the coding sequence of a gene where from transcriptional initiation occurs. It is thought that during the transition from initiation to elongation sigma factors are

released from RNAP core, leading to re-association of sigmas with free, non-transcribing (Burgess, 1971; Hansen and McClure, 1980; Shimamoto et al., 1986; Stackhouse et al., 1989; see figure 2). However, a recent study identified a population of RNAP that retains σ^{70} throughout elongation (Bar-Nahum and Nudler, 2001).

Statistical analysis of promoters recognized by $E\sigma^{70}$, the most abundant holoenzyme (see below), has shown that the typical consensus promoter has two conserved 6-bp DNA sequences located about 35 and 10 base pairs upstream of the transcriptional start site, with an average separation of 17 nucleotides between them (Harley and Reynolds, 1987; Lisser and Margalit, 1993). With respect to gene regulation it is interesting to note that a growing *E. coli* cell contains ~2000 molecules of core RNA polymerase (Ishihama, 1997), which is less than the total number of genes (see above) on the *E. coli* chromosome. Thus, *E. coli* is, in principle, unable to transcribe all its genes at the same time.

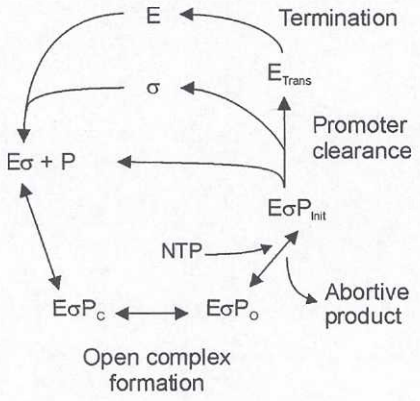

Fig. 2. General mechanism of transcriptional initiation. P denotes promoter; $E\sigma P_C$, closed complex; $E\sigma P_O$, open complex; $E\sigma P_{Init}$, initiation complex; E_{Trans}, elongating core RNAP.

2.3 Sigma factors and anti-sigma factors

Most bacteria have several sigma factors and all, except σ^{54} (Merrick and Edwards, 1995), are structurally similar to the housekeeping sigma factor, σ^{70} (Gross et al., 1992). Each sigma factor recognizes specific promoter sequences. Thus, depending on which sigma factor is associated to core, different sets of genes (regulons) can be transcribed. The number of sigma factors in different bacteria varies greatly; for instance in *Mycoplasma genetalium* there is only one whereas *Bacillus subtilis* contains 17 (Helmann, 1999). *E. coli* has seven different sigma factors, σ^{70}, σ^{N} (also called σ^{54}), σ^{S} (σ^{38}), σ^{H} (σ^{32}), σ^{F} (σ^{28}), σ^{E} (σ^{24}) and σ^{FecI}, which provide the means to an effective and sudden response to extra- or intracellular stimuli. Most housekeeping genes required for growth–related tasks are dependent on a σ^{70} programmed RNAP while $E\sigma^{N}$, among other things, controls genes involved in nitrogen scavenging (Merrick and Edwards, 1995) and some stress induced genes (Carmona et al., 2000; Shingler, 1996). Further, σ^{S}, the stationary phase sigma factor is required to induce several stress response genes (Hengge-Aronis, 1993; Loewen et al., 1998). σ^{32} governs the heat-shock regulon, which responds to protein misfolding in the cytoplasm whereas σ^{E} is elevated during protein misfolding in the periplasmic space (Grossman et al., 1984; Jenkins et al., 1991; Morita et al., 1999a; Raina et al., 1995). σ^{F} stimulates expression of flagella and genes involved in chemotaxis (Arnosti and Chamberlin, 1989) and σ^{FecI} is needed for the production of some extra-cytoplasmic proteins (e.g. Angerer et al., 1995).

Competition studies *in vitro* have shown that sigma factors have different affinities for core RNA polymerase and σ^{70} was shown to have the highest affinity (Maeda et al., 2000). *In vivo* measurements, using western blot analysis, suggested that the levels of σ^{70}, σ^{54} and σ^{28} remains approximately constant in cells during exponential growth and stationary phase whereas σ^{S} is undetectable during growth, but reaches about 30 % of the levels of σ^{70} in stationary phase (Jishage et al., 1996). Similarly, it has been shown that the levels of σ^{H} and σ^{E} are very low in exponential phase and the accumulated levels in stationary phase are still lower than the levels of σ^{70} (e.g. Ishihama, 2000).

For the reason that none of the alternative sigma factors seems to reach the levels of the primary house-keeping sigma factor, σ^{70}, it is intriguing how the alternative sigma factors can compete for core RNA polymerase. However, it has been shown that during conditions inducing a heat-shock response (accumulation of σ^{H}), σ^{70} is inactivated, leading to more available core RNA polymerases for σ^{H} (Blaszczak et al., 1995).

Alternative sigma factors are subjected to regulation in a complex manner in order to be activated when required. One kind of regulatory phenomenon involves anti-sigma factors. Anti-sigma factors are proteins that specifically recognize and bind sigma factors and thereby inhibit E binding; e.g. FlgM (anti-σ^{28}) in *S. typhimurium* (Kutsukake and Iino, 1994; Ohnishi et al., 1992) and RseA (anti-σ^{E}) in *E. coli* (De Las Penas et al., 1997; Missiakas et al., 1997)). For the record, it should be mentioned that the first demonstration of an anti-sigma factor was done during experimental studies of RNA polymerase modifications during phage T4 infection. The anti-σ^{70} factor, AsiA, encoded by T4, facilitates transcription of the phage-encoded genes necessary for the life cycle of the phage by switching the RNA polymerase specificity such that the host σ^{70} is substituted by the T4 specific sigma factor, σ^{gp55} (e.g. Malik et al., 1987; Orsini et al., 1993).

In addition to blocking interaction with core RNA polymerase, some anti-sigma factors function as deliverers of sigmas to proteolytic complexes (i.e. proteases). Many of the proteases found in *E. coli* are well conserved in both prokaryotes and eukaryotes. In *E. coli*, protein degradation plays important roles providing amino acids during starvation, in regulating the levels of specific proteins, and in eliminating damaged or abnormal proteins (e.g. during heat-shock or oxidative stress). Proteases also have regulatory tasks, such as controlling levels of other regulators (i.e. σ's) that are required during a short transient time or when no requirements of activity of these are called for. Recently, RssB/SprE (Muffler et al., 1996; Pratt and Silhavy, 1996) was shown to be an anti-σ^{S} factor, and when bound to σ^{S}, the protease ClpPX, efficiently degrades σ^{S} (Becker et al., 1999; Zhou et al., 2001). In a similar fashion, one of the best-characterized anti-sigma factors in *E. coli*, DnaK, regulates the levels of σ^{32} in association with DnaJ-GrpE and FtsH (e.g. Liberek et al., 1992; Straus et al., 1990; Yura and Nakahigashi, 1999).

Interestingly, like phage T4, *E. coli* cells harbors a gene, *rsd*, coding for an anti-σ^{70} factor. It has been shown that Rsd accumulates in stationary phase in a ppGpp dependent manner and that Rsd interacts with σ^{70} *in vitro* (Jishage and Ishihama, 1998; Jishage and Ishihama, 1999). Accordingly, overproduction of Rsd resulted in reduced and increased transcription from σ^{70}- and σ^{S}-dependent promoters, respectively (Jishage and Ishihama, 1999). This finding adds to the picture of how alternative sigma factors, despite their low levels compared to σ^{70}, may compete for core RNA polymerase.

