On the role of small regulatory molecules in the interplay between σ^{54}- and σ^{70}-dependent transcription

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Education's purpose is to replace an empty mind with an open one.
Malcolm Forbes (1947-), American Publisher

The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!', but 'That's funny...'
Isaac Asimov (1920-1992), American Writer

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PAPERS IN THIS THESIS

This thesis is based on the following articles that are referred to in the text by their roman numerals.

I. Bernardo LM, **Johansson LU**, Solera D, Skärfstad E, and Shingler V (2006). The guanosine tetraphosphate (ppGpp) alarmone, DksA, and promoter affinity for RNA polymerase in regulation of σ^{54}-dependent transcription. *Mol Microbiol.* 60(3):749-64.

II. Szalewska-Palasz A**, Johansson LU**, Bernardo LM, Skärfstad E, Stec E, Brännström K, and Shingler V (2007). Properties of RNA polymerase bypass mutants: Implications for the role of ppGpp and its co-factor DksA in controlling transcription dependent on σ^{54}. *J Biol Chem*. 282(25):18046-56.

III. Bernardo LM*, **Johansson LU***, Skärfstad E, and Shingler V (2009). σ^{54}-promoter discrimination and *in vivo* regulation by ppGpp and DksA. *J Biol Chem*. 284(2):828-38.

IV. Johansson LU, Solera D, Bernardo LM, Moscoso J, and Shingler V (2008). σ^{54}-RNA polymerase controls σ^{70}-dependent transcription from a non-overlapping divergent promoter. *Mol Microbiol.* 70(3):709-23.

* The first two authors contributed equally to this work.

LIST OF ABBREVIATIONS

AAA+	ATPases associated with diverse cellular activities
Acetyl CoA	Acetyl Co-enzyme A
ADP	adenosine diphosphate
ATP	adenosine triphosphate
B. subtilis	*Bacillus subtilis*
bEBP	bacterial Enhancer Binding Protein
C23O	catechol-2,3-dioxygenase
C-terminal	carboxy terminal
Dmp	dimethylphenol
DNA	deoxyribonucleic acid
DksA	DnaK suppressor A
ECF	extracytoplasmic function
E. coli	*Escherichia coli*
FIS	factor for inversion stimulation
GDP	guanosine diphosphate
GTP	guanosine triphosphate
HTH	helix-turn-helix
Hfq	Host Factor Q
H-NS	heat-stable (or histone-like) nucleoid-structuring protein
HU	heat-unstable nucleoid protein
IHF	Integration Host Factor
mRNA	messenger RNA
NCR	non-conserved region
NMR	nuclear magnetic resonance
N-terminal	amino terminal
NTP	nucleoside triphosphate
ppGpp	guanosine tetraphosphate
pppGpp	guanosine pentaphosphate
P. putida	*Pseudomonas putida*
RelA	ppGpp synthetase I
RNA	ribonucleic acid
RNAP	RNA polymerase
rRNA	ribosomal RNA
SpoT	ppGpp synthetase II
tRNA	transfer RNA
T. thermophilus	*Thermus thermophilus*
UAS	upstream activating sequence
UV	ultra violet

ABSTRACT

Signal responsive transcriptional control in bacteria is mediated through both specific and global regulatory circuits to attune promoter output to prevailing conditions. Divergent transcription of a regulatory gene and a cognate promoter under its control provides an opportunity for interplay between transcription dependent on RNA polymerases utilizing various σ-factors, each of which programs the holoenzyme to recognize different classes of promoters. The work presented in this thesis analyses the consequences and mechanisms behind interplay between σ^{54}- and σ^{70}-dependent transcription within the *dmp*-system of *Pseudomonas* sp. CF600. The *dmp*-system confers the ability to grow at the expense of (methyl)phenols and is controlled by two promoters that drive non-overlapping divergent transcription from a common intergenic region: i) the σ^{54}-Po promoter, which controls an operon encoding a suit of specialized catabolic enzymes, and ii) the σ^{70}-Pr promoter, which controls production of the aromatic sensor DmpR – a mechano-activator whose transcription-promoting activity is obligatory for activity of the σ^{54}-Po promoter.

The σ^{54}-Po promoter and its dependence on two non-classical transcriptional regulators – the alarmone ppGpp and its co-factor DksA that directly target RNA polymerase – are the focus of the first part of the thesis. These studies utilized ppGpp and DksA deficient strains, mutant RNA polymerases that bypass the need for ppGpp and DksA, reconstituted *in vitro* transcription systems, and a series of DmpR-regulated hybrid σ^{54}-promoters with different affinities for σ^{54}-RNAP, together with analysis of protein levels of key transcriptional components. Collectively with previous work, these studies provide the experimental support for a robust but purely passive mechanism for ppGpp and DksA global regulation of σ^{54}-transcription, which is likely to also be pertinent for transcription mediated via any alternative σ-factor (Papers I-III). The second part of the thesis focuses on additional roles of ppGpp and DksA through their direct and indirect effects on the activity of the σ^{70}-Pr promoter. These studies unexpectedly revealed that the σ^{70}-Pr promoter is regulated by a novel mechanism in which σ^{54}-RNA polymerase occupancy and activity at the σ^{54}-Po promoter stimulates σ^{70}-Pr output. Evidence is presented that ppGpp and DksA, through DmpR levels, control a feed-forward loop to reinforce silence of the σ^{54}-Po promoter under high energy conditions with robust transcription from σ^{54}-Po when the catabolic enzymes are needed. The interplay outlined above effectively places a σ^{70}-dependent promoter under dual control of two forms of RNA polymerases, and also makes it subservient to regulatory signals that elicit activity of σ^{54}-RNA polymerase. The possibility that such dual sensitivity may be a prevalent, but previously unappreciated, mechanism by which bacteria integrate diverse and/or conflicting signals to gain appropriate transcriptional control is discussed.

INTRODUCTION

1. BACTERIA AS BIOREMEDIATION TOOLS

Many aromatic hydrocarbons are produced naturally in the environment, but hand in hand with industrialization, they have accumulated in soil and waters to toxic levels. Furthermore, continuously increasing use of aromatic hydrocarbons with unusual substitutions on the highly stable benzene ring, for example in wood preservatives and precursors of dyes and plastics, has increased pollution problems associated with this class of compounds. Traditional methods for cleaning contaminated areas, involving vacuum and chemical extraction and/or removal of the contaminated soil, simply move the problem elsewhere. However, a cheaper and more environmental friendly way of achieving the same goal is bioremediation - processes that use the metabolic capacities of microorganisms to transform the polluting compound to simpler molecules. Incorporation of bioremediation strategies in environmental clean-up is expanding, and has the added advantage that the carbon locked in stable aromatic compounds is efficiently fed back into the carbon-cycle (Dua et al, 2002; Timmis & Pieper, 1999).

The evolution and adaptation of soil dwelling biodegradative bacteria occur under their harsh living conditions with lack of nutrients, drought, presence of toxic compounds and changes in osmolarity, temperature and pH being part of their everyday life. To be able to persist, they have evolved a wide variety of sophisticated mechanisms to counteract physicochemical assaults. Adaptation is most often achieved through signal-responsive changes in gene expression, leading to higher or lower levels of the gene product(s). Counteractive adaptation mechanisms include changes in e.g. cell membrane permeability, production of biomolecular efflux pumps that remove compounds from the cell, and the ability to metabolize the toxic compounds as a means of both removing them from their surroundings and at the same time to use them as nutrient sources.

There are several examples among the highly adaptable gram negative Pseudomonads bacteria that efficiently degrade different groups of toxic aromatic compounds (Nelson et al, 2002). Interestingly, these also include compounds that have only been produced and introduced into the environment over the last 30-100 years, indicating a relatively rapid evolution of the metabolic systems carried by these degradative strains. Aspects that facilitate such rapid evolution include the frequent location of these auxiliary catabolic systems on extra-chromosomal DNA (e.g. plasmids) or mobile DNA (e.g. transposons), which makes it easier to mix the genetic information between different strains to create new gene combinations and thus new biochemical abilities (van der Meer et al, 1992).

Today a lot of research is carried out within the field of bacterial metabolism of aromatic compounds. Complete genome sequences are available for a few strains within the *Pseudomonas* genus, and many of the biochemical steps during degradation of aromatic compounds are known in molecular detail. Production of the catabolic enzymes involved in the metabolic pathways is mostly regulated at the transcriptional level. To be able to take efficient advantage of these degradative bacteria within bioremediation, however, there is still a lot to learn about how transcriptional regulation is integrated under different environmental conditions to allow the bacterium to respond appropriately to multiple, and sometimes opposing, regulatory signals. This thesis focuses on transcription regulation of the (methyl)phenol catabolic *dmp*-system of *Pseudomonas* sp. strain CF600, with an emphasis on the mechanisms that underlie coupling of production of the catabolic enzymes to the energy status of the cell. The remaining part of this section thus introduces the different players involved in bacterial transcription and the regulation thereof.

2. TRANSCRIPTION IN BACTERIA

Transcription is the process where RNA is made as a copy of DNA. In bacteria this procedure is performed by DNA dependent RNA polymerase (RNAP), the structure and function of which is further delineated in sections 2.2-2.6. Initiation of transcription by RNAP is a primary access point for controlling the flow of genetic information to the production of small non-coding regulator RNAs (nRNAs), ribosomal RNA (rRNA) and transfer RNA (tRNA) that are involved in the translation process from messenger RNA (mRNA) into proteins. Regulatory components involved in modulating the transcriptional process respond to very diverse signals from the surroundings to mediate appropriate responses as expanded on in the following section.

2.1. Gene expression is subject to both specific and global regulatory input

Signal-responsive control of gene expression can be brought about at the level of transcription (as mentioned above), at the post-transcriptional level (e.g. via translational regulation by nRNAs or RNase-mediated regulation of RNA stability), and at the post-translational level for instance by the requirement of a co-factor, correct folding of the peptide, or by covalent modifications of proteins. The different levels of regulation are mediated by both specific and global stimuli, and can act on either individual genes or multiple genes simultaneously.

To get several related genes coordinately regulated in response to the same stimuli, bacteria have developed the polycistronic operon. Within an operon, several genes situated next to each other are transcribed from the same promoter. In this way a number of genes can be controlled simultaneously by one single regulator whose activity in

turn is regulated by a given stimulus from the surroundings. The specific signals that control the activities of individual regulators are often related to the function of the operon(s) they control. For instance, in the case of a metabolic pathway-encoding operon, the signal to the regulator is usually a substrate or an intermediate of the pathway; which thus places the operon under specific-signal control. Multiple poly- or mono-cistronic operons can also be differentially but coordinately regulated on a specific level when forming a regulon where the same protein negatively or positively affects multiple promoters. This contrasts a stimulon, where a group of genes and/or operons are responsive to a defined signal without consideration of the regulatory proteins that cause the signal-responsive effect (reviewed in Cases & de Lorenzo, 2005).

Within bacteria, specific regulation of a gene, an operon or a regulon, is frequently dominated by so called global regulation. Global regulation enables the bacterium to sort between the vast numbers of stimuli it is exposed to and prioritize its responses to place the bacterium in as an advantageous position as possible in relation to the other bacteria in its vicinity. The factors involved in global regulation often work through the activity of individual promoters, and this activity is dictated by the levels and/or activity of individual forms of RNAP and regulatory proteins. These, in turn, are ultimately dictated by metabolic signals resulting from the adaptation of the bacterium to its current environment (reviewed in Cases & de Lorenzo, 2005; Shingler, 2003).

2.2. RNA polymerase and its subunit structure during the transcription cycle

The multi-subunit RNAP constitutes one of the largest proteins within bacteria. The catalytic part of this enzyme is called core RNAP and consists of two α-subunits and the β-, β'- and ω-subunits, which together form a thermodynamically stable complex of approximately 400 kDa. The α-subunits function as a scaffold, on which β and β' are assembled with the aid of the ω-subunit (Ghosh et al, 2001). Within the core enzyme, β and β' form a crab-claw like structure, which encompass the Mg^{2+} ion-containing active site where the actual RNA catalysis is performed (Murakami et al, 2002).

The transcriptional process can be divided into four distinct steps; promoter localization, initiation, elongation and finally termination (Fig. 1), out of which the core RNAP can perform the later three on its own. The core RNAP can initiate transcription at random from nicks, ends and single stranded parts of the otherwise double stranded DNA. However, for specific promoter localization, the additional and exchangeable sigma (σ) subunit is needed. A σ-factor bound to the core RNAP forms a σ-RNAP holoenzyme, which is guided to distinct

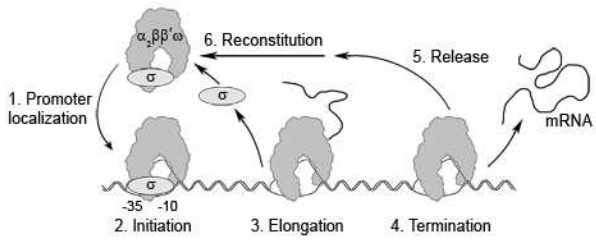


Fig. 1. The distinct steps of the transcription cycle mediated by the RNA polymerase are 1) promoter localization, 2) initiation, 3) elongation and 4) termination.

promoter sequences by the DNA-binding properties of the σ-factor. The role of the σ-factor in the context of the σ-RNAP holoenzyme stretches further than functioning as a guide. It also participates in the initiation process by aiding the separation of double stranded DNA into single stranded DNA (a process called melting or isomerization) to form the so called open RNAP-promoter complex (Gross et al, 1998; Wösten, 1998), and also functions as the target for some transcriptional activators. The σ-factor is stochastically released from the holoenzyme during the elongation phase (Raffaelle et al, 2005). The transcription elongation phase is terminated by either the helicase activity of the termination factor Rho, or by the formation of a nascent RNA hairpin structure. After termination, the released core RNAP can re-associate with a σ-factor, and the transcription process from a promoter can start all over again (Richardson & Greenblatt, 1996).

2.3. The specificity RNA polymerase subunit σ

In general it can be said that the nature and living conditions of a given bacterial species are reflected by the number of σ-factors encoded in its genome (Kill et al, 2005). For instance, a dedicated intracellular pathogen living under relatively constant conditions often only encodes one σ-factor (e.g. *Mycoplasma genetalium*), the gut-commensal *Escherichia coli* encodes seven different σ-factors, the highly adaptable *Pseudomonas putida* encodes 24 different σ-factors, while *Streptomyces coelicolor* encodes more than 60 (Martinez-Bueno et al, 2002). A higher number of σ-factors provides abundant opportunities for rapid and dedicated adjustments of transcriptional patterns because different σ-factors direct binding of the holoenzyme σ-RNAP to different promoter DNA signature sequences. Promoters can thus be divided into different classes where each class is preferably recognized by a given σ-factor (Lonetto et al, 1992). Each promoter class often controls production of gene products that are involved in similar processes (Table 1). Thus, by controlling the levels and/or activity of a σ-factor, a whole class of promoters and its cognate set of genes can be turned on or off, or tuned up and down.

Table 1. σ-factors and their functions within the bacterium. Adapted from (Wösten, 1998).

