

Regulatory Roles of MarA in Flagellar Biosynthesis in *Escherichia coli*: A Novel Mechanism for Transcription Initiation

By

Ksenia Klimova

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Abstract

Multiple antibiotic resistance is one of the main threats to human health in the modern world. Multiple antibiotic resistance (*mar*) locus provides resistance to a wide range of antibiotics and phenolic compounds in gram-negative bacteria *Escherichia coli*. The transcription factor MarA encoded in this locus is mainly required for activation and expression of the AcrAB-TolC encoded efflux pump, which plays a primary role in active antibiotic transport out of the cell. However, recent studies suggest that MarA is also involved in lipid trafficking, DNA repair and biofilm formation in *E. coli*.

The current work shows that MarA can also regulate flagellar gene expression, suggesting an interplay between flagellar biosynthesis and intrinsic response to antibiotics. New σ^{28} -dependent promoter regions were found upstream of the *flgBCDEFGHIJ/KL* and *flgAMN* operons, which were known as σ^{70} - and FlhDC-dependent only. Both operons are located in close proximity to each other on opposite DNA strands, and share an FlhDC binding site. Another σ^{28} -dependent promoter was also found upstream of the *flgJ* gene, which is a part of the *flgBCDEFGHIJ/KL* operon. A new σ^{70} -dependent promoter was found upstream of the *flgM* gene, which also has a σ^{28} -dependent promoter, and can also be transcribed as part of the *flgAMN* operon. *Marboxes* were identified upstream of the *flgA*, *flgB*, *flgJ* and *flgM* genes.

MarA has been known as an activator that assists only σ^{70} -dependent transcription in a Class I or Class II manner. In the current study, MarA activates both *flgA* and *flgB*

σ^{70} -dependent promoters in an FlhDC-dependent manner *in vivo* via an unknown indirect mechanism. However, *in vitro* MarA assists the novel σ^{28} -dependent *flgB* promoter in a Class III manner. MarA binds between the -10 and -35 elements of σ^{28} -dependent *flgB* promoter, and distort conserved -11A base, where unwinding of the open complex nucleates. At the same time, MarA represses open complex formation on *flgA* promoter.

This regulatory mechanism suggests that MarA plays a role in redirecting transcription to favor partial flagellar biosynthesis during antibiotic stress, potentially enabling *E. coli* to escape hazardous environments. The novel finding that MarA can assist RNAP σ^{28} expands our understanding of transcription regulation and suggests a broader regulatory interactions in bacterial adaptive responses.

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List of Abbreviations

A	Adenine
APS	Ammonium persulfate
AMP	Adenosine monophosphate
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
°C	Degrees Celsius
CIP	Calf intestine alkaline phosphatase
Cryo-EM	Cryogenic electron microscopy
CTD	C-terminal domain
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytosine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
DAEC	Diffusely adherent <i>E. coli</i>
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
ddDNA	Double-stranded DNA
ddH ₂ O	Deionised and distilled water
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Diaminoethanetetra-acetic acid

EAEC	Enterοaggregative <i>E. coli</i>
EHEC	Enterohaemorrhagic <i>E. coli</i>
EIEC	Enteroinvasive <i>E. coli</i>
EPEC	Enteropathogenic <i>E. coli</i>
ETEC	Enterotoxigenic <i>E. coli</i>
G	Guanine
GMP	Guanosine monophosphate
H-NS	Histone-like nucleoid structuring protein
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HTH	Helix-turn-helix
IM	Inner membrane
kb	Kilobase
LB	Luria-Bertani
LC/MS	Liquid chromatography/mass spectrometry
LPS	Lipopolysaccharide
Mar	Multiple antibiotic resistance
MarA	Activator of multiple antibiotic resistance
MarA	Repressor of multiple antibiotic resistance
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
Mg ²⁺	Magnesium ion
mRNA	Messenger RNA
NTD	N-terminal domain
NTP	Nucleoside triphosphate
OD	Optical density
OM	Outer membrane
ONPG	Ortho-nitrophenyl-β-galactosidase
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
ppGpp	Guanosine tetraphosphate
RNA	Ribonucleic acid
RNAP	RNA polymerase
RND	Resistance nodulation division
Rob	Right of origin binding protein
rRNA	Ribosomal RNA
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SoxS	Superoxide stress protein
ssDNA	Single-stranded DNA
ssRNA	Single-stranded RNA
T	Thymine
T4 PNK	T4 polynucleotide kinase
TBA	Tris-borate EDTA
TEMED	N,N,N',N'-Tetramethyl ethylenediamine
T_m	Melting temperature
TNSC	Transcription buffer
Tris	Tris (hydroxymethyl) aminoethane
tRNA	Transfer RNA
TSS	Transcription start site
UTI	Urinary tract infection
V	Volts
v/v	Volume/volume
W	Watts
w/v	Weight/volume
WT	Wildtype

Chapter 1

Introduction

Preamble

Bacteria have evolved over 3.5 billion years, from the last universal common ancestor (LUCA), before archaea and eukaryotes appeared on this planet. Both bacteria and archaea are considered prokaryotes due to lack of membrane-bound organelles and a true nucleus. Different sized ribosomes, transcription apparatus and cell wall composition, also identify to these organelles as prokaryotic. Bacterial cells can display five shapes: rod (bacilli), spherical (cocci), curved or comma (vibrios), spiral (spirilla), and corkscrew (spirochaetes).

Bacteria occupied many habitats like fresh and sea water, deep sea hydrothermal vents, soil and arctic snow. As more complex life forms evolved on Earth, bacteria found opportunities to colonize and utilise them as habitats. Over time, bacteria have adapted to life in organisms like plants, mushrooms and other pro- and eukaryotes including us, humans. This co-evolutionary process shaped the trajectory of bacterial adaptation, enabling them to establish successful symbiotic relationships with new hosts. This adaptability is made possible by the intricate and precise regulation of genes, allowing bacteria to express genes essential for their immediate needs while silencing those that are non-essential, thereby saving energy.

A goal of bacterial molecular biology is to gain a deeper understanding of how bacteria effectively manage and adapt to stress. Bacteria are constantly exposed to various stressors, including changes in temperature, pH, radiation, starvation, high osmotic pressure, and the presence of toxic compounds, including antibiotics. This work is focused on the mechanisms through which bacteria *Escherichia coli*, a member of Enterobacteriaceae family, regulates the expression of their genetic material and explore new molecular mechanisms involved in antibiotic resistance.

1.1 Enterobacteriaceae species

Enterobacteriaceae represent large and diverse family of facultatively anaerobic, non-spore-forming, rod-shaped, gram-negative bacteria that contains over 60 genera and more than 250 species [3]. Production of peritrichous flagella, arranged along the length of a bacterial cell is one of the main characteristics of this group with some exceptions found in the genera *Klebsiella*, *Leminorella*, *Moellerella*, and *Shigella* [4]. However, the main molecular indicator of this family is the Enterobacterial Common Antigen (ECA), or a capsular polysaccharide whose precise function remains unclear, though it is believed to contribute to the preservation of the outer membrane's permeability barrier [5].