3 Exponential and Stationary phase

A hallmark of bacterial growth is efficiency and speed. *E. coli* cells can divide every 16 minutes in a rich medium as long as the nutrient supply is in excess and metabolic byproducts do not reach toxic levels. The dominant activity of the bacterial cell is protein production and therefore ribosome biosynthesis is a key event during fast growth. The ribosome synthesis is proportional to the square of the growth rate (Gausing, 1980) and coupled to the cells growth requirements (growth rate-dependent control). As there is a continual alteration in the availability of nutrients in the environment, microbes have developed a highly regulated expression of ribosomal proteins, ribosomal RNA and transfer RNA (tRNA). There are several overlapping pathways regulating ribosome synthesis, yet, most of the regulation is thought to occur on the level of transcription but it is still not exactly clear how the control is brought about. However, there are basically two models describing this regulation, one includes guanosine tetra-phosphate (ppGpp; the stringent response) and the second model proposing a feedback mechanism coupled to the translational capacity of the cell (the ribosome feedback model; e.g. Condon et al., 1995). However, the two models are not mutually exclusive and it has been suggested, that they may work in concert (Hernandez and Bremer, 1990).

Upon depletion of essential nutrients from the medium the growth rate slows down and eventually reaches zero. At this point the cells has entered stationary phase. In stationary phase, *E. coli* cells become rounder and smaller, and the production of stable RNA and ribosomal proteins shut off (e.g. Nomura et al., 1984).

In addition, in contrast to bulk RNA, the half-life of mRNA increases more than two-fold, regardless of whether production of the transcript is repressed or stimulated (Albertson and Nyström, 1994). Moreover, stationary phase cells are more resistant than growing cells to a number of damaging agents such as, H_2O_2 (Jenkins et al., 1988), alkylating agents, ethanol, acetone, toluene (e.g. Hengge-Aronis, 1996), and acidic or basic pH conditions (Lee et al., 1994; Siegele and Kolter, 1992). Even though the resistances obtained in stationary phase can differ depending on the condition leading to stationary phase (e.g. nitrogen vs. phosphate starvation; Ballesteros et al., 2001), it has been proposed that the factors induced in the transition between exponential and stationary phase provide the cells with general protective features (e.g. Matin, 1991; Reeve et al., 1984). Several specific stress-inducible regulons controlled by the regulators SoxRS, OxyR, FadR and RpoH, contribute to the increased stress resistance in stationary phase (Dukan and Nystrom, 1998). However, perhaps the most important factors governing stasis-induced gene expression are the general stress response sigma factor, σ^S, and the alarmone, ("magic spot") ppGpp (e.g. Cashel et al., 1996; Hengge-Aronis, 2000).

4 Regulons and their regulation

4.1 Sigma S regulon

The starvation or stationary phase sigma factor, σ^S (encoded by *rpoS*), is the master regulator of the general stress response in *E. coli*. σ^S has been shown to play a central role in programmed switches of gene regulation leading to physiological and morphological changes that occur upon a number of diverse stresses in bacteria. For example, σ^S directs transcription of genes and operons whose products are involved in prevention of oxidative damage (e.g. Loewen et al., 1985; Mulvey et al., 1990), osmoprotection (Giaever et al., 1988), ethanol resistance (PAPER II), virulence (Krause et al., 1991), acid shock (Atlung et al., 1997), heat-shock/thermotolerance (e.g. Muffler et al., 1997a; Rockabrand et al., 1998), and cell-wall synthesis (Lange and Hengge-Aronis, 1991a). Further, mutants lacking σ^S exhibit an accelerated die-off during conditions of growth arrest (Lange and Hengge-

Aronis, 1991a), and markedly elevated levels of oxidized proteins (Dukan and Nystrom, 1998; Dukan and Nyström, 1999) as well as "superinduction" of genes requiring other sigma factors (PAPER III).

4.1.1 Regulation of σ^S

The regulation of σ^S concentration is complex and is controlled at the levels of *rpoS* transcription, *rpoS* mRNA translation, and σ^S stability. Many different stress conditions result in σ^S accumulation, and each one them appear to affect the control of σ^S synthesis differently. *rpoS* is the second gene in an operon with *nlpD*. The majority of *rpoS* mRNA originates from one promoter (P*rpoS*) located within *nlpD*, whereas some very low basal level of transcription comes from two promoters located just upstream of *nlpD-rpoS* (e.g. Lange et al., 1995; Lange and Hengge-Aronis, 1994; McCann et al., 1993; Takayanagi et al., 1994). Observations using P*rpos-lacZ* fusions has shown that *rpoS* is induced as the cells enters stationary phase (Lange and Hengge-Aronis, 1991b; Schellhorn and Stones, 1992). Some reports have indicated cAMP as a possible effector of *rpoS* transcription. However, one group found that a Δ*cya* mutant abolished transcription of *rpoS* (McCann et al., 1991), whereas another group observed elevated levels of transcription in a Δ*cya* mutant (Lange and Hengge-Aronis, 1991b). Another signal that may affect transcription of *rpoS* is ppGpp (Lange et al., 1995), and strains lacking ppGpp (i.e. Δ*relA* Δ*spoT*) have some phenotypic similarities to *rpoS* mutants, including a reduced viability in stationary phase and salt sensitivity (Hengge-Aronis, 1993). However, others claim that the correlations between ppGpp and/or cAMP and *rpoS* transcription are artificial and that *rpoS* mRNA levels actually decrease during growth arrest and that σ^S accumulation is solely due to stabilization of the sigma protein (Zgurskaya et al., 1997). Nonetheless, it is clear that σ^S accumulation requires ppGpp (Gentry et al., 1993; Lange et al., 1995; Zgurskaya et al., 1997).

Lately, the regulatory role of small RNAs has begun to be recognized. Although the occurrence of antisense RNAs have been known for many years, the participation of small RNAs in diverse regulatory contexts, such as protein tagging for degradation, stimulation of transcription, and modulation of RNA polymerase are

relatively recent discoveries (e.g. Wassarman et al., 1999). Hfq, also called HF-1, was discovered as an *E. coli* protein required for synthesis of bacteriophage Qβ RNA (Franze de Fernandez et al., 1968) and it turns out that Hfq binds *rpoS* mRNA *in vitro* (Hengge-Aronis, 2000) and is required for *rpoS* translation *in vivo* (Brown and Elliott, 1996; Muffler et al., 1997b). Further, Hfq has been shown to associate with ribosomes as well as the nucleoid and small RNAs (Azam et al., 2000; Kajitani et al., 1994; Wassarman et al., 2001). Hfq inactivation causes a variety of phenotypic changes indicating that Hfq acts as a global regulator (Muffler et al., 1997b; Tsui et al., 1994). With respect to *rpoS* regulation, it is thought that Hfq directly interact with the *rpoS* message and regulatory RNAs simultaneously. For instance, DsrA, which contains regions of sequence complementary to at least five different genes (*hns*, *argR*, *ilvIH*, *rpoS*, and *rbsD*; Lease et al., 1998) has been demonstrated to regulate both *hns* and *rpoS*, by RNA-RNA interactions (Lease et al., 1998; Majdalani et al., 1998). By binding to *rpoS* mRNA (probably under influence by Hfq), DsrA opens a stable stem-loop (Brown and Elliott, 1997; Lease et al., 1998; Majdalani et al., 1998) and stabilizes the *rpoS* transcript (Lease and Belfort, 2000). This enables access to the Shine-Dalgarno sequence of *rpoS*, which facilitates translation. In contrast, the small RNA, OxyS, of the oxidative stress response, negatively regulates translation of *rpoS* (Altuvia et al., 1997). Again, Hfq is required for this repression of *rpoS* translation (Zhang et al., 1998). Similarly, the nucleoid histone-like protein, H-NS, has a negative effect on Hfq-mediated stimulation of *rpoS* translation either by inhibiting synthesis of Hfq or by binding to Hfq (reviewed in Nogueira and Springer, 2000). In addition, DsrA inhibits translation of *hns* mRNA, by blocking translational initiation (Lease et al., 1998). Thus, many factors contribute to the complex translational regulation of *rpoS* mRNA. Even though it is not clear how the regulation occurs, one important fact is that *hfq* is epistatic to mutations affecting all the other known factors (i.e. *hns*, *dsrA* and *oxyS*) that modulate *rpoS* mRNA translation.