Group	Examples	Functions
Primary σ-factors	σ^{70}/σ^{D}: Gram-negative bacteria σ^{A}: Gram-positive bacteria MysA: Mycobacteria HrdB: *Streptomyces*	House-keeping σ-factor, main σ-factor in exponentially growing cells
Stationary phase σ-factors (non-essential)	σ^{S}/σ^{38}: Enterobacteria, *Pseudomonas* SigB-E: Cyanobacteria	σ-factor induced upon entry into stationary phase and/or during hyper-osmotic or acid stress σ-factor induced during circadian responses, carbon and nitrogen limitation and stationary phase
Flagella σ-factors	σ^{F}/σ^{28}: Enterobacteria WhiG: *Streptomyces* σ^{D}: *Bacillus subtilis*	Expression of late flagellar components, chemotaxis and/or early sporulation genes
Extracytoplasmic function (ECF) σ-factors	σ^{E}/σ^{24}: *E. coli*, Mycobacteria CarQ: *M. xanthus* AlgU: *P. Aeruginosa* SigV-Z: *B. subtilis*	Expression of genes involved in alginate biosynthesis, iron uptake, antibiotic production, induction of virulence factors, outer membrane proteins and extreme heat shock
Heat shock and related σ-factors	σ^{H}/σ^{32}: Gram-negative baceria SigB: *M. xanthus* SigC: *S. aurantiaca* σ^{B}: *B. subtilis*	Expression of genes involved during stress counteraction, fruiting body formation, maturation of myxospores and late sporulation
Sporulation σ-factors	σ^{H}, σ^{F}, σ^{E}, σ^{G}, σ^{K}: *Bacillus, Clostridium*	Involved in sequential expression of genes required for sporulation
σ^{54}-class of σ-factors	σ^{54}/σ^{N}: Enterobacteria, *Rhizobium, Pseudomonas, Bacillus, Caulobacter, M. xanthus*	Expression of genes involved in e.g. nitrogen fixation, nitrate utilization, glutamate synthesis, aromatic degradation and fimbriae and flagellar synthesis

Regulation of σ-factors is mediated at several different levels and can be highly complex. Some σ-factors are only produced under certain conditions while others are kept in an inactive state in the cell until they are needed. One example of a σ-factor that is only synthesized in response to specified signals is the stress and starvation σ^{S}-factor, encoded by the *rpoS* gene. In *E. coli,* transcription of this gene is stimulated by the global regulatory nucleotide ppGpp, which is discussed in section 3.1. Translation of the *rpoS* mRNA is also regulated, being stimulated by Hfq and HU through the mRNA secondary structure. In addition, phosphorylated RssB modifies the availability of σ^{S} by delivering this protein to the ClpXP protease for degradation (reviewed in Hengge-Aronis, 2002). Another way of controlling σ-factor availability is through a specific anti-σ-factor that sequesters a cognate σ-factor so that it is maintained in the cell but in a state where it cannot associate with core RNAP (e.g. σ^{E} and its cognate anti-σ-factor RseA; Alba & Gross, 2004). Signals that cue the need for promoter activities controlled by the σ-factor, lead to inhibition or degradation of the anti-σ-factor and consequent release of the σ-factor to allow association with core RNAP and thus transcription.

Control of different promoter classes through cognate σ-factors is also influenced by their binding properties for core RNAP that vary in 1 to 10 nM range. These determine the relative levels of the different σ-RNAP holoenzymes and thus occupancy of the different promoter classes (Grigorova et al, 2006 and references therein). Recently it has been found that auxiliary proteins can influence holoenzyme formation. For example formation of σ^{S}-RNAP and σ^{70}-RNAP is facilitated by the Crl protein in *E. coli* (Gaal et al, 2006).

In addition to the hierarchies between promoter classes created through varying holoenzyme pools, the innate affinities of different promoters within a given class also vary, creating promoter hierarchies within a class upon which signal-responsive transcriptional activators and repressors act. Taken together, the various and complex regulation of the levels and activities of different σ-factors and regulators that result from internal and external cues, combined with differing affinities of promoters for their cognate σ-RNAP constitute an immense repertoire for signal-responsive control of transcription of genes to ensure that each is transcribed at a level appropriate to prevailing conditions.

2.4. The σ^{70}-class of σ-factors

The different σ-factors can be divided into two distinct classes based on sequence and structure resemblance; namely the large σ^{70}-class of σ-factors and the structurally distinct σ^{54}-class (Fig. 2). The σ^{70}-class is named after the housekeeping σ-factor in e.g. *E. coli* and *P. putida*, which is the σ-factor that is involved in recognizing promoters controlling genes responsible for growth and proliferation of the bacterium. The name σ^{70} originates from the molecular weight of the *E. coli* protein (70 kDa). Another name for the same protein is σ^{D}, derived from the name of the *rpoD* gene that encodes σ^{70} (Lonetto et al, 1992). In the context of the σ^{70}-holoenzyme, σ^{70} recognizes and binds to specific promoter sequences located at -35 and -10 with respect to the transcription +1 start site. The consensus of these sequences is TTGACA-N17-TATAAT, where N is any nucleotide (Records et al, 1996).

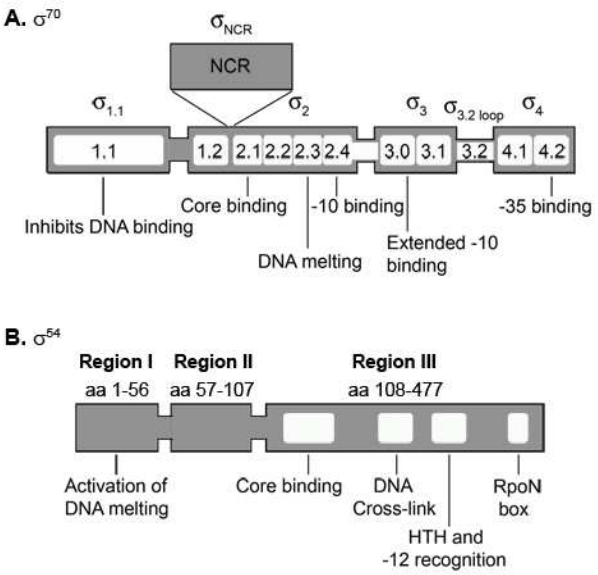


Fig. 2. Schematic illustrations of the exchangeable RNA polymerase subunits σ^{70} (A) and σ^{54} (B). NCR: non-conserved region, HTH: helix-turn-helix. Adapted from (Murakami & Darst, 2003) and (Gallegos & Buck, 1999), respectively.

Based on phylogenetic relatedness, the σ^{70}-class of σ-factors has been divided into four different groups (Lonetto et al, 1992; Lonetto et al, 1994), with group 1 containing the primary housekeeping σ-factor orthologs (e.g. σ^{70} in *E. coli*, and σ^{A} in *Thermus aquaticus* and *Bacillus subtilis*). σ-factors of group 2 are present in some species and are dispensable for growth. These are closely related to group 1 and the most extensively studied member of this group is the *E. coli* stress factor σ^{S} (Hengge-Aronis, 2002). Group 3 σ-factors are less conserved than the σ-factors in group 1 and include the heat shock (e.g. *E. coli* σ^{32}) and sporulation (e.g. *B. subtilis* σ^{F}) σ-factors. FecI, dedicated to controlling iron acquisition, is one member of group 4 into which the extracytoplasmic function (ECF) σ-factors are sorted. The σ-factors in this last group show relatively little sequence similarity to the other σ^{70} groups (reviewed in Gruber & Gross, 2003).

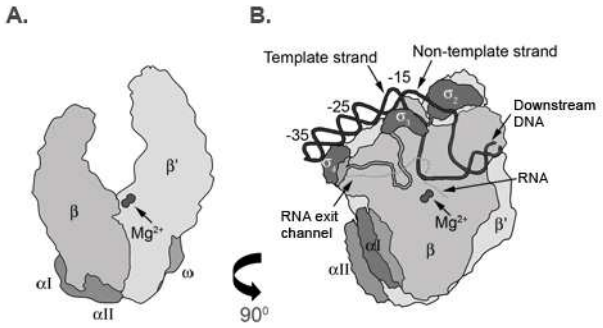


Fig. 3. Schematics of (A) a side view of the core RNA polymerase and (B) a front view of the σ^{70}-RNA polymerase holoenzyme during open complex formation. Subunits as labeled in the figure. Nascent RNA (not yet synthesized in the open complex) is indicated by a light grey line. Adapted from (Hsu, 2002).

The σ^{70}-class of proteins contain up to four distinct regions, which are further divided into sub regions (Fig. 2A), with each region ascribed distinct functions in binding to the core RNAP and the promoter DNA (Gross et al, 1998). These regions are spread across the upstream surface of the core RNAP crab-claw, as illustrated for the σ^{70}-RNAP in Fig. 3B. The highly negatively charged region 1.1 (domain $\sigma_{1.1}$) is only present in group 1 σ^{70}-factors and auto-inhibits DNA binding of free σ^{70}. In the context of the holoenzyme, region 1.1 is initially situated within the RNAP active site cleft of the closed RNAP-DNA promoter complex where it mimics DNA, but is moved out of this site when the open complex is formed to allow entrance of the template strand DNA (Camarero et al, 2002; Dombroski et al, 1992; Mekler et al, 2002). Region 2.4 of domain σ_2 recognizes the -10 promoter element, while region 2.3 of the same domain is essential for DNA melting. σ_2 forms one of the primary interfaces with the core RNAP (Campbell et al, 2002 and references therein), and in some housekeeping σ-factors of group 1, an acidic non-conserved region (NCR) is inserted within the σ_2 domain. The NCR has been suggested to sterically inhibit promoter-σ^{70} interaction and repel the negatively charged DNA in the absence of binding to core RNAP (Malhotra et al, 1996). However, the significance of this suggestion has been debated (Callaci & Heyduk, 1998). Region 3.0 of domain σ_3 recognizes extended -10 promoter elements. The σ_3 domain is lacking in group 4 σ-factors that therefore cannot identify this type of promoters. Region 3.2 (the $\sigma_{3.2}$ loop) protrudes into the

active site cleft of the RNAP and is believed to directly or indirectly be involved in binding of the initial nucleoside. Finally, region 4.2 of the σ_4 domain recognizes and binds to -35 DNA promoter elements, and when doing so bends the DNA to allow contact between the C-terminal domain of the α-subunits and the upstream DNA. The σ_4 domain also interacts with the β-flap region of core RNAP and is a common target for transcriptional regulators (reviewed in Murakami & Darst, 2003).

2.5. The structurally distinct σ^{54}-class

In contrast to the σ^{70}-class that contains multiple alternative σ-factors, the second class of σ-factors only contains orthologs of the *E. coli* σ^{54}-protein, which received its name from its molecular weight (54 kDa). This protein is encoded by the *rpoN* gene, also referred to as *ntrA*, and another name for the same σ-factor is therefore σ^{N} (Merrick, 1993). σ^{54} was discovered for its role in nitrogen assimilation, but is also involved in other processes, for example alginate biosynthesis, assembly of motility organs and utilization of different carbon sources (reviewed in Valls et al, 2004). The promoter sequences recognized by σ^{54} are very different from the σ^{70} recognition sequences. The consensus is TGGCAC-N5-TTGCa/t, situated at positions -24, -12 relative to the transcription start, with the underlined bases conserved in almost every σ^{54}-promoter investigated (Barrios et al, 1999). Again contrasting σ^{70}, σ^{54} can bind to certain promoters on its own; however, binding of σ^{54}-RNAP holoenzyme is always much more efficient (Merrick, 1993).

One major functional difference between the two σ-factor classes is that σ^{70}-like proteins form holoenzymes that can spontaneously isomerize from a closed RNAP-DNA promoter complex into an open complex where the DNA is melted and the RNAP can access the template DNA strand for transcription initiation. The σ^{54}-RNAP holoenzyme, however, is locked in a thermodynamically stable closed complex at the promoter and needs an activator that through ATP hydrolysis remodels the complex to facilitate DNA melting and open complex formation (reviewed in Buck et al, 2000; Studholme & Buck, 2000). These activator proteins (also known as bacterial Enhancer Binding Proteins, bEBPs) belong to the family of ATPases associated with diverse cellular activities (AAA+) and are further discussed in section 2.7.

To my knowledge, the only existing structural information regarding the σ^{54}-factor are NMR structures of the RpoN box in region III of σ^{54}, both alone and in complex with the -24 promoter element (Doucleff et al, 2005; Doucleff et al, 2007), and cryoelectron microscopy reconstruction of a bEBP-bound σ^{54} and the σ^{54}-RNAP holoenzyme, either alone and in complex with a bEBP (Bose et al, 2008; Rappas et al, 2005). However, additional information about σ^{54} has been gained through alignments of the highly conserved amino acid sequence of this protein from different species. The σ^{54}-factor is composed of three different regions (Fig. 2B), where region I (amino acids 1-56) interacts with bEBPs, with the -12 promoter box and the core RNAP (Bordes et al, 2003; Sasse-Dwight & Gralla, 1988). This region is located close to or at the position where DNA is loaded into the active site, thereby repressing open complex formation by physically blocking entry of the promoter DNA into the active site (Bose et al, 2008). The varying region II (amino acids 57-107) links region I to region III and is sometimes absent. This region has been suggested to be involved in DNA binding and σ^{54}-RNAP stabilization (Cannon et al, 1999), and to affect open complex formation (Southern & Merrick, 2000). Region III (amino acids 108-477) is involved in core RNAP binding and DNA interaction, and contains the C-terminal RpoN box that binds to the -24 promoter motif (Burrows et al, 2003; Doucleff et al, 2007).

2.6. The transcription initiation process

The process of transcription initiation can be divided into several distinct steps (Fig. 4), at which considerable changes in the structures of both DNA and RNAP occur. These steps are similar for transcription initiation with RNAP holoenzymes containing any σ-factor; with the major difference of the absolute dependence of σ^{54}-RNAP for an activator. Below, changes between these steps are first summarized for σ^{70}-like RNAPs, with differences for σ^{54}-RNAP subsequently highlighted.

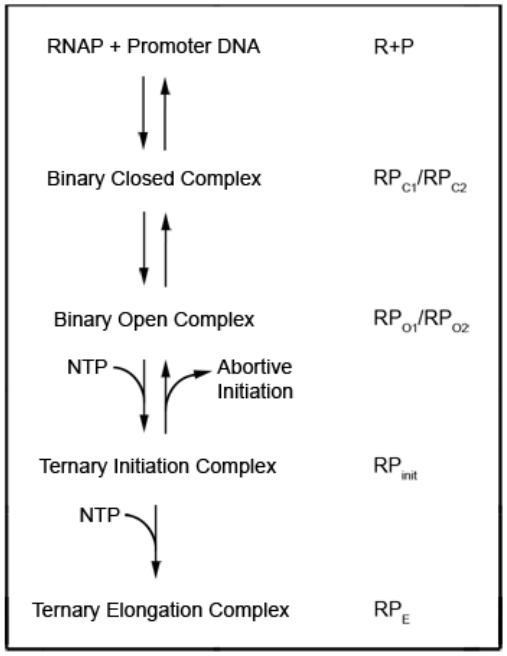


Fig. 4. The multistep transcription initiation process. R: RNA polymerase, P: promoter DNA.