Members of Enterobacteriaceae family have been discovered in soil, fresh and sea water, and have formed associations with various living organisms, like plants, insects, and vertebrates, including humans [6]. Members of this family can act as commensals, intestinal pathogens, or opportunistic extraintestinal pathogens in humans. In clinical research, Enterobacteriaceae have been known as pathogens including *Salmonella*, *Escherichia coli* (O157, non-O157), *Shigella*, and *Yersinia enterocolitica* [7], [8]. Certain disease patterns within this family are quite distinctive in the context of human infections, including conditions like diarrhea, sepsis, respiratory and urinary tract infections as well as soft tissue infections, osteomyelitis, endocarditis and many other.

1.1.1 *Escherichia coli*

Escherichia coli is one of the best studied members of the Enterobacteriaceae family. *E. coli* is a Gram-negative rod-shaped facultative anaerobe, able to inhabit the lower intestine (colon) of warm-blooded animals as their primary habitat and water, sediment, plants and soil as their secondary one [9, 10].

E. coli is a commensal of a healthy human gut microbiome and colonises the mucous

layer of the colon. However, some other strains of *E. coli* are pathogens of intestinal and urinary tract diseases [11]. These strains of *E. coli* have acquired virulence characteristics (such as enterotoxins, invasion proteins, adhesion factors, etc.), enabling them to adapt to new environments, including human hosts, and induce a wide range of diseases in healthy individuals. Traits, associated with virulence, are often encoded on genetic elements that can be transferred to other strains, forming new combinations of virulence factors. Such genes may be transferred as genetic elements that were previously mobile but successfully integrated into the genome. The most successful combinations of these virulence factors persists, giving rise to specific pathotypes of *E. coli* with the capacity to cause such diseases as diarrhea, urinary tract infections (UTIs), sepsis, bloody diarrhea (hemorrhagic colitis), meningitis and hemolytic uremic syndrome (HUS), which is linked to acute kidney failure, and are implicated in severe clinical outcomes. There are six well-described categories among those pathogenistic bacteria: enteropathogenic *E. coli* (EPEC), enterohaemorrhagic *E. coli* (EHEC) (with a Shiga toxin-producing *E. coli* (STEC) strains), enterotoxigenic *E. coli* (ETEC), enteroaggregative *E. coli* (EAEC), enteroinvasive *E. coli* (EIEC) and diffusely adherent *E. coli* (DAEC) [12, 13, 14, 15].

Even though some *E. coli* strains cause serious illnesses, most of what is known about life at the molecular level has been gained through the study of this bacterium. Strain *E. coli* K-12 has been known as a model organism in biotechnology and molecular biology for many years due to its rapid cell division, easy maintenance, and well-characterised genome [16, 17, 18, 19]. This organism has recently become a platform for genome design [20, 21, 22]. Strain K-12, isolated in 1922 from the stool of a convalescent diphtheria patient, has been studied around the globe ever since [23]. Currently, we have many databases that represent knowledge of the *E. coli* K-12 MG1655 genome. These include EcoCyc [19] and EcoGene [24], structure of its proteins on UniProt [25] and PDB [26], and its specific classes of gene products on RegulonDB [27]. Despite being the most studied bacterial strain, in *E. coli* K-12

genome almost 35% of all genes lack experimental evidence of function [28]. It is important to note that the *E. coli* K-12 laboratory strains commonly used as model organisms today are non-pathogenic. They lack the O antigen on their lipopolysaccharide layer in the outer membrane and are incapable of infecting humans [29]. While *E. coli* K-12 serves as an excellent model organism, its use is limited for studying pathogenicity. In such cases, other strains may be more appropriate.

1.1.2 Stress response in bacteria

The environments inhabited by *E. coli* and other members of Enterobacteriaceae are varied and often challenging, particularly when those bacteria are associated with hosts in the gastrointestinal systems. In this setting, *E. coli* must continually monitor external signals and react to any changes in temperature, pH, osmolarity, starvation, presence of toxic or signalling chemicals and other factors to redirect their behaviour and metabolism accordingly [30, 31]. The broad spectrum of sensing and response systems provides a significant evolutionary advantage, allowing bacteria to tune into severe conditions.

The first defense system against molecules causing stress or damage in Gram-negative bacteria is the outer membrane (OM). OM locates outside of the peptidoglycan (PG) cell wall and inner membrane (IM), all together comprising a bacterial cell envelope. Bacteria maintain its integrity in many ways, for example, employing two-component systems (TCSs), which regulate gene expression via various sensor kinases and response regulators [32]. Sensing the stress factors allows bacteria to synthesise second messengers such as cyclic AMP (cAMP), cyclic GMP (cGMP), cyclic di-GMP (c-di-GMP), cyclic di-AMP (c-di-AMP), guanosine tetra- and penta- phosphates (p)ppGpp, inorganic polyphosphate (polyP) etc. [33]. Those effector molecules, that are produced under many different stressful circumstances for bacteria, can affect transcription of genes important in a the stringent response

[34, 35]. The latter is a stress response that occurs in bacteria due to amino-acid starvation, fatty acid limitation, heat shock, and other stress conditions. Other genes that are under control of effector molecules, also involved in quorum sensing, biofilm formation, virulence, starvation, sporulation and persisters cells formation [36, 37, 38, 39]. Stress response system to toxic chemicals in *E. coli* is a *mar* regulon, which is regulated by MarA transcriptional regulator, which once triggered by toxins, activates many genes responsible for an innate resistance [40]. In addition, all of those processes are co-regulated by alternative σ -factors, that redirect transcription to specific sets of genes required during stress [41, 42, 43].

1.2 Gene expression in bacteria

Bacteria possess an incredible capability to adjust their biology in response to changes in their surroundings. This adaptive response involves the precise control of gene expression. Hence, bacteria up-regulate the production of proteins and enzymes, required at certain time points, and shut them down, to conserve metabolic and energetic resources. Bacterial gene expression is tightly regulated at steps including transcription initiation, transcript elongation, translation, messenger RNA stability, and protein turnover [44].

1.2.1 The central dogma of molecular biology

According to the central dogma of molecular biology, genetic information in all living cells flows from deoxyribonucleic acid (DNA) to messenger RNA (mRNA) and further to protein [45]. Transcription is catalysed by RNAP, and the ribosome catalyses translation [46, 47]. In addition, both DNA and RNA can be replicated by DNA-dependent RNAP and RNA-dependent DNA polymerase, respectively (DNA and RNA replication). Information coded in mRNA can also be transferred to DNA by reverse transcriptase (reverse transcription), (Figure 1.1).