The protease-complex involved in σ^S degradation is ClpXP (Schweder et al., 1996). ClpP is the protease, and the ATP-dependent substrate-recognizing chaperone ClpX unfolds and transfers σ^S to the proteolytic center of ClpP (Kim et al., 2000; Singh et al., 2000). However, to efficiently accomplish this, the two-component response regulator protein RssB (SprE, MviA) must bind and deliver σ^S to the ClpXP complex (e.g. Bearson et al., 1996; Muffler et al., 1996; Pratt and Silhavy, 1996), see

figure 3. In addition, it is thought that RssB may be σ^S specific since no observations so far have indicated the involvement of RssB during other ClpXP proteolytic reactions e.g. proteolysis of λO protein (Zhou and Gottesman, 1998). It has been shown that acetyl phosphate phosphorylates the D58 residue of RssB (Bouche et al., 1998) and that this greatly enhances the affinity of RssB for σ^S (Becker et al., 1999). Moreover, studies has shown that K173 in region 2.5 of σ^S is essential for RssB binding *in vitro* and for σ^S degradation *in vivo* (Becker et al., 1999). Interestingly, region 2.5 of σ^S has been shown to be involved in promoter recognition and thus, binding by RssB to σ^S may prevent $E\sigma^S$ promoter interaction as well as $E\sigma^S$ formation.

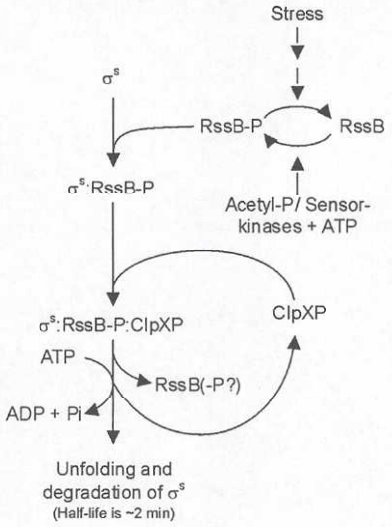

Fig. 3. Degradation of sigma S (Adapted from Hengge-Aronis, 2000).

Because sigma factors must associate with core before transcriptional initiation there are further steps where σ^S activity can be modulated, i.e. formation of holoenzyme and holoenzyme-promoter interaction. As pointed out, under stress conditions, σ^S accumulates up to levels corresponding to only 30 % of those of σ^{70} (Jishage et al., 1996). In addition, several promoters dependent on sigma factors other than σ^S are activated in stationary phase e.g. P*uspA* (σ^{70}) (Nyström and

Neidhardt, 1992), Pu (σ^{54}) (de Lorenzo et al., 1993), and P*dnaK* (σ^{32}) (Jenkins et al., 1991). Consequently, sigma factors have to compete for core RNA polymerase, (e.g. Zhou et al., 1992; and PAPER III). Interestingly, *in vitro* affinity measurements have shown that σ^{70} has a several-fold higher affinity for core than σ^{S} (Maeda et al., 2000), indicating that some factor or factors are crucial for the formation and activity of σ^{S}-containing holoenzyme ($E\sigma^{S}$). One such factor may be ppGpp. It was shown that σ^{S}-dependent promoters require ppGpp even in the presence of high levels of σ^{S} produced ectopically (PAPER IV). Also, many regulatory factors such as, IHF, H-NS, Fis, and cAMP have been shown to modulate (both negatively and positively) σ^{S} activity on specific genes. However, these regulators primarily act as common activators or repressors (often in concert) to control expression from a single σ^{S}-dependent promoter, reviewed in (Hengge-Aronis, 1999) and are not expected to affect σ^{S} binding to core.

4.2 Heat shock regulon

The heat shock response, comprises expression of a number of evolutionary well conserved proteins involved in several processes, including modulation of unfolded or misfolded proteins, repair or turnover of damaged proteins, and assembly of proteins. Many heat-shock proteins (HSPs) are molecular chaperones or ATP-dependent proteases and play crucial roles during both stress (i.e. heat and ethanol) and nonstress conditions. So far, two major heat-shock regulons have been discovered. The "classical" heat shock regulon is governed by sigma factor H, σ^{32} (or σ^{H}; encoded by *rpoH*), which directs RNA polymerase to transcribe genes dealing with aberrant proteins in the cytoplasm, while the σ^{E} regulon, encoding periplasmic proteases and folding enzymes, is specifically induced by extracytoplasmatic stresses.

When *E. coli* cells grown at 30°C are shifted to 42°C, the transcriptional activity at the promoters of the heat-shock regulon is increased as a result of accumulation of σ^{32} (Grossman et al., 1984; Straus et al., 1987). Transcriptional regulation of sigma 32 is rather complex and the regulatory region of *rpoH* contains three σ^{70}-dependent promoters and one promoter requiring σ^{E}. However, it is

thought that the transcriptional regulation has minor effects on σ^{32} synthesis (e.g. Arsene et al., 2000). Instead, heat induced accumulation of σ^{32} is primarily regulated at the level of translation and protein stability (Kamath-Loeb and Gross, 1991; Nagai et al., 1991; Straus et al., 1990; Tilly et al., 1989). The secondary structure of *rpoH* mRNA is coupled to and dependent on temperature, leading to degrees of accessibility for the translational machinery (e.g. Morita et al., 1999b). In addition, during steady-state growth (i.e. non-stress conditions), σ^{32} has an extremely short half-life (< 1 min) and it is argued that the heat shock chaperone complex DnaK-DnaJ-GrpE may bind and deliver σ^{32} to the heat shock protease FtsH (HflB) for proteolytic degradation (e.g. Tatsuta et al., 1998). During a heat shock, the initial (and transient) stabilization of sigma 32 is most probably due to σ^{32} being sequestered away from the DnaK-DnaJ-GrpE chaperones, which at the time becomes occupied in re-folding unfolded or misfolded proteins (Bukau, 1993; Craig and Gross, 1991; Straus et al., 1990). Subsequently, σ^{32} becomes available for core RNA polymerase interaction, leading to production of heat shock proteins that ultimately brings about a negative feedback control of σ^{32} by inhibiting *rpoH* translation and destabilizing σ^{32} (see figure 4).