The first step in transcription initiation is the binding of σ-RNAP holoenzyme (R) to the double stranded promoter DNA (P) to form the first form of at least two variants of a binary closed complex (RP_{C1}). When the RP_{C2} is formed, DNA both upstream and downstream of the transcriptional start site is brought into contact with the RNAP, preparing the complex for isomerization of the DNA (Campbell et al, 2002; and reviewed in deHaseth et al, 1998). Binary open complex formation (RP_O) is the next step in the transcription initiation process. As for RP_C, RP_O also has at least two intermediates, and the formation of a binary open complex is characterized by structural changes in the σ-RNAP (Murakami et al, 2002). These changes lead to formation of a transcription bubble, where the DNA is unwound and separated into single strands from approximately position -10 to +2 with respect to the transcriptional start site. The single stranded DNA template is transferred to the RNAP active site as soon as the strand separation has extended downstream of the +1 position. Nucleoside triphosphate (NTP) substrates are supplied to the active site via the secondary channel of the RNAP, and RNA synthesis is initiated to give rise to a ternary initiation complex (RP_{init}). For the growing nascent RNA to be able to exit through the RNAP exit channel under the β-flap, the $\sigma_{3.2}$ loop placed within the active site has to be displaced by the elongating RNA chain. Unless this happens, abortive transcription occurs where the short RNA transcript dissociates from the complex and is released (Murakami & Darst, 2003). The step where abortive transcription can potentially take place ends when the $\sigma_{3.2}$ loop is dislodged, and for this an approximately 12 nucleoside long RNA chain has to be synthesized. It is believed that the exit of the RNA chain through the RNA exit channel aids in destabilizing the σ_4-β-flap interaction, leading to a destabilization in the binding of σ_4 to the -35 promoter element. Promoter escape by the RNAP is thus initiated, and the RNAP is released from the promoter and can move downstream while elongating the RNA. The σ-factor is stochastically released from the elongation complex through sequential breaking of the numerous interactions between the σ-factor and the core RNAP, giving rise to a ternary elongation complex (RP_E; Raffaelle et al, 2005).

The thermodynamically stable closed complex formed by σ^{54}-RNAP bound to its -24 and -12 promoter elements can not proceed to open complex formation. For this, interaction with an activator protein of the AAA+ family is required. ATP hydrolysis by the activator relocates region I of the σ^{54}-factor from its natural conformation where it inhibits open complex formation by blocking the entry of the template DNA into the RNAP active site. Once this conformational change in region I has taken place, transition into an open RNAP-DNA complex can occur (Bose et al, 2008; Buck et al, 2000). Firstly, a heparin-sensitive open complex is formed (RP_{O1}) and further aid by the activator is needed for a transition into a heparin-stable complex that can bind NTPs (RP_{O2}; Wang et al, 1997). After this step, the pathway for transcription initiation from a σ^{54}-dependent promoter is very similar to the σ^{70}-RNAP pathway. One difference, though, is that abortive transcription occurs more frequently for σ^{54}-RNAP complexes, with abortive products of only 2 nucleosides compared to the 3-12 nucleosides for σ^{70}-RNAP (Hsu, 2002; Tintut et al, 1995).

2.7. Bacterial Enhancer Binding Proteins and their function as σ^{54}-transcription activators

Classical bacterial transcriptional activators typically act by binding to DNA regions in or just next to a promoter to affect the recruitment of the RNAP to the promoter and/or to augment a rate-limiting step during transcription initiation (reviewed in Browning & Busby, 2004). Transcriptional activators controlling σ^{54}-dependent transcription differ from classical activators in the sense that they bind more than 100 base pairs upstream or downstream from the promoters they control (hence the name bacterial Enhancer Binding Proteins or bEBPs), and they only act on pre-bound σ^{54}-RNAP (reviewed in Wigneshweraraj et al, 2008).

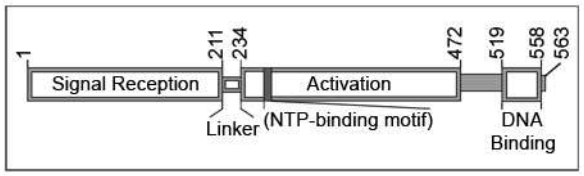


Fig. 5. Schematic illustration of the domains of a typical bacterial Enhancer Binding Protein, here the σ^{54}-activator DmpR. Adapted from (Studholme & Dixon, 2003).

The activity of the bEBPs is controlled in response to different environmental and/or physiological stimuli, leading to changes in the behavior of the cell. These stimuli can activate bEBPs to cause modifications in for instance the utilization of alternative carbon sources (e.g. DmpR and XylR), nitrogen assimilation and utilization (e.g. NtrC and NifA) or production of virulence determinants (e.g. HrpS) (reviewed in Shingler, 1996). The general domain structure of the bEBP family is outlined in Fig. 5. These proteins usually consist of three distinct domains that are involved in signal reception, activation of transcription and DNA binding, respectively. The C-terminal domain contains a helix-turn-helix DNA-binding motif, and mediates binding to distally located DNA sequences. Because these binding sites are usually upstream of the promoter, they are referred to as upstream activating sequences (UASs) and can be moved as much as 1 kb upstream from the promoter, without major loss of transcription activation (Reitzer & Magasanik, 1986). The central domain is the most highly conserved region and is

absolutely required for bEBP function. This domain contains the AAA+ module required for ATPase activity, and is often sufficient for activating transcription from σ^{54}-dependent promoters, indicating that some bEBPs can activate σ^{54}-transcription from solution (Berger et al, 1995; Jovanovic et al, 1999). Walker A and B motifs of this domain are associated with nucleotide binding, while a GAFTGA motif, situated on a mobile loop, is involved in productive engagement with the σ^{54}-RNAP and energy transfer from the ATP hydrolysis that supports the conformational change into an open σ^{54}-RNAP-promoter complex (Bordes et al, 2003; Rappas et al, 2005). The N-terminal region is the signal reception domain that in the absence of an activating signal (e.g. a small ligand, phosphorylation or by an interacting protein) represses the activity of these proteins (reviewed in Shingler, 1996). The signal-sensing domain and the AAA+ module are coupled via a flexible linker, which has been shown to be involved in repression of ATPase activity of the aromatic-responsive bEBP DmpR (O'Neill et al, 2001).

The bEBPs are usually present in an inactive dimeric state, and oligomerization (usually into hexamers) is required to stimulate ATPase activity and permit interaction with the σ^{54}-RNAP (e.g. Rappas et al, 2005; Wikström et al, 2001). Since the UASs are distally situated, DNA looping has to occur for the bEBP to be able to interact with the σ^{54}-RNAP bound on the promoter (Su et al, 1990; Wedel et al, 1990). This can either take place via an intrinsic bend in the intervening DNA or be aided by the DNA bending protein Integration Host Factor (IHF; see Fig. 6B; reviewed in Pérez-Martin & de Lorenzo, 1997). ATP hydrolysis by the bEBP when contacting the σ^{54}-RNAP leads to conformational changes that relieve the repressive effect by region I of σ^{54} so that open complex formation can occur (Fig. 6C).

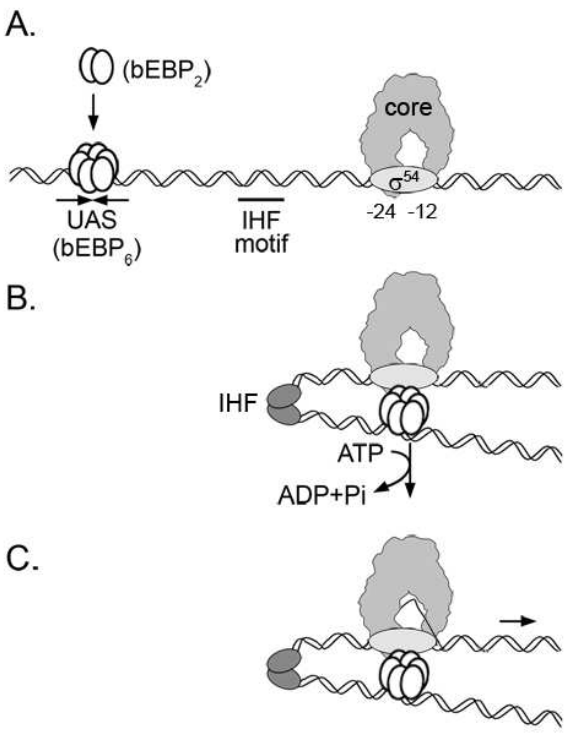


Fig. 6. Illustration of transcription activation from a σ^{54}-dependent promoter by a bEBP.
(A) σ^{54}-RNAP binds to specific promoter motifs positioned -12 and -24 relative to the +1 transcriptional start, forming a closed σ^{54}-RNAP-promoter complex. Active bEBPs bind to UASs upstream of the promoter.
(B) The bEBP multimer contacts the σ^{54}-RNAP-promoter complex by DNA looping, often aided by IHF.
(C) ATP hydrolysis by the AAA+ domain of the bEBP results in formation of an open σ^{54}-RNAP-promoter complex with melted DNA. Adapted from (Schumacher et al, 2006)

3. GLOBAL BACTERIAL TRANSCRIPTION REGULATORS

Global regulators simultaneously up- and down-regulate large sets of genes to help co-ordinate transcriptional patterns to prevailing conditions. Three global regulators that are of particular importance to the experimental system used in my research are introduced below (sections 3.1-3.3).

3.1. The alarmone ppGpp

The unusual nucleotide guanosine tetraphosphate (ppGpp), also known as the magic spot, is the major regulator of the stringent response where rRNA biosynthesis and ribosome production is down-regulated. The stringent response is engaged when ppGpp is produced to high concentrations under conditions of amino acid starvation. ppGpp, however, is also produced when carbon, nitrogen and phosphate are limiting and/or in response to physical-chemical stresses, which all cause a decrease in growth rate and/or growth arrest (reviewed in Potrykus & Cashel, 2008). Broadly stated, the role of the alarmone ppGpp is to herald stress to enable the cell to pre-load itself with proteins to help combat harsh conditions that lie ahead. In general, ppGpp negatively regulates transcription of genes that are involved in cell proliferation and growth, while it positively regulates transcription of genes involved in maintenance and stress survival (reviewed in Nystrom, 2004; Traxler et al, 2008). Because ppGpp is involved in controlling production of other global regulators (e.g. IHF, σ^{S} and σ^{H}), it influences gene expression patterns to a very high degree (Fig. 7; Eymann et al, 2001; Lange et al, 1995). In addition to being found in the most types of bacteria, ppGpp is also produced in the chloroplasts in plants, where it is induced upon stresses like wounding, heat shock, UV irradiation and draught (Takahashi et al, 2004).

In *E. coli* and *P. putida,* ppGpp is synthesized from ATP and GTP by the ppGpp synthetase I (RelA) and

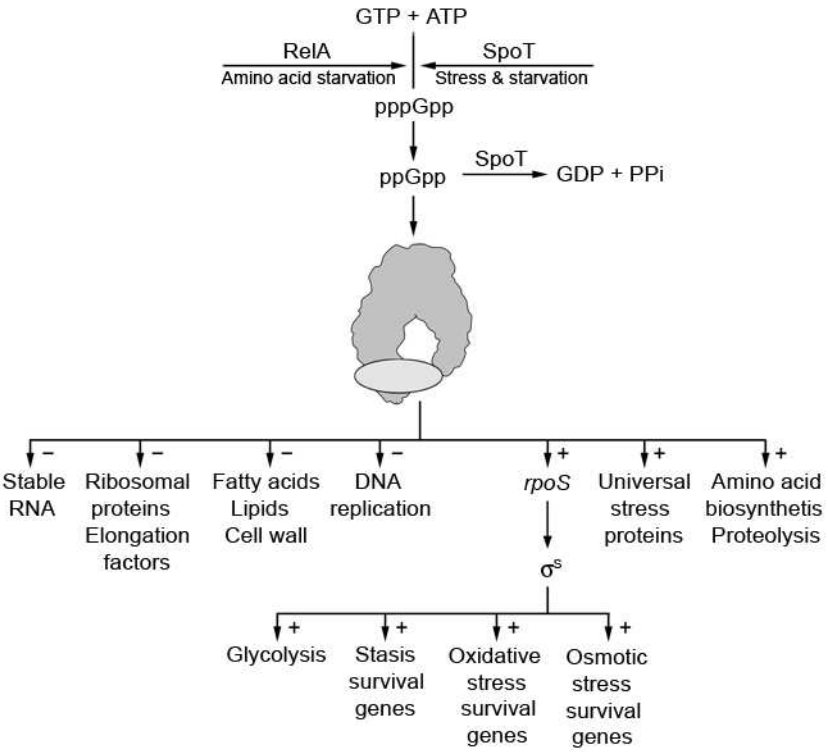


Fig. 7. The stringent response alarmone ppGpp is synthesized in response to starvation and stress. ppGpp directly targets the RNAP and redirects transcription from genes involved in cell proliferation and growth to genes implicated in counteracting stress and starvation. Adapted from (Magnusson et al, 2005).

synthetase II (SpoT) (Cashel et al, 1996; Sze et al, 2002). First pppGpp is produced, which is then rapidly converted to ppGpp by guanosine pentaphospate hydrolase (Kuroda et al, 1997). Deletion of both the *relA* and *spoT* genes renders a strain incapable of synthesizing ppGpp (a $ppGpp^0$ strain). A $ppGpp^0$ strain is essentially blind to amino acid starvation and exhibits a "relaxed response" during which rRNA and ribosomes are continuously produced, rather than being down-regulated as during the ppGpp-mediated stringent response (Cashel et al, 1996). Production of ppGpp by RelA is stimulated when RelA associates with a stalled ribosome with uncharged tRNA in the A-site (Wendrich et al, 2002), an event that occurs with greatly increased frequency when cells are starved for amino acids or approach the stationary phase of growth in rich media. SpoT appears to be responsible for ppGpp production in response to stresses other than amino acid starvation. In addition to being able to synthesize ppGpp, SpoT can also hydrolyze it into GDP, and is thus dual functional (Cashel et al, 1996). It is most common for bacteria to only contain one single protein which, like SpoT, has dual ppGpp synthetase and hydrolyzing activities (Mittenhuber, 2001). The two catalytic sites required for these activities are usually situated in the amino terminal region of the protein, and allosteric changes through the carboxy terminal region are suggested to control the switching between the two opposing catalytic activities (Hogg et al, 2004). It has been proposed that an interaction with a central co-factor in fatty acid synthesis (the Acyl-carrier protein) might be a mediator that controls the opposing activities of SpoT (Battesti & Bouveret, 2006).