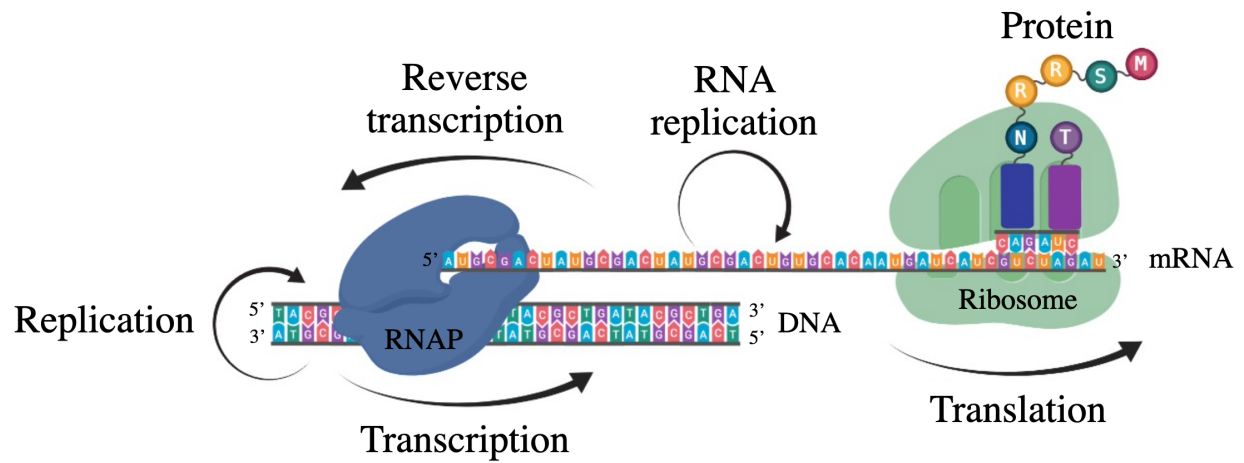


Figure 1.1: The central dogma of molecular biology.

The figure depicts the flow of genetic information in cells from DNA to messenger RNA (mRNA) (transcription) to protein (translation). RNAP is in blue, ribosome is in green, amino acids are depicted as colored circles, with gray peptide bonds separating them. Created in BioRender.

1.2.2 Transcription cycle

The bacterial transcription cycle is catalysed by RNAP core enzyme that consists of five subunits $\alpha_2\beta\beta'$ and ω . The transcription cycle has four stages. At the first stage, a promoter is recognised by RNAP holoenzyme that includes an additional σ subunit, which is crucial for promoter recognition [48, 49]. There are seven σ factors that recognise different promoters in *E. coli* [50]. At the second stage, the promoter open complex is formed, and abortive RNA synthesis initiates [51]. After RNAP leaves the promoter, the σ factor is typically released, allowing the formation of a transcription elongation complex, and processive RNA synthesis begins. The fourth stage of the transcription cycle is termination (Figure 1.2).

Under stress conditions, bacteria rapidly adjust their gene expression to survive. This adaptation involves the activation of specific stress response genes, which can be regulated by different σ -factors or the regulatory proteins. Regulation modifies the transcription cycle to prioritise transcription of specific genes, ensuring that the proteins necessary for stress resistance are produced quickly.

1.2.3 Structure of bacterial RNA polymerase

Bacterial RNAP is a multi-subunit enzyme with a total molecular mass of around 400 kDa, that catalyses all transcription in bacteria. There are 2,000 molecules of RNAP core enzyme per *E. coli* cell [52, 53]. Transcription can only be initiated specifically by a holoenzyme, which is comprised of the core subunits and a σ factor, that recognises and melts promoter DNA [54] (Figure 1.3).

Overall, two biggest subunits, β and β' , shape RNAP holoenzyme in crab claw. Within the crab claw locate an RNA exit channel, that leads nascent RNA out of the polymerase, an internal channel, with an enzyme active site, that accommodates the dsDNA during transcription and a secondary channel that allows incoming NTPs (nucleoside triphosphates)

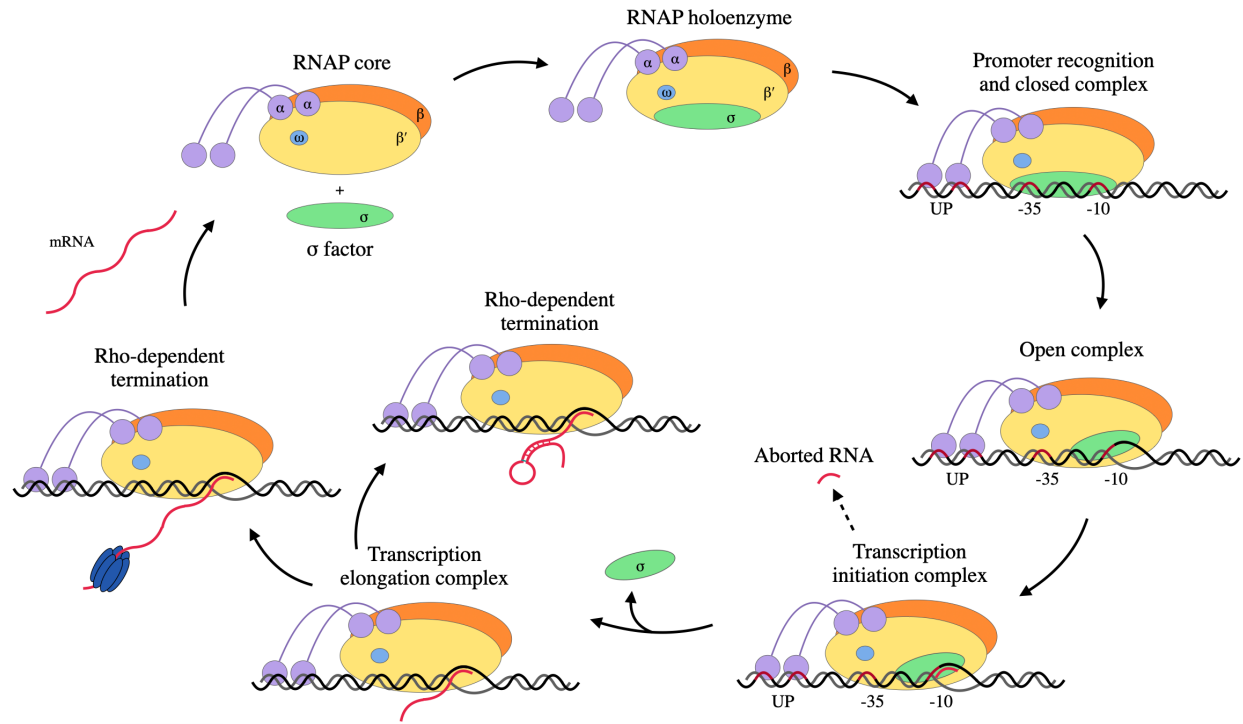


Figure 1.2: The bacterial transcription cycle.

Schematic showing transcription cycle in bacteria including initiation, elongation and termination. RNAP holoenzyme recognises the promoter, forming a closed complex. The DNA unwinds to form the open complex, and the addition of initiating NTP leads to formation of the initiating complex. RNA synthesis starts with the formation of phosphodiester bonds between nucleotides. The RNA can either be released during abortive transcription or continue to the elongation phase. Transcription can be terminated in a rho-dependent or rho-independent manner (adapted from [55]).