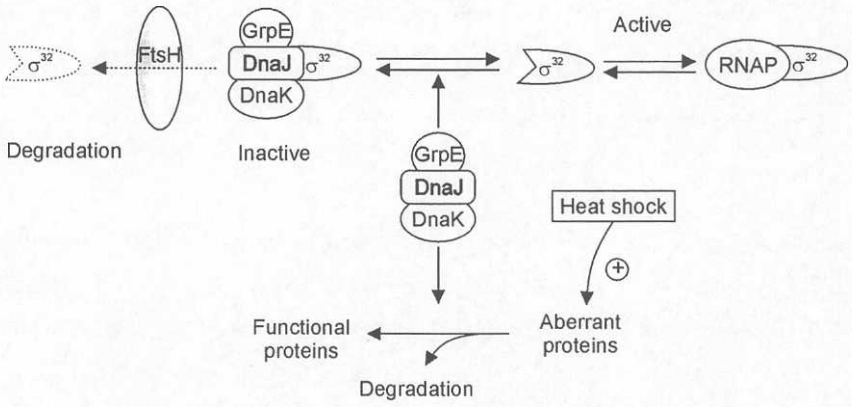

Fig. 4. Homeostatic regulation of sigma 32. σ^{32} is in equilibrium between an active form that can interact with RNAP during stress, and an inactive form, which is bound to the chaperone complex DnaK-DnaJ-GrpE.

4.3 Stringent response

The stringent response is elicited by nutrient starvation. The hallmark of the stringent response is a sudden accumulation of the nucleotide guanosine 3', 5'-bispyrophosphate (ppGpp; e.g. Baracchini and Bremer, 1988; Cashel and Gallant, 1969; Lazzarini et al., 1971; Ryals et al., 1982a; Ryals et al., 1982b; Ryals et al., 1982c), followed by a rapid inhibition of rRNA synthesis (e.g. Cashel and Gallant, 1969; Sands and Roberts, 1952; Stent and Brenner, 1961; Travers, 1976). In *E. coli*, synthesis of the nucleotide, ppGpp, is mediated by the ppGpp synthetases, PSI and PSII, encoded by the *relA* and *spoT* genes, respectively (Xiao et al., 1991; see figure 5).

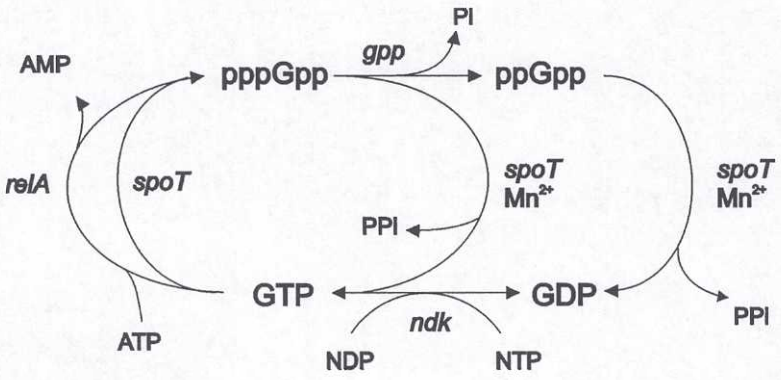

Fig. 5. Metabolism of ppGpp (Adapted from Cashel et al., 1996).

The RelA mediated synthesis of ppGpp is ribosome dependent and is triggered by amino acid starvation. The sensing/signaling mechanism is an uncharged tRNA in the A-site of a translating ribosome (Haseltine and Block, 1973; Haseltine et al., 1972). SpoT, on the other hand, is responsible for (i) ppGpp synthesis during a large variety of conditions (except amino acid starvation) that reduce the growth rate of cells and (ii) ppGpp degradation when cells confront a nutritional up shift, but the exact signaling pathways for SpoT are not clear (Cashel et al., 1996; Hernandez and Bremer, 1991; Murray and Bremer, 1996; Xiao et al., 1991). There is an inverse

correlation between steady state growth rate and ppGpp concentrations (e.g. Baracchini and Bremer, 1988; Hernandez and Bremer, 1990) but as a stress is imposed on the cells (leading to inhibition of growth) a rapid increase in ppGpp concentration occurs, whereafter the levels decreases again to a new steady state (Cashel, 1969; Fiil et al., 1972; Lund and Kjeldgaard, 1972).

The stringent response has mainly been studied in *E. coli* but there are also reports describing effects of ppGpp on different activities in other bacteria, including spore formation in *Myxococcus xanthus* (Harris et al., 1998), induction of virulence of *Legionella pneumophilia* (Hammer and Swanson, 1999) and *Mycobacterium tuberculosis* (Primm et al., 2000), antibiotics production in *Streptomyces coelicolor* (Kang et al., 1998) and *Streptomyces antibioticus* (Hoyt and Jones, 1999), and DNA replication and endospore formation in *Bacillus subtilis* (Eymann et al., 2001; Levine et al., 1995). In addition, a RelA/SpoT homologue has been found recently in the plant *Arabidopsis thaliana* (van der Biezen et al., 2000).

The alarmone ppGpp binds the carboxy-terminal domain of the β (Chatterji et al., 1998; Reddy et al., 1995) and the amino-terminal domain of the β' (Toulokhonov et al., 2001) subunits of RNA polymerase and by doing so accomplishes an immense differentiation in gene expression. Gene expression studies during amino acid starvation (RelA-dependent ppGpp accumulation) by 2D-gel analysis indicated that 50% of all the proteins where affected by the lack of ppGpp, see (e.g. Cashel et al., 1996). Similar studies (2D-gel analysis) with strains either overproducing ppGpp, from an IPTG inducible P*tac-relA* promoter fusion, or strains lacking ppGpp underline the pleiotropic effects of the stringent response (e.g. Jones et al., 1992; Schreiber et al., 1991). One important discovery made is that a strain lacking ppGpp, requires a supplement of amino acids in the media for growth (e.g. Xiao et al., 1991). Consequently, mutants that suppress the polyauxotrophy of a ppGpp^0 strain were identified and isolated (e.g. Cashel et al., 1996). Most commonly, such mutations are found in the genes *rpoB* or *rpoC* encoding the β and β' subunits of RNA polymerase (Cashel et al., 1996). However, some mutations are localized in *rpoD*, encoding σ^{70} (Cashel et al., 1996), suggesting that ppGpp may have a role in Eσ^{70} holoenzyme function. In addition to suppressing amino acid auxotrophy, several of these suppressors have also been shown to suppress the loss of typical stationary phase characteristics of ppGpp^0 strains, including accumulation of σ^S in stationary phase

(Hernandez and Cashel, 1995), induction of the universal stress proteins, UspA (PAPER I and V), UspB (PAPER IV), UspC, D and E (Gustavsson et al., 2002), down-regulation of rRNA promoters (Barker et al., 2001a; Bartlett et al., 2000), and induction of the σ^{54}-dependent, Po promoter (Sze and Shingler, 1999).