The alarmone ppGpp has been under investigation for four decades since it was first discovered (Cashel & Gallant, 1969), but it is still not certain where ppGpp precisely binds to RNAP to mediate its effect on transcription. Cross-linking studies using ppGpp analogues have shown that ppGpp binds close to the interface of the β- and β'-subunits of the RNAP, and that residues of both subunits are involved (Chatterji et al, 1998; Toulokhonov et al, 2001). Mutations within the genes encoding β (*rpoB*), β' (*rpoC*) and σ^{70} (*rpoD*) that affect transcription regulation by ppGpp have been found (Hernandez & Cashel, 1995). In 2004, a co-crystallization of ppGpp bound to *Thermus thermophilus* RNAP was published; illustrating ppGpp bound in either of two orientations close to, but not overlapping, the active site. It was suggested that the binding of ppGpp to this site might affect the interaction of NTPs with RNAP by changing the configuration of the active site, and that ppGpp might interact with the non-template strand in the open complex, just upstream of the transcription start site (Artsimovitch et al, 2004). However, a recent publication has shed doubt on the biological relevance of the proposed structure, since *T. thermophilus* RNAP does not respond to ppGpp and mutations of the equivalent residues of *E. coli* RNAP that were proposed to ligand ppGpp in *T. thermophilus* RNAP did not affect ppGpp-regulated transcription (Vrentas et al, 2008). This contrasts the original finding (by Artsimovitch et al, 2004) that an analogous mutation in the β'-subunit of *E. coli* RNAP did apparently negatively influence ppGpp regulated transcription. Vrentas et al (2008) suggest this contradictory data might be due to the use by Artsimovitch et al (2004) of *E. coli* RNAP unsaturated with the ω-subunit, which is a subunit necessary for ppGpp effects through RNAP and thus on transcription *in vitro* (Vrentas et al, 2005).

ppGpp has been shown to directly affect several steps of transcriptional initiation from different σ^{70}-dependent promoters *in vitro*. It is likely that the different negative regulatory mechanisms put forward work together or independently depending on the intrinsic kinetic limitations of a given promoter. Examples are 1) as mentioned above, at the level of formation rate of the

ternary initiation complex through competition between ppGpp and NTPs at the active site of RNAP (Artsimovitch et al, 2004; Jores & Wagner, 2003). This would particularly affect promoters that are sensitive to the levels of initiating NTP levels. 2) Destabilization and decreased life-time of open RNAP-promoter complexes, which would negatively affect promoters which have open complex stability as a rate limiting step (e.g. rRNA operon promoters; Barker et al, 2001; Paul et al, 2004a; Zhou & Jin, 1998), and 3) to negatively affect promoter clearance, as proposed to underlie the ppGpp-mediated down-regulation of transcription from the λP_R promoter (Potrykus et al, 2002). In the case of promoters that are directly stimulated by ppGpp, such as those directing synthesis and transport of some amino acids, it has been proposed that ppGpp accelerates promoter activity by lowering the transition energy required to form some rate limiting intermediate in the pathway to open RNAP-promoter complex formation (Paul et al, 2005). While ppGpp is thought to primarily act at the level of transcriptional initiation, it can also influence elongation rates by increasing pausing during transcription elongation (Kingston et al, 1981).

Positive ppGpp-regulation of promoters recognized by some alternative σ-factors (e.g. σ^{54}, σ^{S} and σ^{H}) has been proposed to be mediated in an indirect manner, mainly via modulating the outcome of σ-factor competition for a limiting pool of core RNAP (Jishage et al, 2002; Laurie et al, 2003; and Paper I and II). A major part of this thesis is based upon studies leading to a passive model for ppGpp-mediated effects on σ^{54}-dependent transcription, which is expanded upon in the Results and Discussion section.

3.2. The RNA polymerase binding protein DksA

DksA is an RNAP binding protein that was first discovered as a multicopy suppressor of an *E. coli dnaK* deletion mutant (Kang & Craig, 1990). Later on DksA has been shown to be involved in the transcription regulation of numerous genes. Like ppGpp, DksA destabilizes the open RNAP-promoter complex on several promoters (Paul et al, 2004a), and its loss renders pleiotropic effects such as deficiencies in cell division, amino acid biosynthesis, quorum sensing and virulence (e.g. Bass et al, 1996; Jude et al, 2003; Mogull et al, 2001; Paul et al, 2005). DksA is not as widely spread among different bacterial species as ppGpp (Paul et al, 2004b; Perederina et al, 2004). However, DksA and ppGpp frequently act together to both stimulate and inhibit transcription from many promoters *in vivo* and/or *in vitro* (e.g. Aberg et al, 2008; Costanzo et al, 2008; Paul et al, 2004a; Paul et al, 2005). The phenotypes of DksA null and $ppGpp^0$ *E. coli* strains do not totally overlap (Aberg et al, 2008; Brown et al, 2002; Magnusson et al, 2007). It is therefore possible that these two regulators in some cases act together and in other cases act on their own.

DksA is a relatively small protein (17 kDa, 151 amino acids) with structural (but not sequence) similarities to the transcription factor GreA that belongs to a growing family of secondary channel regulators (Artsimovitch et al, 2004; Perederina et al, 2004). The Gre factors function in reactivating stalled RNAP by stimulating the intrinsic endonucleolytic transcript cleavage activity of the RNAP. A coiled-coil domain in the Gre factors protrudes into the secondary channel of the RNAP to position acidic residues on the tip of the coiled-coil structure close to the active site where they are proposed to aid in coordinating one of the two catalytic active site Mg^{2+} ions (Fish & Kane, 2002). Perederina and colleagues (2004) suggest that the coiled-coil structure of DksA, like that of Gre factors, would protrude into the secondary channel of the RNAP so that the two aspartate residues at the tip of the coiled-coil could interact with a Mg^{2+} coordinated by ppGpp, and thereby assist and/or stabilize the putative bond between ppGpp and the RNAP (Artsimovitch et al, 2004; Perederina et al, 2004). Although an attractive idea to account for the synergistic effects seen with ppGpp and DksA both *in vivo* and *in vitro*, the recent findings by Vrentas et al (2008) indicate that the synergistic effects are mediated in some other way than by a simultaneous direct interaction of these two regulators with one of the Mg^{2+} ions. A more recent suggestion as to how DksA might negatively control transcription via its coiled-coil structure was published by Rutherford et al (2009). These authors propose that conformational effects by DksA are transmitted by the RNAP bridge helix and trigger loop to the RNAP switch region(s), to affect residues involved in clamp opening/closing and/or residues which directly interact with promoter DNA, ultimately leading to inhibition of transition to an RNAP-promoter complex intermediate (Rutherford et al, 2009). However, it is unclear how these DksA-mediated conformational changes in the RNAP might stimulate transcription from some promoters or allow DksA and ppGpp to act together to control transcription.

3.3. The DNA bending protein IHF

IHF belongs to the group of nucleoid-associated proteins, which are involved in compaction of the bacterial chromosomes and represent the bacterial equivalents to histones. In addition to IHF, the major nucleoid-associated proteins found in *E. coli* are H-NS (heat-stable [or histone-like] nucleoid-structuring protein), HU (heat-unstable nucleoid protein) and FIS (factor for inversion stimulation) (reviewed in Travers & Muskhelishvili, 2005). The levels of IHF are partially under the control of ppGpp in *E. coli* and *P. putida*, and vary over growth with an up to approximately 7-fold increase at the exponential-to-stationary phase transition (Aviv et al, 1994; Ditto et al, 1994; Murtin et al, 1998; Valls et al, 2002). In addition to

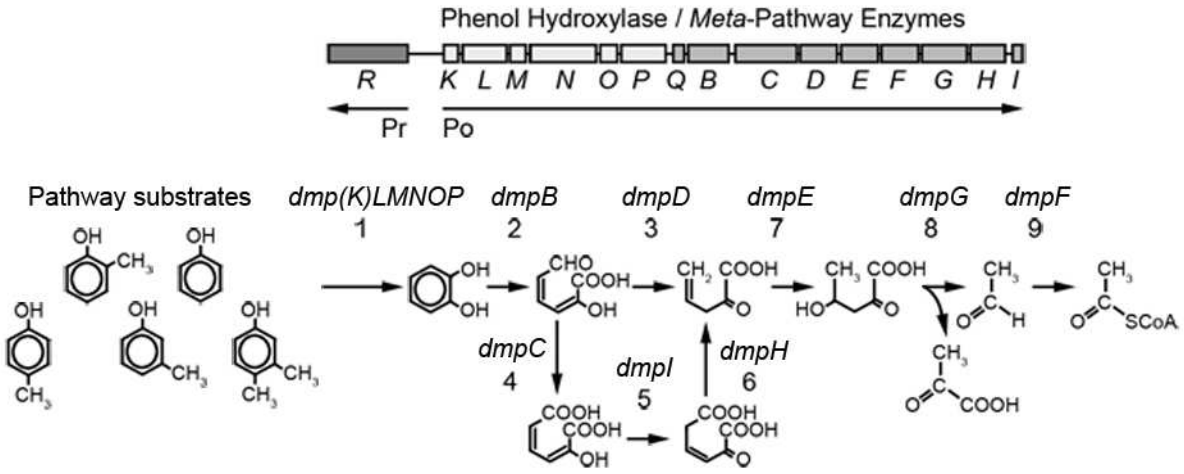


Fig. 8. Genetic organization of the *dmp*-system and the (methyl)phenol catabolic pathway catalyzed by the Dmp enzymes. Phenolic substrates are converted to catechols by the multi-component phenol hydroxylase Dmp(K)LMNOP. Ring-cleavage of the catechols is mediated by the catechol-2,3-dioxygenase DmpB, followed by further degradation of the intermediates to acetyl-CoA and pyruvate by the *meta*-pathway enzymes DmpC-I. Adapted from (Powlowski & Shingler, 1994).

having a role in compacting chromosomal DNA, IHF can also function as a transcription factor. The transcriptional regulatory properties are mediated through the ability of IHF to alter DNA topology by bending DNA by more than 160° when bound to its target sequence (Rice et al, 1996). Changed DNA topology caused by IHF (and/or by other nucleoid-associated proteins) can affect transcriptional output from promoters by for instance i) increased or decreased binding of the RNAP and/or transcriptional regulator(s), ii) formation or interference of interactions between the RNAP and transcriptional regulator(s), iii) increased or decreased unwinding of the double stranded DNA, and/or iv) nucleation and formation of higher order structures that lead to transcription activation or repression (reviewed in Dai & Rothman-Denes, 1999).

IHF is particularly important for positive regulation of σ^{54}-dependent promoters, where it facilitates the contact between the promoter-bound σ^{54}-RNAP and the distally bound bEBP. IHF can additionally contribute to control of certain σ^{54}-promoters by stimulating recruitment of i) σ^{54}-RNAP to the promoter (Bertoni et al, 1998), ii) bEBPs to their binding sites (Jovanovic & Model, 1997), iii) by promoting and/or stabilizing the open σ^{54}-RNAP-promoter complex (Sze et al, 2001) and iv) by restricting the transcriptional activation specificity to a single bEBP bound to specific UASs (Claverie-Martin & Magasanik, 1992; Dworkin et al, 1997; Pérez-Martín & De Lorenzo, 1995).

4. METABOLISM OF AROMATICS IS REGULATED ON A GLOBAL LEVEL

The ability of soil micro-organisms like *Pseudomonas, Burkholderia* and *Acinetobacter* to degrade aromatic (e.g. phenols and toluenes) and aliphatic (e.g. alkanes and styrene) compounds has been investigated for many years. The genomes of *Pseudomonas* species contain a very large proportion of regulatory genes and genes that are dedicated to the catabolism, transport and efflux of organic compounds (Nelson et al, 2002). Biodegradation of hydrocarbons and related compounds necessitates specialized enzymes which catalyze the sequential breakdown to central metabolites. The genes for these pathways are commonly organized into operons either on the chromosome, flanked by transposons, or on trans-missible plasmids (Williams & Sayers, 1994).

The ability of bacteria to catabolize hydrocarbons and related compounds through these auxiliary pathways serves as an advantage to the host bacterium when competing with other bacteria in the surroundings, but is not a beneficial feature when the compounds are absent. The production of the specialized suits of enzymes is metabolically expensive. To avoid energy wastage, it is important that the regulation of these catabolic systems is coupled to host physiology so that they are not produced when energetically more favorable carbon and energy sources are available. Systems for the catabolism of aromatic compounds are thus usually controlled both at a specific and a global level, where global regulatory input signals availability of more favorable carbon sources to override the specific regulation (reviewed in Shingler, 2003). This can for instance occur through competition between pathway substrates and central metabolites for binding to a regulator (e.g. ClcR; McFall et al, 1997) or by exchanging the σ-factor in use (e.g. the Pm promoter of pWW0; Marqués et al, 1999). Another example of this kind of signal integration is provided by catabolism of

(methyl)phenols by *Pseudomonas* sp. strain CF600, and is the focus of my thesis.

5. *PSEUDOMONAS* sp. STRAIN CF600 AND THE *dmp*-SYSTEM

The soil bacterium *Pseudomonas* sp. strain CF600 has the ability to degrade phenols and methylated phenols, and to use these compounds as its only carbon and energy source. The potential use of CF600 in bioremediation and/or for the construction of biosensors has prompted dissection of its (methyl)phenol catabolic system over the last 20 years (reviewed in Shingler, 2004; and Shingler, 2009). *Pseudomonas* sp. strain CF600 was originally isolated from coke liquor waste through its ability to grow at the expense of 3,4-dimethylphenol, and subsequently found to also catabolize phenol, 2-, 3- and 4-methylphenols, all of which are toxic substances. The (methyl)phenol degradative ability of this strain is conferred by the *dmp*-system (dimethylphenol; see Fig. 8), which is carried on the self transmissible Inc-P2 megaplasmid pVI150 (Shingler et al, 1989). The *dmp*-system is comprised by the regulatory gene *dmpR* and the *dmp*-operon, containing fifteen structural genes (*dmpKLMNOPQ / BCDEFGHI*) encoding the nine enzymes involved in the stepwise degradation of (methyl)phenols into the central metabolites pyruvate and acetyl-CoA (Shingler et al, 1992). The *dmp*-system of pVI150 can also be moved to *P. putida* KT2440, enabling this new host to degrade (methyl)phenolic compounds, something it cannot do without carrying the *dmp*-system (Shingler et al, 1989).

The promoter controlling transcription of the *dmp*-operon is denoted Po. The Po promoter is a typical -24 (GG), -12 (GC) σ^{54}-promoter and is completely dependent on σ^{54}-RNAP for transcription initiation (Shingler et al, 1993). DmpR is the obligatory bEBP needed for activation of transcription from σ^{54}-Po, and transcription of the *dmpR* gene is divergent to that of the *dmp*-operon (Fig. 8). The activity of DmpR is regulated by the binding of phenolic pathway substrates (and some structural analogues) to the amino-terminal domain of the protein. Binding of these aromatic effector molecules unlocks the transcriptional promoting ability of DmpR by allowing ATP-binding triggered multimerisation to its active form (O'Neill et al, 1998; O'Neill et al, 2001; Wikström et al, 2001). In its multimeric form, DmpR can efficiently bind to its UAS sites located approximately 170 base pairs upstream of the Po promoter. Binding of IHF to its DNA site located between the UASs and Po, aids the productive interaction between the Po-bound σ^{54}-RNAP and active DmpR bound to the UASs (Sze et al, 2001). The necessity for phenolic effectors to activate DmpR so that it can promote transcription of the *dmp*-operon constitutes the specific regulation of the Po promoter to ensure that the metabolically expensive Dmp-enzymes are only produced when pathway substrates are available.

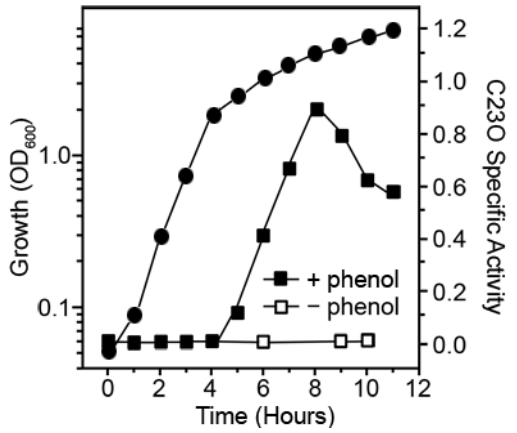


Fig. 9. Monitoring of the expression of the *dmp*-operon in *P. putida* KT2440, by measuring the catechol-2,3-dioxygenase (C23O) activity of one of the *dmp*-operon products, DmpB. Circles indicate growth, while squares represent C23O activity of DmpB in the absence (open squares) and presence (filled squares) of phenol in the rich growth medium. Adapted from (Sze et al, 1996).