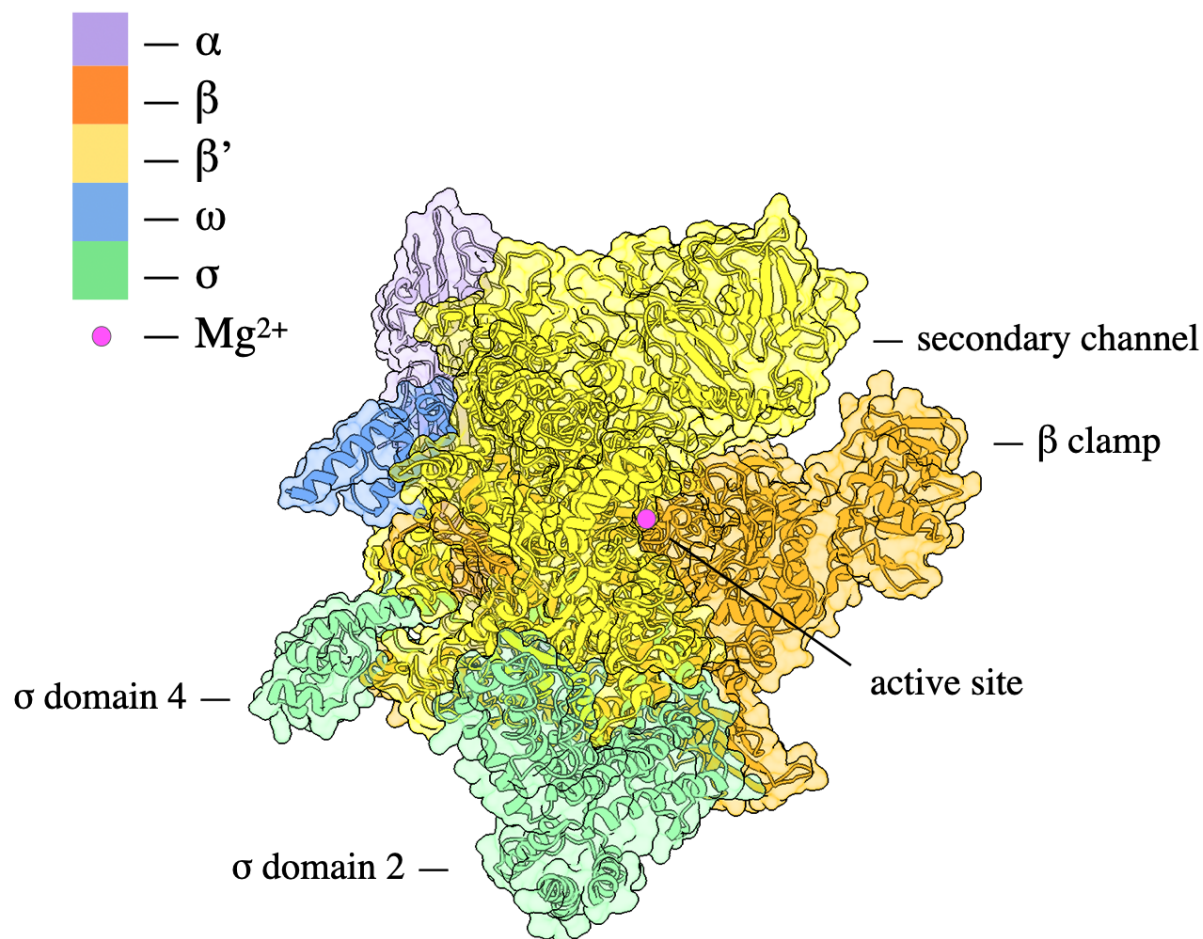


Figure 1.3: Crystal structure of RNAP σ^{70} .

Crystal structure of RNAP σ^{70} with the main features highlighted [56]. Subunits are colored according to the key. Image was generated using ChimeraX.-1.7.

to enter the active site [57]. The cryo-EM structures have shown that the active site cleft is approximately 70 Å deep and 100 Å long, but only 15 Å wide [58], however, the cleft is dynamic, and can extend up to 20–25 Å [59]. The active site possess three invariant aspartate residues on the β' subunit (Asp 739, Asp 741 and Asp 743) [60]. Those aspartates bind two catalytic magnesium ions. First Mg^{2+} is weakly coordinating the 3'OH of RNA and lowering the pKa around the attacking hydroxyl, while second Mg^{2+} stabilises the negative charges [57]. The active site also contains a β' F-bridge helix and a β' G-trigger loop [48]. This G-trigger loop stabilises the correct orientation of incoming NTP in i+1 site. The G-trigger loop also accommodates a surface-exposed domain, called SI3, that modulates pausing and is essential for growth [61]. F-bridge helix acts as the pawl, in a ratchet-like translocation mechanism during transcription elongation, and also plays a role in backtracking [62]. RNAP moves forward only by energy released from binding the correct NTP, along with the reciprocating movement of the F-bridge helix and G-trigger loop, drives the step-wise forward motion of RNAP [57]. Thus, the G(trigger)-loop-F(bridge)-helix unit coupling RNA synthesis and translocation [57]. The β' subunit also binds one Zn^{2+} via non-canonical zinc-binding motif in the residues 814–898 and assists in folding of the β' subunit itself as well as in RNAP assembly [63]. The second zinc ion is bound by a canonical zinc-binding domain in the β' NTD (N-terminal domain) (residues 65–94) and has been shown to function in various transcription processes [64]. For example, when RNAP is poorly positioned on the promoter due to weak -35 element, β' zinc binding domain stretches out from its canonical position and interacts with the upstream non-template strand to facilitate RNAP–promoter closed-to-open complex isomerisation [65].

Two α subunits of the RNAP are identical and comprised of two independently folded α NTD and α CTD (α - carboxy terminal domain) domains that are joined via 13-15 AA long flexible linker [66]. The α subunit dimerises via α NTD, and 'fastens' large subunits β and β' together [49, 57, 67]. The α CTD plays an important role in promoter recognition,

by binding to the upstream promoter DNA, as well as interacting with transcription factors. The α CTD residues 249-329 recognise promoter UP elements, and specific R265 determinant recognises the minor groove of DNA [68]. It is important to note that while the α subunits contribute to promoter recognition, the presence of the σ factor is essential for sequence-specific transcription initiation.

The ω subunit, although the smallest in RNAP, with just 91 residues, is conserved but non-essential across bacteria. This subunit functions as a chaperone, facilitating the proper folding of the β' subunit [69, 70, 71]. *In vitro* studies on RNAP from *E. coli* shows that the stringent response signaling molecule (p)ppGpp binds between β' and ω subunit, and that the latter is essential for (p)ppGpp-induced promoter selection [72, 73]. Moreover, transcriptome studies showed that ω subunit influences the selection of σ factors by the RNAP core and impacts promoter selection by the RNAP holoenzyme associated with the primary σ factor [74, 75, 76].