DNA sequences located between the –10 region and the transcriptional start site of stringently controlled promoters have been predicted to be determinants of stringent regulation (Travers, 1980a). Negatively regulated promoters have a conserved GC-rich motif in this region, which if mutated, loses its ability to inhibit transcription by a ppGpp programmed RNA polymerase (e.g. Travers, 1980b; Zacharias et al., 1989). In contrast, some positively regulated promoters exhibit an AT-rich motif in the discriminator region (Travers, 1984) and it has been shown that the *his* promoter has such a motif and is dependent on this motif for stimulation by ppGpp (Riggs et al., 1986). What has become apparent from these and later studies is that the effect of ppGpp on transcription initiation depends on the promoter-RNA polymerase interaction. For instance, based on *in vitro* studies, using mutated RNA polymerases (epistatic to ppGpp deficiency with respect to growth in the absence of amino acids) or addition of ppGpp, it has been suggested that the negatively regulated *rrnB* P1 forms an unstable open complex with RNA polymerase, requiring high levels of the initiating nucleotide to initiate transcription (Bartlett et al., 1998). But, upon a stringent response (ppGpp accumulation) the open complex is further destabilized leading to abortive transcription (Barker et al., 2001a; Barker et al., 2001b; Bartlett et al., 1998; Ohlsen and Gralla, 1992; Zhou and Jin, 1998). Conversely, positively regulated promoters form extremely stable complexes with RNA polymerase, such that initiation is halted, and it is argued that destabilization with ppGpp may, in fact, help such promoters (e.g. Barker et al., 2001a; Barker et al., 2001b). Another model proposed suggests that a ppGpp programmed RNA polymerase will dissociate from stable RNA promoters and as a consequence become available to initiate transcription at positively regulated promoters (Zhou and Jin, 1998). In this scenario, positively regulated promoters are indirectly controlled by ppGpp-dependent alterations in RNA polymerase availability.

While it is generally accepted that ppGpp is responsible for the immediate decrease in rRNA synthesis that occurs when a sudden restriction in nutrient supply occurs (e.g. Cashel and Gallant, 1969; Sands and Roberts, 1952; Stent and

Brenner, 1961; Travers, 1976), it have been, and still is, controversial whether ppGpp governs growth rate-dependent regulation of rRNA synthesis during exponential growth (reviewed in Cashel et al., 1996 and Condon et al., 1995). In essence, two flanks exist and could be described as pro or against ppGpp as a regulator of the phenomenon (e.g. Condon et al., 1995). The Pro-ppGpp model, "RNAP partitioning model" or "promoter selectivity model", (e.g. Baracchini and Bremer, 1988; Ryals et al., 1982b; Travers et al., 1980) argues that there are two forms of RNAP, one with and the other without ppGpp bound to it. If cells grow fast there is low levels of ppGpp, hence, low levels of "stringent" RNAP and therefore high transcriptional activity at rRNA operons. Whereas, if cells are growing slowly there are high levels RNAP programmed with ppGpp and thus little rRNA synthesis. In contrast to the model suggesting ppGpp as the regulator of growth rate-dependent regulation, the "ribosome feedback model" (based on Jinks-Robertson et al., 1983) proposes that the cells are prone to produce as many ribosomes as possible, but not more than is required for the rate of protein synthesis needed at the moment. The feedback signal is not known, but an excess of translational capacity (i.e. extra rRNA) has been proposed to be the sensor and/or signal (reviewed in Condon et al., 1995). In addition, Gaal *et al.* (1997) showed, in an *in vitro* transcription experiment, that the activity at stable RNA promoters correlates with the concentration of the initiating nucleotides (GTP and ATP). In addition, they showed that the GTP and ATP pools increased with increasing growth rates. Based on these results, the authors proposed that the nucleotides act as the feedback signal sensed by the rRNA operons and that they would be responsible for the growth rate-dependent regulation *in vivo*. In contrast, Peterson and Moller (2000) stressed that the NTP pools are independent of growth rate. Yet, given the complexity of rRNA regulation, it seems reasonable that different regulatory mechanisms might operate to varying degrees under different conditions.

Most studies on the effects by ppGpp have dealt with repression/activation of genes requiring σ^{70} but lately alternative sigma factors have come into focus as well. For instance, the Pu and Po promoters, dependent on σ^{54}, are induced only in the presence of ppGpp (Carmona et al., 2000; Sze et al., 2002; Sze and Shingler, 1999). Further, as mentioned above, σ^{S} is dependent on ppGpp for its accumulation

(Gentry et al., 1993; Lange et al., 1995; Zgurskaya et al., 1997) as well as its activity (PAPER IV).

Travers (1985) argued that ppGpp might act by loosening the protein-protein interactions between σ^{70} and RNA polymerase and thereby facilitating replacement of one sigma factor by another. VanBogelen and Neidhardt (1990) suggested that ppGpp might affect different sigma factors' affinity for E based on the sluggish and delayed induction of heat shock genes in a ppGpp^0 mutant (Grossman et al., 1984; Jones et al., 1992; VanBogelen and Neidhardt, 1990). In addition, Hernandez and Cashel (1995) showed that ppGpp drastically reduces the fraction of σ^{70} bound to E and put forward the idea that ppGpp may alter the competition between σ^{70} and alternative sigma factors.

5 Aims, results and discussion

The primary aim of this work was to elucidate the transcriptional regulation of the stationary phase induced universal stress proteins, UspA and UspB. The *uspAB* locus is located at the 77 min region of the *E. coli* chromosome (Nyström and Neidhardt, 1992). *uspA* and *uspB* are divergently transcribed and their translational sequences are separated by 390 bp, see figure 6.

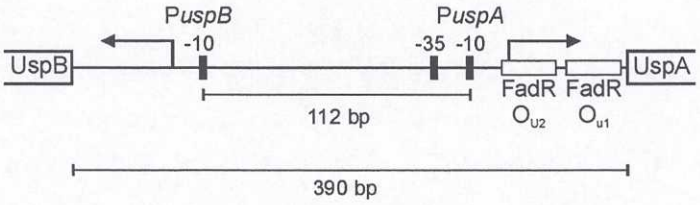

Fig. 7. The *uspA* and *uspB* loci (Not drawn to scale.)

The *uspA* gene is transcribed from a σ^{70}-dependent promoter located 125 bp upstream of the translational start (Nyström and Neidhardt, 1992). There are two FadR binding sites positioned between the transcriptional and translational start sites of *uspA* (Farewell et al., 1996). Expression of *uspB* is dependent on σ^S and there is a σ^S promoter located upstream of the translational start (PAPER II).

5.1 The universal stress protein A, UspA

Nyström and Neidhardt (1992) isolated and cloned the gene encoding a protein, UspA (universal stress protein A) that appeared to be induced during more stress conditions than any other protein observed on 2-D gels. The UspA protein is a serine/threonine phosphoprotein and is important for long-term survival during starvation (Freestone et al., 1998; Freestone et al., 1997; Nyström and Neidhardt, 1993; Riley and Labedan, 1996). UspA belongs to a conserved family of proteins (Usp family), which have been suggested to be ancestors to the developmental, DNA-binding, MADS-box proteins of eukaryotes (Mushegian and Koonin, 1996; Nyström and Gustavsson, 1998). Further, *uspA* homologues have been found in several bacterial species and often in multiple copies (e.g. Diez et al., 2000). *E. coli* harbors six Usps of which, at least four (UspA, C, D and E), seem to share the same pattern of expression (Gustavsson et al., 2002). Recently, it was shown that a *uspA* mutant was less resistant to UV irradiation and mitomycin C exposure and that DNA aberrations transduce RecA-dependent signals to the *uspA* promoter, which, however, only affect P*uspA* during stasis (Diez et al., 2000).