5.1. The *dmp*-system is globally regulated by ppGpp

Expression of the (methyl)phenol Dmp-enzymes can readily be monitored by measuring the catechol-2,3-dioxygenase (C23O) activity of the DmpB enzyme or by using transcriptional fusions between the Po promoter and the *luxAB* luciferase genes. As mentioned above, the presence of (methyl)phenol-activated DmpR is absolutely required for activity of the Po promoter, and thus for production of the catabolic enzymes. However, even in the presence of active DmpR, the Dmp-enzymes are not produced until the transition between the exponential and stationary phase of growth in rich media, i.e. when nutrients start to become limiting for growth (see Fig. 9). This growth phase regulatory phenotype is seen both in the native *Pseudomonas* CF600 host and in *P. putida* KT2440, and also in *E. coli* (Sze et al, 1996). The minimal system required to reproduce this growth phase regulation of Po is the presence of the *dmpR* gene and the Po regulatory region, either in their native configuration or on independent replicons (Sze et al, 1996). The above findings suggested that host-encoded factors involved in this growth phase regulation can be found in both *P. putida* and *E. coli* and that they function in a similar manner, allowing the use of the more easily manipulated *E. coli* to pursue the mechanism underlying this phenomenon. It should be noted that in *P. putida* KT2400, the *dmpR* gene is carried in monocopy on the chromosome to render the same levels of DmpR as in the native CF600 host. In *E. coli*, however, *dmpR* needs to be carried on a 16 copy plasmid to reach the same DmpR levels as in CF600, indicating that there are differences in expression levels in *Pseudomonas* as compared to in *E. coli* (Sze et al, 1996).

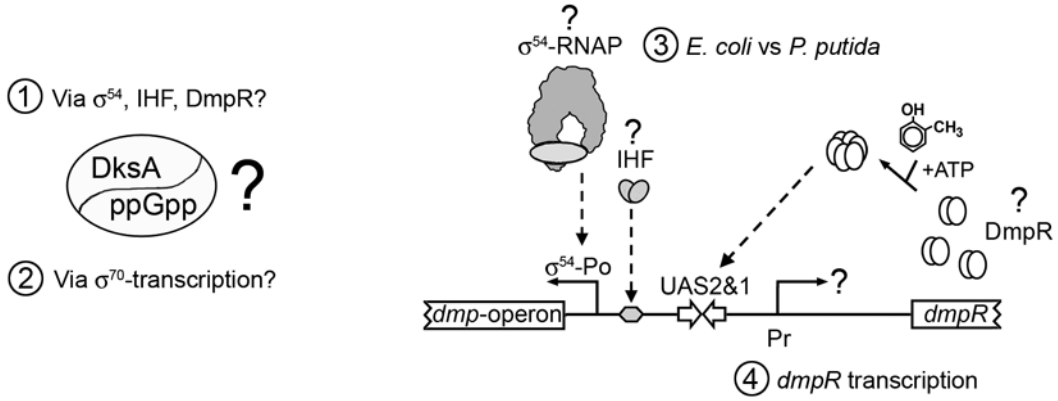
Using *E. coli,* the role of some potential global regulators in mediating growth phase regulation of the Po promoter was investigated and excluded, for instance FIS and σ^S (Sze et al, 1996). The same study showed that the levels of DmpR are of great importance for high-level transcription from the Po promoter. The alarmone ppGpp was subsequently found to be the major global link between activity of the Po promoter and the physiological status of the cell that underlie growth phase regulation (Sze & Shingler, 1999). This alarmone is thus involved in both maintaining silencing of Po activity under high energy conditions and rapid Po induction under low energy conditions where ppGpp levels are elevated (e.g. where nutrients are depleted at the exponential-to-stationary phase growth transition; Sze & Shingler, 1999).

In the Results and Discussion section of this thesis, I present studies performed to investigate the mechanism(s) that underlie the involvement of ppGpp in growth phase regulation of transcription from the σ^{54}-Po promoter, and later the Pr promoter that drives transcription of *dmpR*. These studies were conducted in the light of the relatively recent finding of DksA as a potent co-regulator of ppGpp-mediated responses.

AIMS

The main aims of my research have been to investigate how ppGpp and DksA mediate their control of σ^{54}-dependent transcription in *Escherichia coli* and *Pseudomonas putida*, and later on how production of the bacterial Enhancer Binding Protein DmpR is regulated. More specifically:

1. To determine whether the effect by ppGpp and DksA on σ^{54}-dependent transcription is mediated directly and/or indirectly through any of the components involved in specific regulation of σ^{54}-transcription
2. To investigate if ppGpp and DksA mediate their effect on σ^{54}-dependent transcription through σ^{70}-transcription
3. To elucidate whether σ^{54}-dependent transcription in *E. coli* and *P. putida* is regulated similarly
4. To resolve how expression of the σ^{54}-activator DmpR is regulated, including the potential involvement of ppGpp and DksA



RESULTS AND DISCUSSION

In this section, I present and discuss the main results achieved during my thesis work. Data presented are referred to by the roman letters (I-IV) of the Paper where they were published and the corresponding figure number in the respective Paper.

6. DO ppGpp AND DksA HAVE DIRECT AND/OR INDIRECT EFFECTS ON THE σ^{54}-DEPENDENT Po PROMOTER?

6.1. σ^{54}-dependent transcription from the Po promoter is dependent on ppGpp and DksA in *Escherichia coli* and *Pseudomonas putida*

The stringent response alarmone ppGpp has previously been shown to be necessary for efficient *in vivo* transcription from the σ^{54}-dependent Po promoter (Sze et al, 2002; Sze & Shingler, 1999). However, the putative involvement of DksA in regulation of σ^{54}-dependent transcription had not been investigated prior to the study in Paper I. Since our research on σ^{54}-Po promoter regulation is conducted in both *E. coli* and *P. putida* backgrounds, a comparison between *E. coli* and *P. putida* strains devoid of ppGpp and/or DksA was performed. Phenotypically these two organisms react differently to loss of these regulatory molecules. The enteric bacterium *E. coli* acquires polyauxotrophic amino acid requirements when lacking the ability to produce ppGpp and/or DksA (Paper I and Cashel et al, 1996). In contrast, the nutritionally and environmentally adaptable *P. putida* remains prototrophic in strains devoid of either of these molecules (Paper III and Sze et al, 2002).

Lack of either ppGpp or DksA in both *E. coli* and *P. putida* renders the σ^{54}-Po promoter defective in transcription to a similar extent (Paper I, Fig. 1A; and III, Fig. 2A), while the simultaneous loss of both of these regulatory molecules almost completely abolishes transcription from Po in *E. coli* (Paper I, Fig. 1A). The dependency of Po promoter activity on both ppGpp and DksA is thus great. The cooperative effects upon loss of both molecules fit with a previous suggestion that DksA stabilizes ppGpp binding to the core RNAP (Perederina et al, 2004 and references therein). However, recently it has been shown that the previously proposed binding site for ppGpp (Artsimovitch et al, 2004) is unlikely to be the biologically relevant one (Vrentas et al, 2008). Thus, whether DksA and ppGpp actually co-operate through stabilization of ppGpp binding remains an open question.

When grown in rich medium, the levels of ppGpp dramatically increase at the transition point between exponential and stationary phase of growth in both *E. coli* and *P. putida* (reviewed in Magnusson et al, 2005; Sze & Shingler, 1999). To investigate whether production of DksA was regulated in a similar manner in the two species, the transcription profile of the *dksA* promoter and the DksA protein levels in *E. coli* and *P. putida* were monitored. Transcription from the P_{dksA} promoters is similar in both cases with a peak in mid-exponential phase of growth (Paper III, Fig. 2D). The transcriptional differences over growth, however, only result in an approximate 2-fold difference in DksA protein levels (Paper III, Fig. 2E). Regulation of σ^{54}-dependent transcription from the Po promoter is thus primarily mediated by changes in the levels of ppGpp in the cell.

One plausible explanation for the reduced transcription from Po seen upon loss of ppGpp and/or DksA was that these molecules are needed for efficient production of σ^{54}. However, the levels of σ^{54} are constant over growth and are not affected in either *E. coli* or *P. putida* strains devoid of ppGpp or DksA (Paper I, Fig. 1B; and III, Fig. 2B-C). The effect by these molecules on σ^{54}-Po output is thus not mediated through changes in the cellular σ^{54}-levels.

6.2. ppGpp and DksA have no direct effect on σ^{54}-dependent transcription *in vitro*

ppGpp and DksA have been implicated in direct stimulation and inhibition of some σ^{E}- and σ^{70}-promoters (Aberg et al, 2008; Costanzo et al, 2008; Paul et al, 2004a; Paul et al, 2005). By employing the by ppGpp and DksA positively regulated σ^{70}-P*thrABC* promoter and the negatively regulated σ^{70}-*rrnB* P1 promoter (Paul et al, 2004a; Paul et al, 2005) as controls, the putative direct effect by these regulatory molecules on σ^{54}-Po activity was examined using *E. coli* transcriptional components. Under conditions where both positive (P*thrABC*) and negative (*rrnB* P1) regulation were recapitulated, no effect was seen on *in vitro* transcription from Po (Paper I, Fig. 2A-B), indicating that Po is not directly stimulated by ppGpp and DksA. The putative effect of ppGpp and DksA on *in vitro* transcription from Po has also been tested with purified *P. putida* σ^{54}-RNAP and DksA. As for the case with *E. coli* transcriptional components, no significant effect was seen upon addition of ppGpp and DksA to the reaction mix (Paper III, Fig. 5B).

The possibility that an effect by ppGpp was masked due to the presence of a high concentration of ATP needed for DmpR-activity was also investigated. However, experiments performed to pursue this issue showed that this was not the case (Paper I, Fig. 2C). These results also showed that the σ^{54}-Po promoter is not affected by the nature or the level of the initiating nucleotide.

ppGpp and DksA destabilize the competitor-resistant open complexes on all promoters tested so far. To investigate whether ppGpp and DksA target σ^{54}-RNAP at all, the effect of these molecules on the stability of the open complex formed on the Po promoter was tested. It was found that ppGpp and DksA by themselves do reduce the lifetime of the Po open complex, and also that there is

a cooperative effect of their simultaneous presence. However, the effect by DksA either alone or together with ppGpp was only seen in the presence of a true competitor DNA, and not when the DNA-mimic heparin was used as the competitor (Paper I, Fig. 3A; and II, Fig. 6A). Despite these findings, open complex stability does not appear to be a rate-limiting step for transcription from the σ^{54}-Po promoter *in vitro*, since the presence of ppGpp and DksA does not result in reduced σ^{54}-transcription from Po (Paper I and III).

The above *in vitro* transcription results indicate that ppGpp and DksA do not mediate their effect on transcription from σ^{54}-Po via a direct action on this promoter, and that an indirect mechanism is employed *in vivo*. The possibility that these two regulatory molecules work via the levels or activity of other players closely involved in the regulation of the σ^{54}-Po promoter was therefore examined as described in the following two sections.

6.3. ppGpp and DksA do not primarily mediate their effect on σ^{54}-Po through changes in IHF levels

The DNA bending protein IHF is involved in the regulation of the Po promoter by assisting physical contact between σ^{54}-RNAP and DmpR bound to their respective binding site (Sze et al, 1996). The levels of IHF increase in both *E. coli* and *P. putida* when cells enter the stationary phase of growth, and have been shown to be partially under ppGpp control in *E. coli* (Aviv et al, 1994; Ditto et al, 1994; Valls et al, 2002). The possibility that ppGpp and DksA mediate their growth phase regulatory control of σ^{54}-Po activity through changes in IHF levels was thus investigated. To avoid pleiotropic effects, a Po reporter construct with a mutated IHF recognition site was used instead of monitoring Po activity in IHF null backgrounds. In wild type *E. coli*, loss of IHF binding capacity leads to an approximate 5-fold reduction in transcription from Po, while the temporal expression remains the same (Paper I, Fig. 8C-D). In wild type *P. putida*, the reduction in σ^{54}-Po activity is greater, with an approximate 10-fold lower transcription from Po upon loss of IHF binding capacity and thus stimulation by IHF (Paper III, Fig. 3A). However, even when IHF is removed from the equation by using the Po reporter carrying a mutated IHF binding site, transcription from Po was decreased in a similar manner as in the presence of the native IHF recognition sequence in both *E. coli* and *P. putida* strains devoid of ppGpp or DksA (Paper I, Fig. 8D; and III, Fig. S1).

The effect on σ^{54}-Po output by over-expression of IHF has also been tested in both these species. However, higher levels of IHF did not have any significant effect on stationary phase expression from the Po promoter in either *E. coli* or *P. putida* and did not relieve exponential silencing of Po (Sze & Shingler, 1999; and Paper III, Fig. 3B). It can thus be concluded that while IHF is of importance for optimal output from the Po promoter, ppGpp and DksA do not mediate their control of transcription from Po through their effects on the levels of IHF.

6.4. ppGpp and DksA do not primarily mediate their effect on σ^{54}-Po through DmpR levels

Another possibility for ppGpp and DksA regulation of transcription from σ^{54}-Po is via the levels of the bEBP DmpR, which is absolutely necessary for activity of the Po promoter. In *E. coli*, *P. putida* KT2440 and in the native *Pseudomonas* CF600 host, DmpR expression is growth phase regulated with an increase in DmpR levels as cells enter the stationary phase of growth in rich media (Paper I, Fig. 1B; and IV, Fig. 1B-C). Western analysis also revealed that the DmpR levels are reduced to approximately half in ppGpp^0 *E. coli* and *P. putida* as compared to the wild type parents, while they are unaffected in strains devoid of DksA (Paper I, Fig. 1B; and III, Fig. 2C).

To investigate whether the reduced amount of DmpR in the cell is the cause of the decrease seen in σ^{54}-Po activity in a ppGpp^0 background, transcription from Po was monitored during over-expression of DmpR in *E. coli* and *P. putida*. Over-expression of DmpR to slightly higher than wild type levels in ppGpp^0 *E. coli* did not affect Po output (Paper I), and lower DmpR levels are thus not the link between lack of ppGpp and decreased Po output in this bacterium. DmpR over-expression in wild type *P. putida* did allow transcription from the Po promoter during exponential phase of growth, where Po is normally silent. However, a swift increase in Po activity could still be seen at the transition point between exponential and stationary phase of growth (Paper IV, Fig. 4B), indicating that in *P. putida* there are two different check points for σ^{54}-dependent transcription from Po; one checkpoint that works via the low levels of DmpR in exponential phase of growth, while the other one is mediated via the action by DksA in conjunction with the elevated levels of ppGpp produced at the exponential-to-stationary phase growth transition. From this study it could thus be concluded that even though ppGpp is involved in regulating the levels of DmpR, and that the levels of DmpR are obviously important for Po activity, a major effect of ppGpp and DksA must occur at some additional level to that controlling the levels of DmpR.