1.2.4 Architecture of promoter sequence

The architecture of bacterial promoters plays a crucial role in regulating gene expression during the transcription cycle. Bacterial promoters are DNA sequences located upstream of the transcription start site (TSS), recognised by the RNAP holoenzyme, which includes the core enzyme and a σ -factor. The σ -factor confers promoter specificity to the RNAP, allowing it to initiate transcription accurately at the start site of genes. RNAP core enzyme is able to non-specifically bind DNA, and initiate RNA synthesis, but cannot initiate transcription at specific promoters [77, 78]. Thus, promoter sequences are essential for initiating transcription and maintaining cellular homeostasis.

Promoters in *E. coli* typically have two highly conserved hexamer sequences known as the -10 and -35 elements [79, 68]. These recognition regions located approximately 10 and 35 base

pairs upstream of the TSS, respectively [80]. The -10 and -35 elements are recognised by a housekeeping σ -factor called σ^{70} and have a consensus sequence of TATAAT and TTGACA, respectively. The spacer region between the -10 and -35 elements is typically around 17 ± 1 base pairs. Both the length and sequence of the spacer significantly influences promoter strength and efficiency [79].

The UP-element is an additional promoter sequence recognised by α CTD of RNAP. The element locates upstream of the -35 region, between -59 to -38 base pairs upstream of the TSS, and have a consensus sequence nnAAA(A/T)(A/T)T(A/T)TTTTnnAAAAnnn, where 'n' is any base [68, 81]. Promoters might also have additional extended -10 element with a consensus sequence TT/C, located just upstream of the -10 element [82, 83]. There also might be a $6 \text{ bp} \pm 1 \text{ bp}$ long GC rich discriminator sequence, which is located between the TSS and the -10 element, usually in stable RNA (tRNA, rRNA) promoter regions [82, 84, 85]. The discriminator also influences the stability of the RNAP-DNA complex by regulating the open complex lifespan [86]. It is also involved in recognition by regulatory signals as a response to environmental conditions. For example, promoters with AT-rich discriminator sequences are more sensitive to regulatory proteins like DksA and ppGpp, which are involved in the stringent response [87, 88]. A promoter schematic is shown on Figure 1.4.

Overall, the main function of promoter sequences is to determine the direction of transcription and specify which DNA strand will serve as the template for mRNA synthesis. Promoter elements define the level of gene expression: strong -10 or/and -35 elements can have a high level of basal activity. Respectively, weak -10 or/and -35 elements can have a low level of basal activity, or prevent gene from being transcribed at all [83]. Additional promoter elements, such as the extended -10 or UP-element, with strong consensus sequence, can increase promoter activity [89, 90, 91]. The strength of promoters influences the allocation of RNAP across the genome, thereby affecting the level of gene expression. This regulatory mechanism is static, ensuring proper gene expression under ordinary conditions. However,

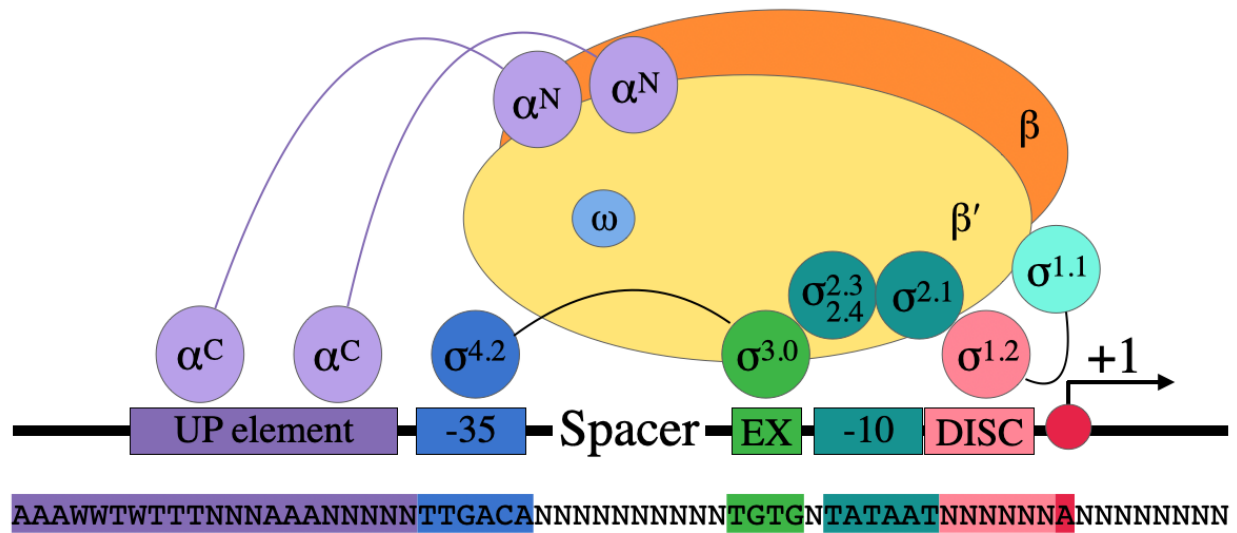


Figure 1.4: Schematic of RNAP and promoter organisation. Structure of a promoter DNA and sequence-specific interactions between RNAP and regions of the promoter. The conserved consensus sequence of each promoter element is shown under the promoter schematic, as well as their relative position compared to the TSS.

it does not facilitate adjustments in gene expression in response to environmental changes.

1.2.4.1 Transcription initiation

In the first step of transcription initiation, RNAP holoenzyme recognises and binds a promoter sequence with its σ subunit, and an initial closed complex is formed [86]. Domains σ_2^{70} and σ_4^{70} recognise the -10 and -35 elements of the promoter, respectively. In the real-time DNA footprinting in the closed complexes DNA is protected from OH attack from base -80 to base +20, and DNA upstream -35 element is bent [92]. In this model, promoter DNA have the UP element region, and when α CTDs interacts with DNA between -35 and -60, DNA is bending almost 100° , which facilitating nonspecific interactions between RNAP and DNA upstream of base -60 [92, 86]. This observations suggest that the upstream promoter duplex in closed complex is positioned along the “back” surface of RNAP. The σ -factor interacts with the region downstream of the -10 element at positions -2 to +2, and bends promoter DNA by 17° , positioning the downstream promoter DNA above the DNA-binding cleft of RNAP [51].

The transition from the closed to the open complex begins when σ_2^{70} specifically untwists and capture the conserved adenine at position -11 (nt strand, non template), thereby initiating nucleation of open complex formation [93, 94, 51]. Then, σ subunit of RNAP melts approximately 13 bp of DNA, from position -11 to +4 to form the open complex [95]. At the same time, the template-strand is loaded into the RNAP active site located between the β and β' subunits of RNAP. The non-template strand is held by domain 2 of the σ -factor [96]. The transition from the closed complex to open complex can occur either in a single step or through a short-lived intermediate state. The intermediate state arises due to a temporary incompatible conformation that prevents the template strand from properly loading into the active site cleft [94, 97].