5.2 *uspA* requires ppGpp for induction

The induction of *uspA* is related to growth inhibition and the expression is primarily regulated at the level of transcription (Nyström and Neidhardt, 1992). The fact that many conditions that induce *uspA* are known to accumulate ppGpp made us examine if ppGpp could have a role in activation of *uspA* expression. Two-dimensional gel electrophoresis analysis of cells carrying a plasmid with the IPTG inducible P*tac-relA*' construct, pSM11 (Schreiber et al., 1991), demonstrated that UspA levels was elevated upon elevating ppGpp levels (PAPER I). Thus, elevated

levels of ppGpp are sufficient to elicit UspA production under otherwise non-stress conditions. In addition, a $ppGpp^0$ strain failed to produce UspA or to induce a P*uspA-lacZ* fusion in stationary phase and the *rpoB*3449 allele (A532Δ), that is epistatic to defects exhibited by a $ppGpp^0$ strain (i.e. growth in minimal media; e.g. Cashel et al., 1996; Zhou and Jin, 1998) suppressed this defect (PAPER I). It should be noted that previous results have indicated that *uspA* may be ppGpp independent (Farewell et al., 1996). However, it has now been demonstrated that the $ppGpp^0$ strain in this study harbored a suppressor mutation. It is not clear how ppGpp exerts its effect on positively regulated promoters requiring $E\sigma^{70}$. It has been proposed that the positive regulation of σ^{70}-dependent promoters by ppGpp is linked to ppGpp-dependent effects on RNAP availability. The accumulation of ppGpp is suggested to result in the dissociation of RNAP from stringent promoters (Bartlett et al., 1998; Zhou and Jin, 1998) resulting in more RNAP becoming available to initiate transcription at promoters that have a relatively poor ability to recruit RNAP. It cannot be excluded, however, that some σ^{70}–dependent promoters are directly regulated by ppGpp and examples of such positive effect of ppGpp on gene expression have been achieved using a coupled *in vitro* transcription-translation assay (Choy, 2000; Primakoff and Artz, 1979; Riggs et al., 1986; Stephens et al., 1975).

5.3 FadR and *uspA* regulation

uspA is a member of the FadR regulon and has two FadR binding sites downstream the promoter in the non-coding region (Farewell et al., 1996). FadR represses not only *uspA*, but also *fad* genes (genes involved in fatty acid degradation), which are induced in stationary phase both in *E. coli* (Farewell et al., 1996) and *S. typhimurium* (Spector et al., 1999). These observations led to the assumption that FadR is inactivated as a repressor when cells enter stasis (Farewell et al., 1996; Spector et al., 1999). However, since ectopic overproduction of ppGpp during exponential phase caused UspA accumulation (PAPER I), and a strain carrying the *rpoB*3449 allele (epistatic to ppGpp deficiency) showed markedly higher P*uspA-lacZ* expression under conditions that do not inactivate FadR (data not shown), we wondered if ppGpp, or the mutant RNA polymerase, could overcome FadR repression of *uspA* and whether FadR is really inactivated in stationary phase. FadR

has been shown to be inactivated by binding long-chain fatty acyl-CoAs (Raman and DiRusso, 1995), which are produced from fatty acids by the gene product of *fadD*. Yet, we observed no difference between a wt and a *fadD* null mutant with respect to *uspA* expression pattern or induction levels (unpublished). Therefore, unless long-chain fatty acyl-CoAs are produced via a hitherto unknown pathway, this result suggests that FadR is not inactivated in stationary phase. To elucidate this possibility, we introduced the strongest FadR operator known (the *fadB* O_B operator, see (Farewell et al., 1996)) centered at +9 with respect to the transcriptional start site of *uspA*. The β-galactosidase activity from this construct was greatly reduced in stationary phase cells containing functional FadR, but not in cells lacking FadR (PAPER I). Thus, we suggest that FadR is active in stationary phase and that a "stringent" RNA polymerase (i.e. presence of ppGpp or mutated RNAP) can override repression by FadR if the operator is sufficiently "poor".

5.4 The universal stress protein UspB

A search in the database for genes similar to *uspB* yielded only one (86% homology) candidate (PAPER II). The homologue, an uncharacterized ORF, was found in *Y. pestis*. As in *E. coli*, upstream (~700 bp) the *uspB*-like sequence in *Y. pestis* a divergently transcribed UspA homologue (89%) was found. Thus, the organization of this locus may be conserved between these species. However, a recent search for proteins with similarity to UspB (BLAST @ NCBI) generated some homologues in species such as *Salmonella typhimurium* and *Vibrio cholerae*. Likewise, a divergently transcribed gene similar to *uspA* was found to be localized upstream of the homologue of *uspB* in *S. typhimurium*. But, since the list of newly sequenced genomes is still growing, more homologues are expected to be revealed.

UspB is composed of 111 amino acids and has the electrophoretic mobility of a 14 kDa protein. Examinations by protein sequence analysis programs suggested that UspB contains two putative membrane-spanning domains and that the proximal one could be a signal peptide separated from the second domain by a putative signal peptide cleavage site. Notably, UspB does not belong to the new Usp family

of proteins, but was originally named UspB because it is induced during a large number of stress conditions (see below).

5.5 Phenotypic analysis of *uspB* mutants

A strain harboring a *uspB* null mutation was compared to its otherwise isogenic wild type parent with respect to growth and survival in a number of environmental conditions, including growth in minimal medium and LB, survival in glucose starvation and LB stationary phase, recovery from long-term starvation, survival during osmotic, heat, and oxidative stress, growth on various carbon sources (ribose, glycerol, acetate and glucose), and growth under anaerobiosis, but no differences were observed. However, the *uspB* null mutant did not develop as high resistance to ethanol as the wild type strain in stationary phase but no difference was observed during exponential growth (PAPER II). Ethanol is well known to affect protein stability as well as membrane integrity (Gross, 1996; Ingram and Vreeland, 1980). Intriguingly, no difference was observed, between the Δ*uspB* and wt strains, during a heat shock, which is, as ethanol, known to disturb protein stability and membrane integrity (Gross, 1996; Sanchez and Charlier, 1989). Thus, UspB might act through a separate pathway than heat-inflicted damages of the membrane in stationary phase. In addition, recent studies (A. Farewell, personal communication) have shown that UspB might be involved in resistance to UV irradiation and mitomycin C exposure. Overproduction of UspB is detrimental to cell viability, and high copy number plasmids containing the entire *uspB* gene are extremely unstable in *E. coli* (PAPER II). The reason for this is still unknown.

5.6 Expression pattern of *uspB*

By using a P*uspB-lacZ* fusion recombined into λ phage and integrated at the λ *att* site in the chromosome as a single copy we determined the expression pattern of *uspB*. In contrast to *uspA*, which is σ^{70}-dependent (Nyström and Neidhardt, 1992), *uspB* induction depends on the stationary phase sigma factor, σ^{S} (PAPER II). During exponential growth in LB the expression of *uspB* is very low but is induced about 50-fold as cells enter stationary phase. Similarly, we found that when cells were grown

in minimal medium limiting for various nutrients, such as glucose, phosphate and nitrogen, P*uspB-lacZ* expression was induced as cells entered stationary phase in agreement with the dependence on σ^S. Further, in addition to starvation conditions, the P*uspB-lacZ* fusion was induced during exponential growth if environmental challenges such as NaCl, sucrose (osmotic shock), H_2O_2 (oxidative stress) or 4% ethanol were imposed upon cells. To determine the mode of regulation of *uspB* a number of mutations in known global regulators were introduced into the strain carrying the P*uspB-lacZ* fusion. Mutations in the nucleoid protein H-NS, known to increase σ^S levels in log phase (Yamashino et al., 1995), showed a seven-fold increased expression of *uspB* in log phase and a two-fold increase in stationary phase. The sequence in the region 85 bp upstream of the transcriptional start of *uspB* resembles an IHF binding site (Nash, 1996). Overproduction of IHF from a plasmid caused a three-fold reduction of P*uspB-lacZ* expression. However, a deletion of the putative IHF binding site did not change the effect of overproduction of IHF. We concluded that IHF has an indirect effect on *uspB* expression unless IHF binds to a hitherto unknown DNA sequence in the *uspB* region. Finally, *uspB* does not seem to be autoregulated since a Δ*uspB* mutation did not alter the expression pattern of P*uspB-lacZ* under any conditions tested. In conclusion, although some known global regulators (IHF, H-NS and CRP; PAPER II) are involved in regulation of *uspB* none of them seems to be essential for growth phase dependent expression of *uspB*.