6.5. Naturally occurring bEBP levels determine σ^{54}-dependent transcription in exponential phase of growth

The finding that over-expression of DmpR in *P. putida* did allow some transcription from Po in the exponential phase of growth raised the question whether naturally occurring levels of a bEBP can allow σ^{54}-transcription in this growth phase. XylR, the activator of the Pu promoter of the pWW0 plasmid, is highly homologous to DmpR. It can

bind to the UASs of Po and activate transcription from this promoter in response to its natural effector molecules (Fernández et al, 1994; Pérez-Martín & de Lorenzo, 1996). In the same way as DmpR is growth phase regulated with an increase in protein levels when entering the stationary phase of growth, so are the levels of XylR (Fraile et al, 2001). The latter, however, is naturally produced in higher levels compared to those of DmpR. Transcription from σ^{54}-Po in a *P. putida* strain synthesizing XylR to approximately 65% of the levels produced from the native pWW0 plasmid was monitored. The results show that when XylR was used as regulator, transcription from Po was relatively higher in a ppGpp^0 background as compared to when DmpR was the regulator. Further, the otherwise very tightly regulated Po activity was also slightly elevated during exponential phase of growth (Paper III, Fig. 4C). These data suggest that some σ^{54}-dependent systems could potentially be expressed in *P. putida* also during conditions where a lot of nutrients are present (i.e. when ppGpp levels are low), so long as the amount of bEBP in the cell is sufficiently high.

6.6. σ^{54} competes with the other σ-factors for a limiting pool of core RNAP

Until rather recently, the number of σ-factors present in the cell was estimated to only slightly exceed the amount of core RNAP available for association to form σ-RNAP holoenzymes. In 2006, however, new assessments of the numbers of σ^{70} and core RNAP in *E. coli* grown in rich media were made. An estimated number of approximately 13,000 molecules of core RNAP are available for holoenzyme formation with approximately 17,000 molecules of σ^{70} (Grigorova et al, 2006). The levels of σ^{54} in the cell are always lower than those of σ^{70}, and are retained at approximately 16-20% of the σ^{70}-levels (Jishage et al, 1996). Further, the levels of σ^S have to be accounted for when cells enter the stationary phase of growth, where σ^S rapidly increase to approximately 30% of the levels of σ^{70} (Jishage & Ishihama, 1995). Under *in vivo* conditions, the competition between the different σ-factors for core RNAP is thus likely very fierce.

Previously it has been shown that the levels of different σ-factors in the cell markedly affect σ^{54}-dependent transcription from the Po promoter. Deletion of the σ^S-encoding *rpoS* gene, overproduction of σ^{54} or underproduction of σ^{70} all result in higher transcription levels from σ^{54}-Po (Laurie et al, 2003; Sze et al, 1996). These findings experimentally demonstrate that there is competition between the different σ-factors for a limiting pool of available core RNAP, and that this competition results in a limitation in σ^{54}-dependent transcription. Competition between σ^{54} and σ^{70} for core RNAP can be examined *in vitro*. There is no cross-recognition between the σ^{54} and σ^{70} promoter classes, and the simultaneous monitoring of σ^{54}-transcription from Po and σ^{70}-transcription from RNA1 can be performed using a template carrying both of these promoters (Laurie et al, 2003). In *in vitro* transcription assays, σ^{54} and σ^{70} have similar high affinity for core RNAP when assessed in isolation. However, under *in vitro* competition conditions, σ^{70} more efficiently out-competes σ^{54} than the opposite (Laurie et al, 2003).

As brought up under section 6.2, ppGpp and DksA do not have any apparent direct effects on transcription from the σ^{54}-Po promoter *in vitro* (Paper I, Fig. 2A-B). To test whether these regulatory molecules are directly involved in controlling competition between different σ-factors for core RNAP, for instance by changing the affinities of core RNAP for σ^{54} and/or σ^{70}, a multiple round *in vitro* competition assay with σ^{54} and σ^{70} was employed. A fixed concentration of core RNAP and σ^{70} was challenged by increasing amounts of σ^{54} either in the presence or absence of ppGpp and DksA. As seen in Paper I, Fig. 4, the presence of ppGpp and DksA did not make σ^{54} better at out-competing σ^{70}. Neither did they affect the outcome when a fixed concentration of σ^{54} was challenged with increasing concentrations of σ^{70} (Paper I, data not shown). Hence, ppGpp and DksA do not appear to act by favoring core RNAP binding of σ^{54} over that of σ^{70}. This suggests that a purely passive mode of action by these molecules is employed to result in enhanced σ^{54}-dependent transcription.

6.7. ppGpp and DksA do not control output from the σ^{54}-Po promoter through Rsd/PfrA

The production of the *E. coli* protein Rsd is partially under control of ppGpp (Jishage et al, 2001; Jishage & Ishihama, 1998). This protein can sequester both free σ^{70} and actively remove σ^{70} from the σ^{70}-holoenzyme *in vitro* (Ilag et al, 2004; Westblade et al, 2004). Over-expression of Rsd renders an increase in σ^{54}-dependent transcription from the Po promoter, as well as higher levels of σ^S-dependent transcription (Jishage et al, 2002; Laurie et al, 2003; Mitchell et al, 2007). Hence it was considered that Rsd might be involved in the ppGpp-mediated control of transcription from σ^{54}-Po by affecting competition between σ-factors for core RNAP. To further investigate this, null mutants were made of *E. coli* Rsd and *P. putida* PfrA, which is a homologue of the Rsd protein. However, neither the temporal nor maximal expression from Po was affected upon lack of these proteins in either organism (Paper III, Fig. S2). ppGpp and DksA thus do not mediate their growth phase regulatory effect on σ^{54}-Po through Rsd/PfrA, although it can not be excluded that under other growth conditions Rsd/PfrA might have effects on σ-factor competition.

7. THE EFFECTS BY ppGpp AND DksA ON Po ACTIVITY ARE LARGELY MEDIATED THROUGH σ^{70}-RNAP AND THEIR DOWN-REGULATION OF POWERFUL STRINGENTLY REGULATED PROMOTERS

7.1. RNAP-mutants provide a link between ppGpp, DksA and σ^{54}-dependent transcription

Previously it has been shown that some core RNAP and σ^{70} mutants isolated on the basis of restoration of prototrophy in ppGpp^0 *E. coli* (Hernandez & Cashel, 1995) could restore efficient transcription from the σ^{54}-Po promoter in the absence of ppGpp (Sze & Shingler, 1999). To further pursue the issue of how ppGpp control of Po activity could possibly be linked through σ^{70}-RNAP, additional suppressor mutants were isolated, this time on the basis of restoration of transcription from σ^{54}-Po in ppGpp^0 *E. coli* (Laurie et al, 2003). Out of the spontaneous mutants isolated in the new screen, five hyper-suppressor mutants were found with the mutations mapped to active site cleft residues of the core β- and β´-subunits (Paper II, Table 2). These hyper-suppressor mutants restored prototrophy to the otherwise auxotrophic ppGpp^0 *E. coli* strain, and resulted in >15-fold higher output from Po than found with wild type core RNAP in a ppGpp deficient strain (Paper II, Fig. 1A). Further, two independent mutations within the σ^{70}-encoding *rpoD* gene were found in the same screen. The σ^{70} mutants restored Po output in a ppGpp^0 background to 1.5- and 16-fold higher than in a ppGpp^0 strain carrying wild type *rpoD* (Paper I, Fig. 1C; and Laurie et al, 2003). These data suggest that ppGpp-control of transcription from the σ^{54}-Po promoter is mediated through the transcriptional apparatus.

As mentioned in section 6.6, there is no cross-recognition between the σ^{54}- and σ^{70}-promoter classes, making it unlikely that the σ^{70} mutants directly affect σ^{54}-Po promoter output. The identification of mutations in σ^{70} that increased transcription from σ^{54}-Po led back to the data regarding competition between different σ-factors for core RNAP (section 6.6). One possible explanation for how these σ^{70} mutants mediate their positive effect on Po output is thus through the common substrate they share with σ^{54}, namely core RNAP.

7.2. σ^{70} mutants are defective in competition with σ^{54} for core RNAP

The two σ^{70} mutants isolated by restoration of σ^{54}-Po activity ($\sigma^{70\text{-}40Y}$ and $\sigma^{70\text{-}35D}$; Laurie et al, 2003) and two mutants isolated by restoration of prototrophy ($\sigma^{70\text{-}P504L}$ and $\sigma^{70\text{-}S506F}$; Hernandez & Cashel, 1995) of ppGpp^0 *E. coli* had previously been purified (Laurie et al, 2003). In the same study, the abilities of these mutants to compete with σ^{54} for core RNAP were examined by an *in vitro* transcription competition assay. The results showed that the σ^{70} mutants are truly defected in σ-factor competition, with their disabilities correlating very well with their capacity to suppress the need of ppGpp by the σ^{54}-Po promoter ($\sigma^{70\text{-}40Y} > \sigma^{70\text{-}P504L} > \sigma^{70\text{-}S506F} > \sigma^{70\text{-}35D}$; Laurie et al, 2003). The effect of the $\sigma^{70\text{-}40Y}$ and $\sigma^{70\text{-}35D}$ mutants on transcription from the Po promoter in a DksA null *E. coli* strain has also been tested. Again, the $\sigma^{70\text{-}40Y}$ mutant was best at restoring transcription from σ^{54}-Po also in the absence of DksA, while $\sigma^{70\text{-}35D}$ resulted in a more modest increase in Po output (Paper I, Fig. 1D).

The requirement for ppGpp and DksA for efficient transcription from the σ^{54}-Po promoter can thus be bypassed *in vivo* by mutations in σ^{70} that render this subunit defective in competition with σ^{54} (and possibly also with other alternative σ-factors) for core RNAP. These data support the hypothesis brought up in section 6.6 that ppGpp and DksA, at least in part, mediate their control of σ^{54}-transcription via modulating the outcome of σ-factor competition, and that this occurs in a purely passive way.

7.3. β and β´ mutants disclose a link between σ^{54}-transcription and properties associated with down-regulation of stringently regulated σ^{70}-promoters

The five hyper-suppressor β and β´ mutants described in section 7.1 (β-R451C, β-H551Y, β-H1244L, β-Q1264P and β'-L432R) that were identified by their ability to allow robust σ^{54}-transcription from Po in the absence of ppGpp, were also investigated for their ability to restore Po activity in a DksA null *E. coli* background. All the core RNAP (β, β') mutants positively affect Po output in the absence of DksA, but not to the same high extent as they restored transcription in a ppGpp^0 *E. coli* strain (Paper II, Fig. 2). This suggests that DksA might have some extra function(s) in the cell that the β and β' mutants cannot compensate for. Despite the finding that all the core RNAP mutants remained co-responsive to ppGpp and DksA *in vitro* (Paper II, Fig. 5C), they all mediated very similar high levels of transcription in cells possessing or lacking ppGpp (Paper II, Fig. 2). One possible explanation for this is that the maximal possible transcription rate from the σ^{54}-Po promoter is already reached in these strains even when ppGpp is absent.

The possible direct affects of the core RNAP mutants on transcription were first investigated by a comparison between σ^{54}- and σ^{70}-dependent transcription *in vitro* using the cognate mutant holoenzyme RNAPs. σ^{54}-dependent transcription from the Po promoter was up to 1.6-fold more efficient than transcription from the σ^{70}-λP_L promoter, indicating that there might be a deficiency in association by the core RNAP mutants to the σ^{70}-subunit (Paper II, Fig. 3E). The effect of these mutants was also assessed in *in vitro* transcription competition assays, where the output from σ^{54}-Po and σ^{70}-λP_L was simultaneously

measured. The results from this study correlated with the *in vitro* transcription data where σ^{54}- or σ^{70}-transcription was monitored in isolation, with the β-subunit mutants in general favoring association with σ^{54} over σ^{70}, while the β'-subunit mutant only showed a minor effect on its σ-association properties (Paper II, Fig. 4). However, none of the core RNAP mutants had as a pronounced effect on competition as the $\sigma^{70\text{-}40Y}$ mutant, also shown in Fig. 4B in Paper II. These effects on σ-factor competition are not major enough to explain the high *in vivo* restoration of σ^{54}-transcription in a $ppGpp^0$ *E. coli* background (Paper II, Fig. 2), suggesting that the core RNAP mutants are also affected in some other property.

To further study the *in vitro* properties of the core RNAP mutants, the innate stabilities of competitor-resistant open complexes formed on the σ^{54}-Po, σ^{70}-*rrnB* P1 and σ^{70}-λP_L promoters were monitored in relation to the stabilities of the complexes formed by holoenzymes utilizing wild type core RNAP in the presence of ppGpp and DksA (Paper II, Fig. 6). All the β/β' mutants formed complexes with lower stability on the three test promoters compared to the wild type, but to different extent and with different profiles. The pattern for each of the core RNAP mutants on σ^{54}-Po and σ^{70}-λP_L are similar, and although the lifetimes are reduced, they all substantially exceed the lifetime of the wild type RNAP in the presence of ppGpp and DksA (Paper II, Fig. 6D and F). However, with the stringent *rrnB* P1 promoter the pattern differed, and all but the β-Q1264P mutant polymerase formed very unstable RNAP-promoter complexes that are close to as unstable, or even less stable, than those seen with the wild type RNAP in the presence of ppGpp and DksA (Paper II, Fig. 6E).

The above findings regarding the intrinsically unstable competitor-resistant open promoter complexes formed by the mutant RNAPs prompted a re-investigation of the σ^{70} mutants described under section 7.1-7.2. All four σ^{70} mutant RNAPs likewise formed more unstable open complexes on σ^{70}-*rrnB* P1 than wild type σ^{70}-RNAP (Paper II, Fig. 7), with the defects of the σ^{70} mutants lying in the same order as their ability to restore transcription from σ^{54}-Po *in vivo*, as was seen with their defects in σ-factor competition.

Taken together, most of the σ^{70} and core RNAP mutants have two different defects that could combine to account for their *in vivo* phenotypes on σ^{54}-transcription from the Po promoter: 1] adjustment of the association between σ-factor and core RNAP to favor σ^{54} over σ^{70}, and 2] decreased inherent stability of competitor-resistant open RNAP-promoter complexes that mimics the ppGpp and DksA down-regulatory effect on stringently regulated σ^{70}-promoters. These properties support a purely passive model of how ppGpp and DksA are proposed to regulate σ^{54}-dependent transcription as described below.

7.4. A passive model for ppGpp and DksA regulation of σ^{54}-dependent transcription

σ^{54}-dependent transcription from the Po promoter is highly dependent on the presence of ppGpp and DksA *in vivo*, with an essential abolishment of Po output upon loss of both of these regulatory molecules. Any model for regulation of σ^{54}-Po dependent transcription by ppGpp and DksA has to take into consideration the following four aspects, based on the work outlined in this thesis; 1] ppGpp and DksA do not have any apparent direct effects on σ^{54}-transcription from Po *in vitro*, either alone or under σ-factor competition conditions. 2] Indirect effects by ppGpp and DksA on the levels of proteins required for efficient σ^{54}-transcription (e.g. DmpR and IHF), although important, are not the major issues in their regulation of transcription from σ^{54}-Po. 3] σ^{54} is under fierce competition with the other σ-factors for core RNAP under *in vivo* conditions, and 4] mutants of σ^{70} and the core RNAP β- and β'-subunits, which can bypass the *in vivo* requirement for ppGpp and DksA, have deficiencies that favor formation of σ^{54}-RNAP and mimic the ppGpp and DksA effect of negative regulation of stringent σ^{70}-dependent promoters.