Once the open complex is formed, synthesis of the mRNA from the template strand

begins. The initiating nucleoside triphosphate (NTP) positions within the active site to establish the 'initiating complex' [98, 99]. The most common transcription start site base is A or G, less common is T and the most unfavorable is C [100]. RNA synthesis starts with the formation of phosphodiester bonds between adjacent NTPs [101]. As these bonds continue to form, the DNA template strand 'scrunches' into the active site, one base pair at a time [99], and a short RNA transcript is produced. At this point, RNAP may return to the promoter open complex state (abortive initiation) or escape the promoter and continue making a longer transcript (elongation) [102, 103]. During abortive initiation, RNAP synthesises and releases transcripts 2–15 nt in length [104]. This process can repeat several times before the elongation phase is achieved. Once the first 12 nt of RNA have been synthesised, it fills the upstream RNA exit channel that disrupts the binding of σ region 4 to the β flap and release σ subunit, which will be then recycled for another round of transcription [49, 105].

1.2.4.2 Transcription elongation

In the elongation process, RNAP adds new nucleotides to the growing mRNA complementary to the DNA chain. In elongation, RNAP maintains an 8-9 bp RNA-DNA hybrid within the 11-12 bp long transcription bubble [48, 106]. The upstream DNA duplex is weakly associated with RNAP [107].

The transcription elongation complex is highly stable and efficient, allowing the new RNA molecule to be synthesised at a rate of 30 to 100 nucleotides per second [108]. This stability is crucial to ensure that transcription can continue uninterrupted over long stretches of DNA. The elongation rate is regulated by elongation factors including NusA and NusG [109]. NusA is also involved in both termination and anti-termination of transcription [110, 111]. It has been shown that NusA directly interacts with RNAP and the nascent RNA, modulating the transcription rate and stabilising paused elongation complexes [112]. Similar to NusA, NusG has a dual regulatory effect on elongation, and physically interacts with RNAP

[113, 114]. During elongation, NusG increases the rate of RNA synthesis by facilitating the processivity of the RNAP. NusG also participate in anti-termination complex formation by preventing premature termination of RNA synthesis. Moreover, NusG is involved in coupling of translation and transcription [115].

1.2.4.3 Transcription termination

RNAP continues elongation until it encounters a termination signal. That can either be an intrinsic termination signal or a protein terminator dependant on the protein factor Rho [116, 117].

Intrinsic termination relies on the interaction between nucleic acid elements and RNAP (however, such factors as NusA can modulate termination efficiency) [118]. Intrinsic terminators are encoded at the end of a gene and include two sequence motifs: an RNA GC-rich stem-loop (hairpin) and an A-rich tract of 8-10 nucleotides immediately downstream [119, 116]. G and C residues in an mRNA form strong hydrogen bonds and a stable hairpin structure. This causes elongating RNAP complex to temporary pause and removes mRNA from the active site. The following U-rich segment, which always locates 8–9 nucleotides from the end of the RNA, makes a weak RNA:DNA hybrid, so that the transcript is easily dissociated.

Currently, there are three models of intrinsic termination. These include: forward translocation, slippage and allostery [120]. The forward translocation model suggests that the RNAP continues to move forward along the DNA without extension of the RNA [121]. This leads to the separation of the RNA:DNA hybrid and the release of the transcript. Here, the RNA hairpin compensates for binding energy lost as the hybrid shortens. In the slippage model of transcription termination, the RNAP encounters an RNA hybrid region that is prone to sequence misalignment [122]. As the RNAP transcribes through this region, it can slip backward on the DNA template, causing the RNA to misalign, which leads to a locked

complex that blocks further transcription, facilitating the termination. The allosteric model suggests the RNAP is not involved in the nucleic acid movements required in the other models [123]. In this model, transcriptional bubble collapses due to conformational change in the RNAP itself caused by hairpin formation.

Rho-dependent termination requires ATPase-dependent helicase RHO [120]. Rho central channel accommodates mRNA. The helicase binds to the rho utilisation site (rut) in the mRNA, that is rich in cytosine and lacking secondary structures [124, 125, 126]. Rho enzyme moves along the RNA, until it reaches the RNAP, where it disrupts the RNA:DNA interaction, causing transcription to terminate [127]. Termination is enhanced by factors such as NusG, which binds to a specific site of Rho and helps to increase effective termination by altering Rho conformation and RNA binding affinity [115].

Rho can interact with both NusA and NusG during transcription elongation. NusG assists in ignoring intrinsic termination signals in favour of Rho-dependent termination sites [128]. During elongation, the ribosomal protein S10 binds NusG on RNAP, and prevents NusG interaction with Rho termination enzyme. However, in the case of translation failure, NusG binds Rho and stimulate termination. Both NusA and NusG interact with different sites of RNAP and Rho, so they do not compete, but assist elongation or termination cooperatively [113].

There is also an Mfd-dependent termination. Mfd stands for Mutation Frequency Decline protein, which is a highly conserved ATP-dependent DNA translocase [129]. Mfd is mostly known for mediating the process of transcription-coupled repair by inducing forward translocation of backtracked, arrested complexes [130, 131, 132]. Mfd binds both DNA and RNAP, undergoes conformational changes that invoke its translocase activity to assist stalled RNAP in dissociation from DNA [133, 134]. However, Mfd can also bind and move along DNA without interacting with RNAP first. It moves slower than RNAP, and can overtake it only if RNAP is stalled. This triggers the release of the RNAP and recruits proteins

essential for DNA repair [135]. Mfd can induce forward translocation of backtracked RNAP complexes, moving them forward to reposition the RNA 3' end in the active site for resumed transcription [136, 120]. If elongation cannot proceed, perhaps due to a lack of nucleotide substrates, Mfd translocase activity can also dismantle the complex, thereby releasing the transcript.

1.3 Regulation of transcription

Transcription is a highly regulated process that ensures genes are expressed only when they are needed. Transcription can be regulated by mechanisms involve: alternative sigma factors, chromosome structure, small molecules and transcriptional factors.

1.3.1 Sigma factors in *E. coli*

Under optimal *E. coli* growth conditions the primary sigma factor σ^{70} , is responsible for initiating transcription of essential genes [137]. The specificity of RNAP holoenzyme is mainly determined by its σ^{70} subunit that can be replaced with any other six alternative σ -factor [138]. Alternative sigma factors are expressed as a response to stress or environmental stimuli and can redirect transcription to the sets of genes requires under those circumstances. Additionally, at some promoters regulatory regions, sites for different σ -factors can overlap [139, 140, 141]. Thus, the same genes can be regulated by different σ -factors under different conditions.

The alternative σ -factors in *E. coli* are:

σ^{54} (RpoN) activates genes involved in nitrogen metabolism and flagellar synthesis [142].

σ^{38} (RpoS) is a stationary phase σ -factor, operates in starvation periods of a cell [143].

σ^{32} (RpoH) the heat shock σ -factor [144].

σ^{28} (FliA) regulates genes involved in flagellar synthesis and chemotaxis [145].

σ^{24} (RpoE) responses to the effects of heat shock and other stresses on membrane and periplasmic proteins [146].

σ^{19} (FecI) regulate gens for iron transport and metabolism [147].