5.7 *uspB* and *uspA* expression is affected by sigma factor competition

Interestingly, a mutation in *rpoS* not only abolishes induction of σ^S-dependent genes but "superinduces" genes requiring other sigma factors (PAPER III). For instance, the rate of synthesis of UspA (σ^{70}-dependent) and Psp (σ^{54}-dependent) is higher in stationary phase in a Δ*rpoS* mutant compared to an *rpoS*$^+$ strain. One explanation for this may be that if one sigma factor (σ^S in this case) is not expressed, other sigma factors will be favored in core RNA polymerase binding and genes requiring such sigma factors will be "superinduced". Such a hypothesis has previously been put forward based on data demonstrating that in cells harboring a temperature

sensitive σ^{70}, several σ^{32}-dependent genes are "superinduced" during a temperature shift from 30°C to 42°C (Osawa and Yura, 1981). To test if sigma factors compete for core RNAP, we introduced a plasmid-borne P*tac-rpoD* construct (Bedwell and Nomura, 1986) into our test strain and measured expression patterns of the σ^{70}-dependent promoters P*uspA* and P*fadD*. Overproduction of σ^{70} (~2.5-fold) caused an essentially identical superinduction of P*uspA-lacZ* and P*fadD-lacZ* as an *rpoS* null allele (PAPER III). In addition, *lacZ* fusions to the σ^{S}-dependent *bolAp1* and P*uspB* promoters were essentially shut down when σ^{70} were overproduced (western blot analysis showed that the levels of σ^{S} were the same in the strains whether σ^{70} was overproduced or not). Similarly, *uspA* expression decreased when a high-copy-number plasmid, pMMKatF2 (Mulvey et al., 1988), overproducing σ^{S} (~10-fold) was present in the cells. These results strongly support the idea that sigma factors compete for limiting amounts of core RNA polymerase. Therefore, it is fascinating how σ^{S} manages to compete and operate successfully in stationary phase, despite its relatively low levels (30% of σ^{70}; Jishage and Ishihama, 1995) and its weak affinity for core (approximately 16-fold lower than σ^{70} *in vitro*; Maeda et al., 2000).

Another possible explanation for the "superinduction" of *uspA* could be that an *rpoS* null mutant has lower levels of FadR and therefore higher *uspA* expression. To test, this we introduced a *fadR*::Tn10 allele, by P1 transduction, into our test strains (wt or Δ*rpoS*) and monitored P*uspA-lacZ* expression. The *fadR*::Tn10 mutation only affected P*uspA-lacZ* expression during exponential growth, while the *rpoS* mutation only had an effect in stationary phase. Accordingly, the Δ*rpoS fadR*::Tn10 double mutant displayed higher expression of P*uspA-lacZ* in both log and stationary phase, indicating that the repression of *uspA* by σ^{S} and FadR are mediated via different pathways as well as during different growth phases. Another idea of how RpoS could mediate repression of *uspA* transcription was raised by the fact that a putative IHF site is located upstream of *uspA* (PAPER II, III), and that overexpression of IHF affect UspA expression positively (Nystrom, 1995). Previously, it has been shown that IHF can bend DNA so that upstream DNA with bound proteins comes in proximity with downstream regulatory sequences, such as promoters (e.g. Nash, 1996). Thus, if IHF binds the putative IHF binding site upstream of P*uspA* the DNA may bend so that the P*uspB*-Eσ^{S} complex will interfere with sigma 70 programmed

RNAP to initiate transcription from P*uspA*. To approach this possibility, we constructed a mutation in the IHF consensus sequence as well as a deletion of the upstream DNA from the middle of the putative IHF binding site and measured P*uspA-lacZ* expression. As shown in PAPER III, neither of these two mutations of the *uspA* promoter region had any effect on *uspA* expression. In addition, IHF overproduction and an *rpoS* mutation caused an additive effect on *uspA* expression, indicating that the pathways are distinct. Thus, it is clear that *uspA* expression is not dependent on the region from the middle of the IHF consensus sequence and upstream and that the effects of an *rpoS* null mutation and IHF are most likely indirect.

5.8 σ^S and ppGpp is required in concert for induction of σ^S-dependent promoters

σ^S requires ppGpp for its own accumulation (Gentry et al., 1993; Lange et al., 1995; Zgurskaya et al., 1997) and it was suggested that the similar phenotypes found in ppGpp^0 and Δ*rpoS* mutants was due to this fact. However, we found that no activity from the σ^S-dependent promoters tested (P*uspB*, *bolA*P1, P*cfa* and P*fadL*) was detectable in the ppGpp^0 strain even when σ^S levels were ectopically produced to levels corresponding to wild type levels (PAPER IV). The results suggested that ppGpp confers dual control on the RpoS regulon by i) being essential for efficient expression and accumulation of σ^S and, ii) required for σ^S function *per se*. Interestingly, the *rpoB* allele, *rpoB*3449, suppressed the lack of induction of the σ^S-dependent promoters tested in the Δ*relA* Δ*spoT* strain (PAPER IV). Thus, the *rpoB*3449 allele restores both accumulation of σ^S and the function of Eσ^S.

5.9 Competition between alternative sigma factors and σ^{70} is affected by ppGpp

Previously, Hernandez and Cashel (1995) had shown that the amounts of σ^{70} bound to RNAP core is elevated in a ppGpp^0 strain compared to a wt strain. Also, if the *rpoD* mutations *rpoD*S506F and *rpoD*P504L (epistatic to ppGpp deficiency) were

introduced into the ppGpp^0 strain, the levels of σ^{70} bound to core were similar to the levels observed in a wild type strain. This, together with the fact that the *rpoB*3449 allele suppresses the lack of induction of σ^S-dependent promoters in a ppGpp^0 background (PAPER IV), made us investigate if ppGpp is an absolute requirement for σ^S function.