The passive model of regulation of σ^{54}-dependent transcription by the global regulators ppGpp and DksA put forward here (Fig. 10), relies on the ability of these molecules to co-operate to down-regulate the powerful stringently regulated σ^{70}-rRNA operon promoters. Changes in ppGpp levels are the key, since the levels of DksA in the cell do not change more than approximately 2-fold during different growth conditions, while ppGpp levels vary dramatically. During conditions of rapid growth in rich media (Fig. 10A), where the ppGpp levels are low, the powerful σ^{70}-rRNA operon promoters sequester a large proportion (approximately 60-70%) of the transcriptional machinery (Bremer & Dennis, 1996). Thus, the majority of the core RNAP will be occupied in transcription of the rRNA operons, leaving a low amount of free core RNAP for association with σ^{70}, σ^{54} and other alternative σ-factors. In turn, this will lead to a low level of σ^{54}-RNAP holoenzyme, a low occupancy of e.g. the σ^{54}-Po promoter, and thus a low output from this promoter under these growth conditions. Under conditions where ppGpp is produced (Fig. 10B), however, this alarmone will in conjunction with DksA function to down-regulate the stringently regulated powerful σ^{70}-rRNA operon promoters, freeing core RNAP from these operons for association with the σ-factors. With a larger free pool of core RNAP, the levels of σ^{54}-RNAP in the cell will increase, leading to higher σ^{54}-Po occupancy and activity.

The role of the core RNAP- and σ^{70}-mutants within this model would be to 1] mimic the effects of ppGpp and DksA by directly reducing transcription from the strong stringent σ^{70}-rRNA operon promoters leading to an

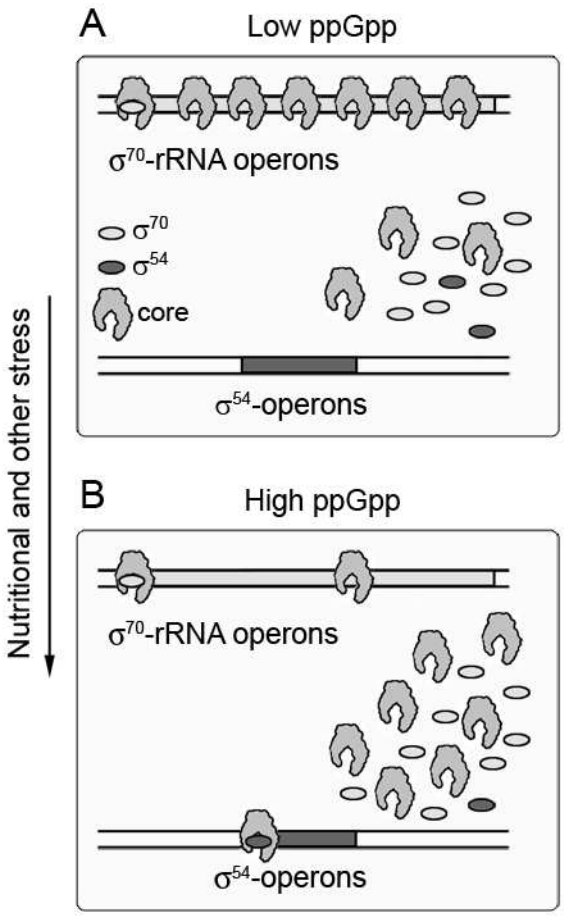


Fig. 10. Passive model for the control of σ^{54}-dependent transcription by ppGpp and DksA. The availability of key transcriptional components is schematically illustrated under different growth conditions.
(A) Growth conditions where ppGpp levels are low. Under these conditions the majority of the transcriptional machinery is involved in catalysis from the powerful rRNA operon promoters, leaving low core RNAP levels available for association with various σ-factors, and thus low σ^{54}-RNAP levels.
(B) Growth conditions rendering high ppGpp levels. ppGpp and DksA mediated down-regulation of transcription from the rRNA operon promoters increases the levels of core RNAP available for holoenzyme formation, leading to increased σ^{54}-RNAP levels and thus higher occupancy of σ^{54}-promoters. Adapted from Paper I.

elevation in the available levels of core RNAP for association with other σ-factors, and 2] to alter the core to σ-factor association properties to favor the formation of σ^{54}-RNAP over that of σ^{70}-RNAP. Further, the hyper-suppressor phenotype in σ^{54}-Po activity mediated by the core RNAP mutants, compared to the milder suppressor phenotype by the σ^{70}-mutants, could possibly be explained by the presence of the other alternative σ-factors in the cell. The σ^{54}-factor is structurally distinct from σ^{70} and other alternative σ-factors that all belong to the σ^{70}-family. Mutations in the β- and β'-subunits of the core RNAP that are defective in association with σ^{70} are thus likely to be altered in their association properties to other σ^{70}-like proteins as well. The σ^{54}-factor might thus be favored in association with these mutant core RNAPs not only over σ^{70}, but also over the other alternative σ-factors, making it better in competing with all other σ-factors in the cell.

7.5. Low affinity σ^{54}-promoters are more susceptible to the loss of ppGpp and DksA than high affinity counterparts

σ^{54}-dependent promoters with lower affinity for σ^{54}-RNAP should theoretically be more dependent on cellular σ^{54}-RNAP levels than high affinity promoters since they would be more difficult to saturate. The proposed increased level of σ^{54}-RNAP in the cell, as the result of ppGpp and DksA action, would thus be predicted to lead to a greater dependency of low affinity promoters on ppGpp and DksA, and they would thereby be more sensitive to the absence of these molecules. To test this hypothesis, a number of hybrid σ^{54}-promoters were constructed. The frame-work of the Po promoter was used to obtain promoters of different affinities for σ^{54}-RNAP that all are activated by DmpR (Fig. 11; and Paper I and III). In addition to reconstructing the Po promoter (designated Po/Po), the promoters used were the *P. putida* derived low affinity promoter Pu (Bertoni et al, 1998), the *Klebsiella pneumonia* derived low affinity promoter PnifH and a mutant derivate thereof named PnifH049 with both increased affinity and transcriptional output (Buck & Cannon, 1992; Morett & Buck, 1989) and two high affinity promoters from *E. coli;* PpspA (Weiner et al, 1995) and PglnA (Popham et al, 1989). The relative affinities of these hybrid promoters for σ^{54}-RNAP were investigated by electro mobility shift assays, and were shown to lie in the order Po/PnifH < Po/Pu < Po/Po < Po/PnifH049 < Po/PglnA < Po/PpspA. These promoters should theoretically be able to recruit σ^{54}-RNAP from the *in vivo* available pool with the same hierarchy (Paper I, Fig. 5B).

Transcription from the hybrid promoters was monitored both *in vivo* and *in vitro*. As was previously seen for the native Po promoter, addition of ppGpp and/or DksA had little or no direct effect on output from the hybrid promoters *in vitro* when using either *E. coli* or *P. putida* transcriptional components (Paper I, Fig. 7D; and III, Fig. 5B). Further, the results from the *in vivo* situation in both *E. coli* and *P. putida* backgrounds were as anticipated, with the lower affinity promoters (Po/PnifH, Po/Pu and Po/Po) showing greater dependency on ppGpp and DksA than the high affinity counterparts (Po/PnifH049, Po/PglnA and Po/PpspA; Paper I, Fig. 7C; and III, Fig. 5A). These data thus fit with the passive model of ppGpp and DksA regulation of σ^{54}-dependent transcription and further strengthens this model.

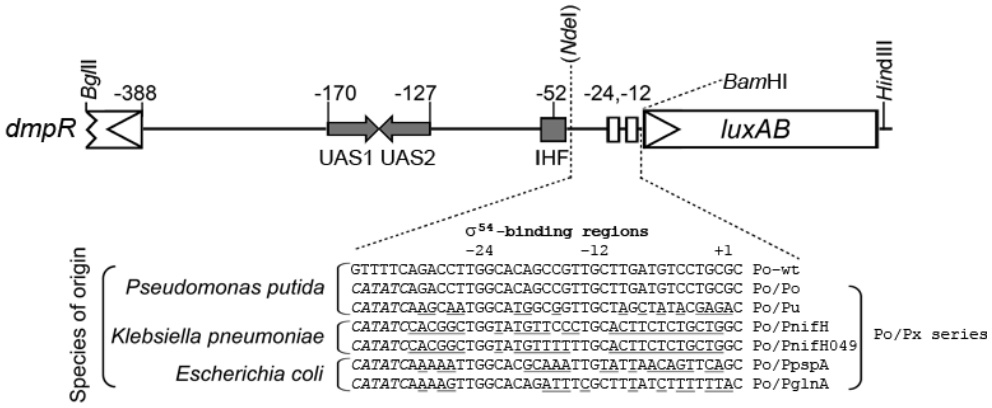


Fig. 11. Hybrid σ^{54}-transcriptional reporters. Schematic illustration (not to scale) of the Po regulatory region together with the locations of *dmpR* and the luciferase *luxAB* reporter genes (open rectangles). Transcription directions are indicated by arrow heads. The DNA binding sites for DmpR (inverted arrows; UAS1 and UAS2), the recognition sequence for IHF (shaded box), and the Po promoter -24, -12 motif (open boxes) are also indicated. Po/Px indicates the origin of the -39 to +2 σ^{54}-RNAP binding region in the context of the Po regulatory region. The lower part of the figure shows the DNA sequences of the -39 to +2 regions of the indicated σ^{54}-promoters, with nucleotides different from the Po promoter underlined. Adapted from Paper III.

8. THE BIOCHEMICAL PROPERTIES OF σ^{54}-RNAP FROM *E. coli* AND *P. putida* DIFFER

8.1. *P. putida* σ^{54}-RNAP discriminates less between different promoters, compared to its *E. coli* counterpart

The studies on transcription from the σ^{54}-hybrid promoters (section 7.5) revealed an interesting difference in σ^{54}-transcription in *E. coli* compared to in *P. putida* that was also seen with purified transcriptional components from these two species. In *E. coli,* the transcriptional output from the different hybrid promoters differed approximately 18-fold between the lowest (Po/PnifH) and the highest (Po/PglnA and Po/PpspA) affinity promoters (Paper I, Fig. 7C). In *P. putida,* however, only a modest 3-fold difference was seen in maximal outputs from the promoters with the highest and lowest affinity for σ^{54}-RNAP (Paper III, Fig. 5A). Further, while the temporal profiles in σ^{54}-transcription is similar in both *E. coli* and *P. putida*, the maximal output differs, with a clear relatively high stationary phase transcription in *P. putida* from even the hybrid promoters with lower affinity. The same pattern with an approximate 3-fold difference in transcriptional output from the high and low affinity promoters in *P. putida* was recapitulated also *in vitro* with *P. putida* σ^{54}-RNAP, while the *in vitro* data with *E. coli* transcriptional components rendered the same 18-fold difference as seen *in vivo* (Paper III, Fig. 5B; and I, Fig. 7D).

To examine if the activities of the two σ^{54}-holoenzymes were equally active, *in vitro* transcription titration assays were performed. *E. coli* or *P. putida* σ^{54} was titrated into reaction mixes with a constant concentration of either *E. coli* or *P. putida* core RNAP, and the output from the Po promoter was determined with either native or heterologous holoenzymes. The σ^{54}-factors from the two species saturated the core RNAP at similar concentrations, indicating that their core association properties do not differ and that the activities of the purified proteins are the same (Paper III, Fig. 5C). However, the absolute transcription output with saturating levels of the σ^{54}-RNAPs differed markedly, with transcription employing σ^{54} from *P. putida* rendering more than 20-fold higher transcription levels than the *E. coli* counterpart (Paper III, Fig. 5D).

Taken together, these data suggest that the biochemical properties of the σ^{54}-RNAP in *E. coli* and *P. putida* differ. The σ^{54}-RNAP from *P. putida* seems to be less discriminative regarding the binding sequence of the promoters than its *E. coli* counterpart, leading to vigorous maximized transcription in the post-exponential phase of growth even from promoters with relatively low affinity for σ^{54}-RNAP.

8.2. Reduced promoter discrimination is conferred via the DNA binding properties of σ^{54}-RNAP

The biochemical properties of the *E. coli* and *P. putida* derived σ^{54} and core RNAP were further investigated using Surface Plasmon Resonance assays (Biacore). Binding of σ^{54}-RNAP to chip-coupled DNA encompassing the following three hybrid promoters was examined: i) The lowest affinity promoter Po/PnifH, ii) the intermediate affinity promoter Po/Po and iii) the high affinity promoter Po/PglnA. The results showed that the binding properties of the σ^{54}-holoenzymes from the two species differ, with the *E. coli* σ^{54}-RNAP showing a more than 5-fold difference in binding to the promoters with lowest and highest affinity, while the *P. putida* counterpart bound to the lowest and highest affinity promoters with less than 2-fold difference (Paper III, Fig. 6). These binding patterns coincide with the transcriptional output seen both *in vivo* and *in vitro*, with less discrimination between promoters with low and high affinity for σ^{54}-RNAP when the

holoenzyme from *P. putida* is employed (Paper III, Fig. 5A-B). Further Biacore studies employing native and heterologous σ^{54}-RNAPs demonstrated that the species differences in binding properties of the σ^{54}-RNAP are largely mediated by the σ^{54}-subunit, because *P. putida* σ^{54} in both the native and heterologous pair showed rapid binding to all three test promoters, while a slower kinetics were seen with the *E. coli* σ^{54}-subunit. In addition, this study revealed that the origin of the core RNAP is also involved in determining the outcome of binding, so that the combination of native *P. putida* derived σ^{54} and core RNAP bound most efficiently to all three test promoters (Paper III, Fig. 7).

These data suggest that in the environmentally adaptive *P. putida,* σ^{54}-transcription, which is often employed for expression of auxiliary metabolic pathways, has evolved to meet the high demands put on soil dwelling bacteria to combine tight regulation during high energy conditions with robust transcription from even low affinity promoters under conditions were nutrient limitation is prevalent.

9. REGULATION OF PRODUCTION OF THE bEBP DmpR PROVIDES A SECOND LINK BETWEEN ppGpp, DksA, σ^{70}-RNAP AND Po ACTIVITY

9.1. The *dmpR*-controlling Pr promoter is regulated by the non-overlapping divergent Po promoter

Prior to the study presented in Paper IV, not much was known about the promoter controlling transcription of the regulatory gene *dmpR*. This promoter, denoted Pr, is a σ^{70}-dependent extended -10 promoter, which is very DNA context dependent (Paper IV, Fig. 2C). Further, the spacing between the extended -10 box of this promoter and the transcriptional +1 start of *dmpR*, mapped by primer extension using both *in vivo* and *in vitro* generated mRNA, is highly unusual (29 base pairs; Paper IV, Fig. 2 and S1). This indicates that the primer extension reaction might have been blocked by some stable secondary structure formed by the *dmpR* mRNA. However, analysis of the sequence in this area does not indicate the presence of any secondary structure. Another more intriguing possibility is that transcription from Pr involves initial scrunching of the DNA within the active site, followed by cleavage of the mRNA by the intrinsic cleavage activity of RNAP that is normally stimulated by the activity of Gre factors. Whatever the mechanism underlying this unusually distal +1 start, it was used for orientation purposes throughout the study in Paper IV.