All of the sigma factors described above fall into one of two families. The σ^{38} , σ^{32} and σ^{28} belong to the σ^{70} family, and share similar structural features in up to four core domains (σ_{1-4}) [137]. High resolution structural analysis showed that σ -factors of the σ^{70} -family initiate transcription in a similar way [148, 149]. Members of this family also recognise similar promoter elements: -10 and -35 regions [150]. Some members of σ^{70} family are called ECF (Extra Cytoplasmic Function) σ -factors [149]. These respond to extracytoplasmic stresses such as envelope stress, oxidative stress, and nutrient limitation. ECFs σ -factors posses only σ_2 and σ_4 domains, and include σ^{19} and σ^{24} factors [151, 147, 152]. All but one of the σ -factors, σ^{54} , is in the σ^{54} family. The σ^{54} -factors family, of which *E. coli* has only one, recognises different promoter elements and have a different mechanism of transcription initiation [153].

Overall, all sigma factors have similar functions [154]:

1. Recognition of promoter sequence on the DNA template.
2. Interaction with transcription activators.
3. Promoter melting and formation of the open complex.

Sigma factors can be regulated by their cognate anti-sigma factors, as well as non-coding RNA molecules, which can block σ from making a complex with RNAP. This allows others σ -factors to bind RNAP [155, 156, 157]. In addition, some σ -factors, such as EcfP in *Vibrio parahaemolyticus*, are inactive until phosphorylated [158].

1.3.1.1 Housekeeping σ^{70} -factor

All bacteria have a housekeeping σ -factor that initiates transcription from most essential genes, in *E.coli* this role is played by σ^{70} . The σ^{70} factor has multiple domains: $\sigma_{1.1-1.2}^{70}$,

σ_{NCR}^{70} , $\sigma_{2.1-2.4}^{70}$, $\sigma_{3.0-3.2}^{70}$, and $\sigma_{4.1-4.2}^{70}$ (Figure 1.5A). Once σ^{70} forms a complex with the RNAP core, its domains— σ_2^{70} , σ_3^{70} , and σ_4^{70} —spread across one face of RNAP, enabling multiple contacts and orienting them with the correct spacing for promoter DNA binding (Figure 1.4), [83]. Among all σ -factors in the *E. coli* cell, σ^{70} has the highest affinity for RNAP and is also the most abundant [159].

Overall, σ^{70} is anchored to the RNAP core through contacts made by σ_2^{70} with the major docking site on the β' subunit and by σ_4^{70} with the docking site on the β subunit, positioning σ_4^{70} near the exterior opening of the RNA exit channel [57]. Domain $\sigma_{3.2}^{70}$ forms a flexible linker between regions 2 and 4, called σ finger. It lies within the active site cleft and interacts with the template DNA strand in the open complex. The σ finger plays a role in positioning the template strand in the active site, binding of initiating NTPs and priming of RNA synthesis [160, 161, 162]. Growing RNA strand clashes with σ finger, leading to abortive initiation, transcriptional pausing or dissociation of the σ factor and promoter escape.

Recognition of discriminator sequence as well as DNA unwinding downstream of the transcription start site in late stages of transcription bubble formation promotes by the upstream end of the $\sigma_{1.2}^{70}$ domain [163, 84, 164, 165]. Domain σ_2^{70} separated from other domains by a non-conserved region (NCR), which participates in DNA interaction around the transcription start site [56]. The σ_2^{70} has three α helices: the second one makes contacts with a coiled-coil domain in the β' subunit of the RNAP core, the third helix is involved in the recognition of the -10 promoter element and DNA melting [166]. Such amino acids in domain $\sigma_{2.4}^{70}$ as Q437, T440, R436, R441, R451, are involved in promoter recognition, whereas aromatic residues F427, Y430, W433, and W434 in domain $\sigma_{2.3}^{70}$ are involved in promoter melting [94, 88]. The last two amino acids W433 and W434 are referred to as the W-dyad in literature [94, 51]. At the upstream -12 position of the -10 element thymine is strongly favored, as it is specifically recognised by Q437 [94]. The most important residue for the promoter melting is the nearly invariant base -11A [167]. Mutations at this position often

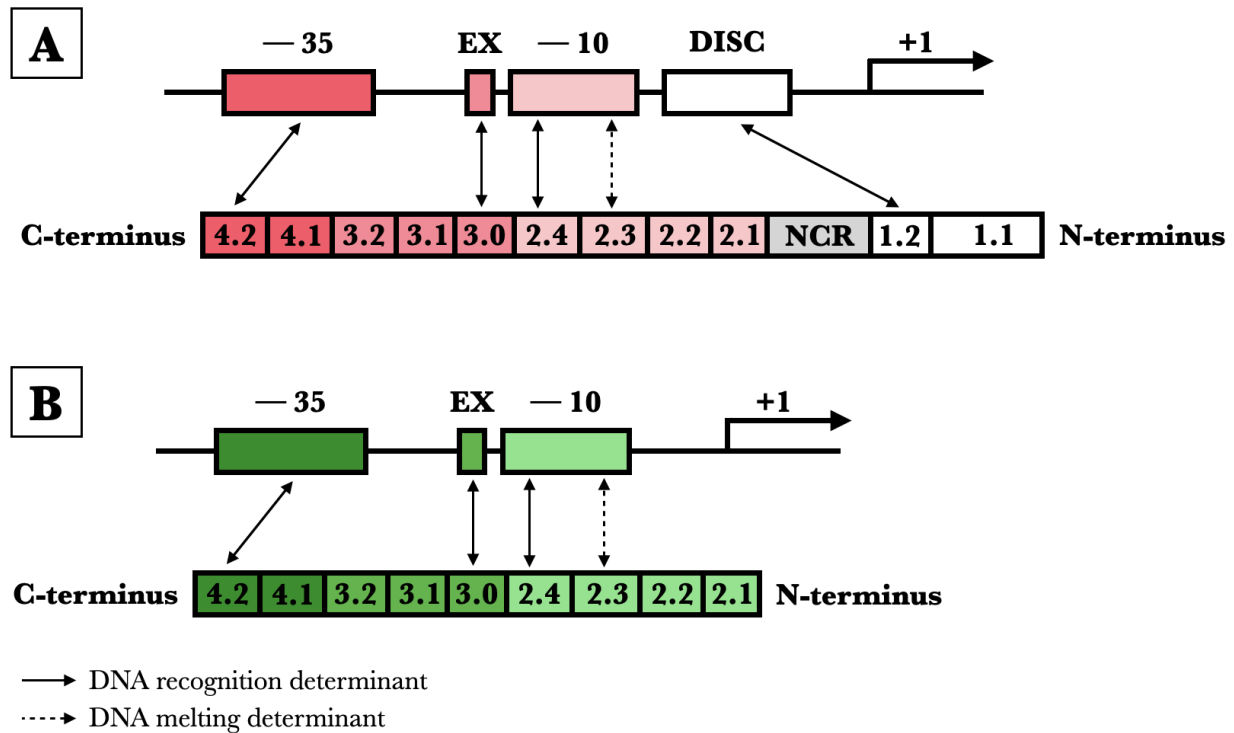


Figure 1.5: General domain structure of σ^{70} and σ^{28} factors.