First, we found that the lack of P*uspB-lacZ* induction in a ppGpp^0 mutant could be alleviated by under-producing σ^{70} or overproducing Rsd (anti-σ^{70}), especially if the cells harbored the multi-copy plasmid pMMKatF2, carrying the structural gene for *rpoS* (PAPER V). This indicated that ppGpp is not an absolute requirement for σ^S function as long as the level of active σ^{70} is reduced. To further investigate this, we made use of two *rpoD* mutants (*rpoD*35 and *rpoD*40) functionally related to *rpoD*S506F and *rpoD*P504L that had been isolated in V. Shinglers laboratory (A. Laurie and V. Shingler, unpublished). These mutations were shown to suppress the ppGpp requirement of the σ^{54}–dependent Po promoter (A. Laurie and V. Shingler, unpublished; Sze and Shingler, 1999). Expression of the model promoter, P*uspB*, was partially rescued by these *rpoD* alleles in a ppGpp^0 background (PAPER V). Thus, *rpoD* mutants that rescue a σ^{54}-dependent promoter, also restore induction of a σ^S-dependent promoter, in a ppGpp^0 background. This prompted us to set up an *in vitro* transcription (IVT) competition assay to test if ppGpp may allow alternative sigma factors to compete more successfully with the housekeeping sigma factor, σ^{70}. Since it is known that σ^{70} and σ^S recognizes similar promoter sequences (e.g. Kusano et al., 1996), and exhibit cross reactivity *in vitro*, we decided to perform *in vitro* competition with σ^{32}, which is known to recognize vastly different promoters than σ^{70} (Gross et al., 1992). To elucidate the relative competitiveness (inhibition of transcription from P*dnaK*) of the wt and mutant σ^{70}, we added increasing amounts of the different σ^{70} proteins to our IVT reaction mix that contained a fixed level of σ^{32}, and measured the relative level of the *dnaK* transcript. Increasing amounts of wt σ^{70} drastically inhibited transcription from *dnaK*. Inhibition by the RpoD35 protein was significantly lower than by wt σ^{70} and the RpoD40 protein was the least effective in inhibiting transcription from the *dnaK* promoter (PAPER V). Next we examined if the behavior of the mutant RpoD proteins would mimic the phenotype of a normal stringent response; i.e. if ppGpp would reduce the

competitive ability of wt σ^{70}. We approached this question by repeating the *in vitro* competition assay with wild type σ^{70} and σ^{32} competing in the presence and absence of ppGpp. The competitiveness of wt σ^{70} was markedly reduced by the addition of ppGpp. Thus, ppGpp has a positive effect on *in vitro dnaK* transcription under conditions of competition between σ^{32} and σ^{70} but have no observable effect when σ^{32} operates alone (ppGpp had no effect on the transcriptional activity from the *dnaK* promoter when no σ^{70} was provided to the system; PAPER V). Next, we used *lac* fusions to σ^{70}-dependent promoters and measured the ability of σ^{S} and σ^{32} to down-regulate these promoters in wt and ppGpp^0 strains. We found that both σ^{S} and σ^{32} were clearly better in down-regulating the σ^{70}-dependent promoters in the presence of ppGpp.

In addition, to determine whether the observed effects were the result of competition for E binding, we repeated the same experiment with ectopic overproduction of the Q80R mutant σ^{32}, which exhibits a drastically reduced affinity for E (Joo et al., 1997). Indeed, overproduction of this mutated sigma totally failed to repress the σ^{70}-dependent promoter P*uspA*, whereas wt σ^{32} was very effective. This confirms that the observed inhibition of *uspA* and *fadD* transcription is an effect of σ^{32} out-competing σ^{70} for E binding. Next, we measured the fraction of total σ^{S} and σ^{32} bound to core in wt and ppGpp^0 strains and observed a substantial lower portion of each sigma factor bound to core in the Δ*relA* Δ*spoT* strain than in the wt.

Thus, it appears that the ability of σ^{S} and σ^{32} to compete with σ^{70} for E binding is facilitated in the presence of ppGpp and that the ppGpp requirement can be suppressed by σ^{70} underproduction. Further, ppGpp-dependent alteration in sigma factor competition for E binding seems to be an integral part of the typical stringent response. Thus, despite the fact that ppGpp accumulation decreases the competitiveness of σ^{70} during stringency, many Eσ^{70}-dependent promoters may well experience an increased Eσ^{70} availability since a large fraction of the holoenzyme is no longer sequestered in transcribing stable RNA operons.

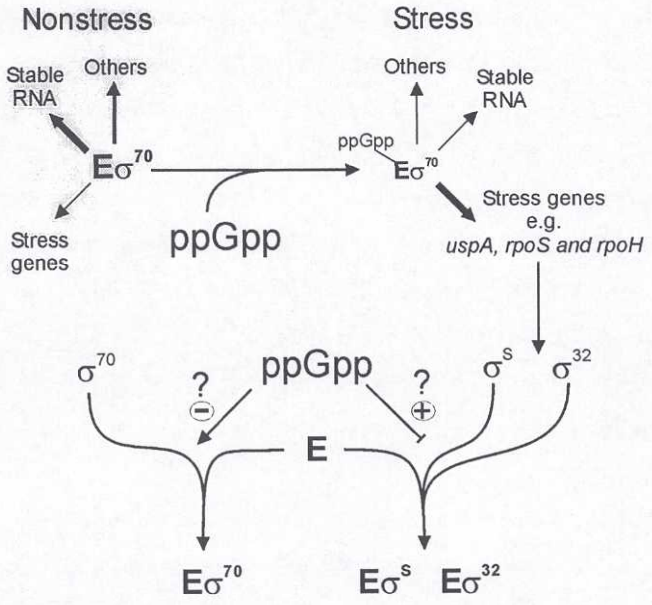

Fig. 7. Model of ppGpp action during stress. ppGpp accumulates and binds core RNAP (E), which is directed away from stable RNA operons to stress genes. Simultaneously, σ^s and σ^{32} will compete better against σ^{70} for E binding.

Additional experiments, such as affinity measurements between σ^S and core RNA polymerase, with or without ppGpp, are required to pinpoint an exact mechanism for the results obtained. For instance, is ppGpp acting by weakening σ^{70} core interaction and/or strengthening $\sigma^S/\sigma^{32}/\sigma^{54}$ core interaction? (see figure 7).

6 Conclusions

A new member, *uspB*, of the sigma S regulon was discovered. The protein, UspB, is involved in stationary phase induced resistance to ethanol. Studies on the transcriptional regulation of *uspB* demonstrates that σ^S is not only dependent on ppGpp for its accumulation in stationary phase, but also for its activity because *uspB* and other σ^S-dependent genes require ppGpp even when σ^S levels are ectopically produced to wild type levels. This requirement can be explained, in part, by the fact that alternative sigma factors (σ^S and σ^{32}) compete better against σ^{70} for core RNA polymerase in the presence of ppGpp both *in vitro* and *in vivo*. For instance, a significantly lower level of σ^S and σ^{32} are bound to core in cells lacking ppGpp and σ^{32}-dependent transcription *in vitro* is facilitated in the presence of ppGpp under conditions of competition between σ^{32} and σ^{70} but have no observable effect when σ^{32} operates alone.

Another novel role of ppGpp was found during analysis of the σ^{70}-dependent *uspA* promoter, in that ppGpp accumulation overcomes repression by an active FadR. This mechanism of regulation is important since the induction of *uspA* and other FadR regulated genes is essential for stasis survival and we called this regulation emergency derepression.

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"Don't mention the war..."
Basil Fawlty

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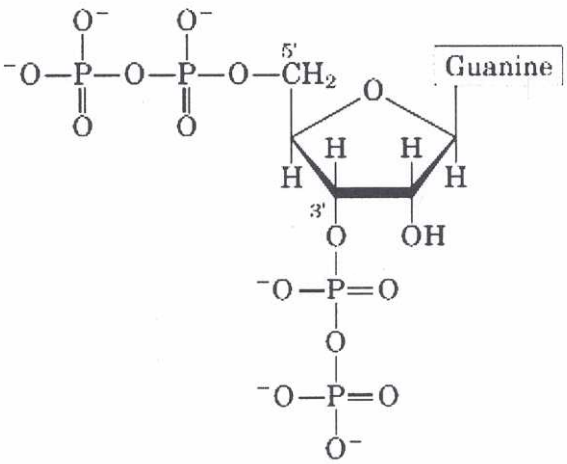

Guanosine 3'-diphosphate,5'-diphosphate
(guanosine tetraphosphate)
(ppGpp)

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