As brought up in section 6.4, the levels of DmpR increase at the transition point between the exponential and stationary phase of growth (Paper I, Fig. 1B; and III, Fig. 2B). Using a transcriptional *luxAB* fusion to the Pr promoter, it was found that the activity of this promoter mirrors the activity of the σ^{54}-Po promoter, with increased transcription upon entering the stationary phase of growth in rich media underlying the higher DmpR levels found in both *P. putida* KT2440 and in the native CF600 host (Paper IV, Fig. 3). The Pr promoter is situated approximately 200 base pairs from the divergent and non-overlapping Po promoter. It was therefore surprising to find that the up-regulation in Pr activity at the exponential-to-stationary phase transition is dependent on the presence of the Po promoter. More specifically, it was found to require occupancy of Po by σ^{54}-RNAP since the elevation in transcription is abolished when the Po promoter is mutated and/or when σ^{54} is absent from the cell (Paper IV, Fig. 6B). According to the passive model of ppGpp and DksA regulation of Po activity (see section 7.4), the levels of σ^{54}-RNAP increase at the exponential-to-stationary phase transition point, leading to a higher occupancy of the Po promoter which, as shown here, would in turn stimulate Pr output at this stage of growth.

In the simultaneous presence of an effector molecule and DmpR in the cell, transcription from Pr is auto-stimulated in the post-exponential phase of growth. Again, this increase is seen at the DmpR protein level in both *P. putida* KT2440 and CF600. Mutational studies and *in vitro* transcription assays utilizing both wild type and constitutively active DmpR derivatives showed that auto-stimulation of Pr activity by effector-activated DmpR is mediated through open complex formation and/or active transcription from the σ^{54}-Po promoter (Paper IV, Fig. 6B and 7E-F). DmpR thus stimulates its own production through promoting transcription from σ^{54}-Po. Since lack of binding and active transcription from Po by σ^{54}-RNAP result in a severe decrease in Pr activity (Paper IV, Fig. 6B), the σ^{70}-Pr promoter is effectively placed under the control of both σ^{70}-RNAP and σ^{54}-RNAP without the presence of a binding site for the σ^{54}-RNAP holoenzyme.

While auto-stimulation of transcription from Pr by effector-activated DmpR-mediated activity of the Po promoter could be reiterated in *E. coli*, stimulation by Po occupancy could not (unpublished data). This result is likely explained by the differing biochemical properties of the σ^{54}-RNAP from *E. coli* and *P. putida* (see section 8.1-8.2). While the *P. putida* derived σ^{54}-RNAP binds strongly to lower affinity promoters (as Po), the *E. coli* counterpart is more discriminative and binds very poorly to low affinity promoters. This property of *E. coli* σ^{54}-RNAP leads to less occupancy of the Po promoter and thus less or no pronounced stimulation of transcription from Pr in *E. coli*.

9.2. Stimulation by non-overlapping divergent transcription: A common regulatory mechanism?

The findings outlined above prompted the question of whether transcription from Pr could be stimulated by transcription from any non-overlapping divergent promoter, or if this feature was limited to the σ^{54}-

dependent Po promoter. To address this question, Po was replaced by the σ^{70}-dependent λP_L promoter, which is independent of the regulators needed for maximized transcription from Po, i.e. σ^{54}, DmpR, IHF, ppGpp and DksA. Since the λP_L promoter is not dependent on any regulator for open complex formation and transcription initiation, the sole influence of occupancy of this promoter on Pr output could not be assessed in the same easy way as for Po occupancy by σ^{54}-RNAP (i.e. by omitting DmpR and/or an effector molecule). Nevertheless, the effect of active transcription from λP_L on Pr output could be evaluated by *in vivo* transcription assays monitoring output from Pr. These assays revealed that Pr indeed could be stimulated by active transcription from a non-σ^{54}-dependent promoter, with an approximate 2-fold increase in Pr output in the presence of the native λP_L promoter as compared to an inactive mutated version of this promoter (Paper IV, Fig. 6B). This finding increases the likelihood that there are other promoters within bacterial genomes and plasmids that, like Pr, are dependent on a non-overlapping divergent promoter for efficient transcription during certain growth conditions.

9.3. IHF stimulates transcription from Pr

Transcription from Po is impaired when binding of the DNA bending protein IHF in the σ^{54}-Po promoter region is prevented by mutation of the IHF binding site, (Paper I, Fig. 8C; and III, Fig. 3A). Reduced Po output should theoretically lead to less stimulation in transcription from the divergent Pr promoter. To evaluate this hypothesis, transcription from the Pr promoter was monitored using constructs with a mutated IHF binding site and either a native or mutated Po promoter motif. The results in Fig. 6B in Paper IV confirm that transcription from the Pr promoter in stationary phase of growth is decreased when IHF cannot bind and stimulate Po output. However, binding of IHF to its recognition sequence also stimulates transcription from Pr when the Po promoter is mutated, indicating that occupancy of the IHF binding site, like occupancy of the Po promoter by σ^{54}-RNAP, promotes transcription from Pr. This contrasts the finding that binding of effector-activated DmpR to the UASs in the Po-Pr intergenic region does not *per se* affect Pr activity, as judged by the lack of any effect when analyzing the impact of DmpR with reporters where the Po promoter is replaced by λP_L (section 9.2; Paper IV, Fig. 6B). The finding that effector-activated DmpR has little or no effect is not unexpected because, in contrast to relatively stable binding of σ^{54}-RNAP and IHF, binding of DmpR-like bEBPs to their UASs is very transient (Valls & de Lorenzo, 2003).

9.4. An additional role for ppGpp and DksA in regulation of the DmpR/Po regulatory circuit

The combination of stimulation in Pr activity by divergent transcription from Po, the requirement of ppGpp and DksA for efficient Po occupancy and activity, and the finding that DmpR levels are reduced in ppGpp^0 *E. coli* and *P. putida* strains prompted the investigation of what direct effect these regulatory molecules might have on transcription from Pr. To this end Pr activity was monitored in parallel in DmpR-expressing *P. putida* wild type, ppGpp^0 and DksA null strains. It was found that ppGpp and DksA stimulate Pr activity both through the activity of the Po promoter, as expected, but also in a Po-independent manner, since transcription from Pr was impaired by lack of ppGpp and DksA when assayed using a reporter construct where the Po promoter was mutated (Paper IV, Fig. 7A). By employing *in vitro* transcription, it was discovered that this extra effect was due to a direct action of ppGpp and DksA on the Pr promoter that resulted in an approximate 3-fold stimulation in Pr output (Paper IV, Fig. 7B). ppGpp and DksA thus play a crucial role in the regulation of the *dmp*-system, with both indirect (σ^{54}-RNAP and IHF levels) and direct (Pr) effects, coupling the production of the energetically expensive Dmp-enzymes to the metabolic status of the cell.

9.5. Higher levels of DmpR increase the sensitivity of the Po promoter to phenolics

Over-expression of DmpR in *P. putida* allows transcription from the σ^{54}-Po promoter during exponential phase of growth (where no activity of this promoter is normally seen), and results in an increased maximal output from Po in the stationary growth phase (see section 6.4; and Paper IV, Fig. 4B). This indicates that DmpR is limiting for Po activity even when DmpR levels are at their highest in the stationary phase of growth. It was of interest to see whether increased levels of DmpR also rendered the Po promoter more sensitive to the presence of (methyl)phenolic compounds. Transcription from Po was thus monitored in the presence of increasing concentrations of 2-methylphenol and either native or over-expressed DmpR levels. This study showed that over-expressed levels of DmpR in the cell maximize transcription from the Po promoter even at very low effector concentrations (Paper IV, Fig. 4C). The natural increase in DmpR levels mediated by Po occupancy when entering the stationary phase of growth, and the further elevated levels of DmpR synthesized due to auto-stimulation through Po activity therefore have a physiological role - more DmpR means more sensitive sensing of pathway substrates. Thus, lower (methyl)phenol concentrations will be sufficient to promote transcription from the Po promoter and hence production of the Dmp-enzymes in the absence of preferred carbon and energy sources.

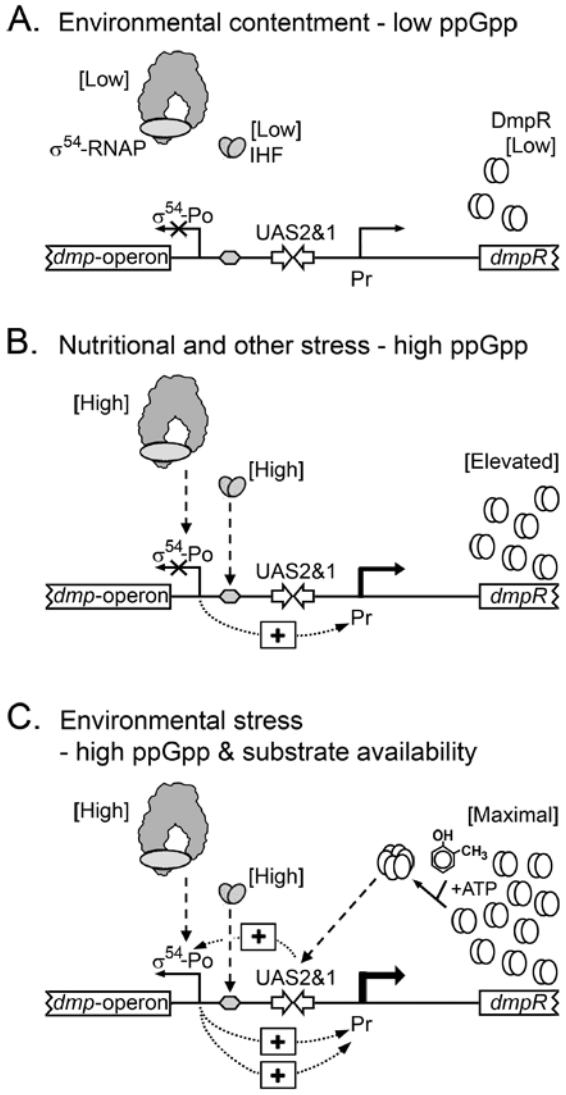


Fig. 12. Model of the regulatory network of the *dmp*-system. Schematic illustration of the consequences of occupancy and activity of the non-overlapping σ^{54}-Po promoter on the divergent σ^{70}-Pr promoter as detailed in the text. The *dmp*-operon and the DmpR encoding gene (open boxes), the IHF recognition sequence (shaded box) and the DmpR binding sites (UAS2&1; inverted arrows) are all indicated with size and distances shown to scale. The Po and Pr promoters are indicated by arrows. Occupancy of DNA binding sites by the respective proteins is shown as dashed arrows, while stimulatory effects (+) are shown as dotted arrows. Adapted from Paper IV.

9.6. Model for the regulatory network of the *dmp*-system

Based on 1] the proposed role of ppGpp and DksA in elevating σ^{54}-RNAP levels (section 7.4), 2] the control of transcription from σ^{70}-Pr via non-overlapping divergent transcription from the σ^{54}-Po promoter (section 9.1), and 3] the direct stimulatory effects of ppGpp and DksA on σ^{70}-Pr activity (section 9.4), a regulatory scheme for graded expression of the *dmp*-system has been proposed (Fig. 12; Paper IV). Within this scheme, ppGpp and DksA are crucial in keeping the *dmp*-operon turned off in the presence of energetically preferred carbon sources, and for the vigorous transcription from the σ^{54}-Po promoter and thus production of the catabolic enzymes when favored carbon sources are absent.

Under conditions where a lot of nutrients are available and the cells are rapidly growing (Fig. 12A), the σ^{54}-RNAP and IHF levels are low, giving rise to low occupancy of the Po promoter and the IHF binding site. As a result, Pr promoter activity is low, and so are the levels of DmpR in the cell. Under conditions where ppGpp is synthesized, i.e. during nutrient or physicochemical stress (Fig. 12B), this alarmone elevates the IHF levels and functions together with DksA to elevate the levels of σ^{54}-RNAP, thus rendering a higher occupancy of their respective binding site. This binding stimulates transcription from the Pr promoter, leading to higher levels of DmpR. A further increase in DmpR levels is achieved by the direct action of ppGpp and DksA on Pr activity. The system is thus primed with σ^{54}-RNAP and IHF bound to their respective binding site, and higher levels of DmpR, which can more efficiently probe the surroundings for (methyl)phenolic compounds that can be used as carbon and energy sources when preferred sources are lacking. Upon encountering an aromatic compound (Fig. 12C), DmpR multimerizes into its active form that can promote transcription from the σ^{54}-Po promoter. Transcription from Po not only renders the production of the (methyl)phenol catabolic enzymes of the *dmp*-pathway, but also further stimulates transcription of *dmpR* from the Pr promoter, maximizing the possibility of having co-occupancy of effector-activated DmpR and σ^{54}-RNAP bound to their respective binding site and thus maximized production of the catabolic Dmp-enzymes when they are truly needed. This feed-forward loop would be held in check by the maximum achievable output from the Po promoter that is established by the co-occupancy by DmpR, σ^{54}-RNAP and by IHF, which facilitates productive interaction between them.

10. POSSIBLE MECHANISMS AND FUTURE PERSPECTIVES

The precise mechanism behind stimulation of transcription from Pr via activity of a non-overlapping divergent promoter is so far unknown. While it is clear that open complex formation and/or active transcription from σ^{54}-Po are necessary, how these processes are coupled to result in stimulation of the activity of σ^{70}-RNAP at Pr remains to be determined. One possibility is prompted by the finding that auto-stimulation of Pr activity can only be recapitulated *in vitro* when a supercoiled DNA template is used, and not with a linear template (Paper IV, Fig. S2). Binding of σ^{54}-RNAP and IHF to their binding sites within

the intergenic region might induce torsional stress, and open complex formation and/or transcription from σ^{54}-Po may further promote this. The torsional stress and consequent alteration in supercoiling status of the DNA could influence either the binding of σ^{70}-RNAP to the Pr promoter and/or any other rate limiting step in the pathway of transcription initiation. Another possibility is that DNA bending, caused by occupancy of Po (by σ^{54}-RNAP) and the IHF binding site (by IHF), might allow upstream DNA to interact with Pr-bound σ^{70}-RNAP to stimulate its activity. Again, this process might be further facilitated by the formation of an open promoter complex and/or active transcription from the σ^{54}-Po promoter.

Whatever the precise mechanism underlying the net stimulation of transcription from σ^{70}-Pr by divergent transcription from σ^{54}-Po, the interplay outlined above effectively places a σ^{70}-dependent promoter under dual control of two forms of RNAP without possession of a cognate σ^{54}-RNAP binding site, and also makes it subservient to regulatory signals that elicit activity of σ^{54}-RNAP. However, the concept with dual sensitivity of a promoter to the levels of two different σ-RNAP holoenzymes need not be limited to σ^{70}/σ^{54}-RNAP holoenzymes. The σ^{54}-Po promoter replacement experiments outlined in section 9.2 indicate that any divergent transcription would likely suffice to impart some level of stimulation of transcription from Pr. Given that i) the activities of many alternative σ-factors are modulated in response to specific signals and ii) that divergent transcription of a regulatory gene and a cognate promoter under its control is a common motif in bacterial regulatory circuitry, it is plausible that other regulatory circuits that involve a σ^{70}-dependent promoter and a non-overlapping divergent promoter dependent on any alternative σ-factor could likewise be made subservient to conditions that elicit activity of the alternative σ-factor through a mechanism analogous to that of the σ^{70}-Pr promoter. Integrated signal-responsive control through divergent transcription of promoters dependent on different σ-factors thus has the potential to be a prevalent, but previously unappreciated, mechanism by which bacteria integrate diverse and/or conflicting signals to gain appropriate transcriptional control.

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