A. Schematic of σ^{70} domains and their cognate promoter elements.

B. Schematic of σ^{28} domains and their cognate promoter elements.

Black solid arrows denote DNA- σ^{70} interactions, and dotted arrow denote regions involved in DNA melting. NCR is a non-conserved region.

lead to complete promoter inactivation and cause severe defects in stability of the open complex [168, 169, 94]. The first tryptophan of the W-dyad initiate the strand separation at the -11A by flipping the base out of the dsDNA into a hydrophobic pocket on the σ_2^{70} [170, 51]. This nucleates DNA melting, and with its progression, residues from both the non-template and template strands interact with the $\sigma_{2.4}^{70}$ domain, allowing the melting to extend from the -11A to +3 downstream of the transcription start site [171, 94].

Domain σ_3^{70} comprised of three α helices where the first one is included in domain $\sigma_{3.0}^{70}$, and its amino acid E458 interacts with the extended -10 promoter element on the DNA major groove, present in some promoters [82, 86]. Domain $\sigma_{4.2}^{70}$ has a helix-turn-helix motif that recognises the -35 element on a major groove with its E585, R588 and R584 amino acids, and due to flexibility of this region, bends the DNA approximately 36° [83, 49]. Both -35 and extended -10 elements are recognised as a double-stranded DNA, to position RNAP correctly for efficient promoter melting. Upon strand separation, the -10 and discriminator motifs are recognised as single-stranded DNA on the non-template strand and held by σ_2^{70} , thereby moving the template strand into the active site of RNAP [138]. When σ^{70} is not in complex with RNAP, $\sigma_{1.1}^{70}$ domain prevents DNA binding, and when in complex with holoenzyme, this domain occupies the active site cleft, until promoter DNA replaces it [86].

1.3.1.2 Flagellar σ^{28} -factor

The σ^{28} -factor is the most widely distributed alternative σ factor among all motile Gram-negative and Gram-positive bacteria, as it controls late stages of flagellum biosynthesis and some other non-flagellar genes [172, 90, 156]. Belonging to the σ^{70} family, σ^{28} shares many sequence and functional similarities. The main difference is that σ^{28} lacks NCR and domain 1, that prevents free σ -factors from binding DNA (Figure 1.5B). Hence, when σ^{28} is not in complex with RNAP, the DNA binding activity of its region 4 is repressed by the anti- σ^{28} FlgM protein [173].

The consensus sequences for the -35 and -10 elements at σ^{28} promoters are TAAAgttt and GCCGATAA, respectively (lowercase letters indicating less highly conserved bases), separated by an 11-bp spacer [174, 175]. The GC nucleotides at the start of the -10 element form an extended -10 motif, which is highly conserved and essential for σ^{28} promoter recognition [176]. This motif is recognised by the R91 residue in the $\sigma_{3,0}^{28}$ domain. The downstream CG at the upstream edge of the -10 element is recognised by residues D81 and R84 in the $\sigma_{2,4}^{28}$. A comparison between σ^{28} and σ^{70} shows that only two of the four aromatic residues in the 2.3 domain are conserved in both sigma factors [177]. Consequently, σ^{28} -dependent promoters have reduced promoter-melting capabilities and rely on strong extended -10 and -10 elements. These regions in σ^{28} -dependent promoters are highly sensitive to substitutions, whereas the -35 element is less sensitive, forming weaker contacts with σ_4^{28} compared to σ_4^{70} , and is often referred to as "weak" in the literature [177, 65]. Residue Q236 in σ_4^{70} does not interact with the -35 element as it does in σ_4^{28} . This observation may suggest that recognition of the -35 element happens differently in those two σ factors. Residues E206, Y214, and E222 in σ_4^{28} interact with the RNAP β -flap region, as do their counterparts in σ_4^{70} , indicating that σ^{28} and σ^{70} bind to the RNAP core in similar ways [178]. The σ finger of the σ^{28} occupies a similar position in the active site cleft to σ^{70} [148, 65].

To compensate weak interactions between -35 region and σ^{28} , the β' zinc-binding domain of RNAP stretches out from its canonical position and interact with non-template strand, which stabilises promoter binding [65]. Zinc-binding domain relocation facilitating the isomerization from closed to open complex.

1.3.2 Chromosomal structure

The bacterial chromosome is organised in the nucleoid in a way which allows every gene to be accessible on demand. In a relaxed form, DNA is in B-conformation, where one complete

turn of the DNA helix is 10.5 bp and negatively super-coiled [179]. Negative super-coiling facilitates DNA unwinding during replication and transcription, as well as compaction of the chromosome in bacteria [180, 181]. The organisation is mediated by DNA-binding nucleoid-associated proteins (NAPs) [182]. NAPs, such as Factor for Inversion Stimulation (Fis) and histone-like nucleoid-structuring (H-NS) protein, regulate chromosomal structure via folding, bridging, and twisting the chromosome both locally, at the level of regulatory elements or individual operons, and over longer distances. This leads to the formation of macro domains and interactions between different chromosomal parts [183]. More specifically, Fis also acts as a transcriptional regulator, binds to the AT-rich DNA, and either activates or represses expression of genes related to growth, metabolism, and stress responses, via interactions with RNAP [184]. H-NS also binds to AT-rich regions, creating loops, thus, causing genome condensation [185]. H-NS considered as a global repressor of virulence and horizontally-acquired genes in bacteria [186], however, in some cases H-NS also can act as an activator of certain genes [187, 188].

1.3.3 Small molecules

Transcription can be regulated by many different types of small molecules, including small ligands, non-coding RNAs, nucleotides and small peptides [87].

E. coli chromosome encodes almost 80 small RNAs (sRNAs), that can affect gene expression in several different ways. For example, sRNAs can influence processing of pre-tRNAs, sequester proteins away from other factors, or form base-paired interactions with their target mRNAs [189]. sRNA synthesis itself is mediated by transcriptional regulators, that respond to environmental stimuli. In turn, sRNAs can regulate expression of its own transcription factors, as well as any other genes required under given circumstances [190].

Small ligands, such as (p)ppGpp, contact RNAP directly and can destabilise the open

complex at some promoters [191]. The (p)ppGpp ligand is involved in the stringent response to nutrient deprivation, and targets promoters of genes whose products are required for ribosome function, thus down-regulating translation of all RNA in the cell [192]. Furthermore, the *E. coli* 6S RNA directly binds to σ^{70} , which prevents its binding to the promoter, increasing transcription requiring σ^{38} [193].

Nucleotide availability can also play a role in gene regulation. For example, the signalling molecule c-di-GMP, can affect expression of genes encoding curli fibres, important for biofilm formation [194, 195]. In addition, a high concentration of NTPs required for transcription of ribosomal RNA (rRNA), otherwise not only transcription of rRNA is inhibited, but also translation of other genes, due to lack of ribosome [196].

1.3.4 Transcription factors

There are at least 314 transcription factors in *E. coli* K-12, of which 35% are activators, 43% repressors and 22% are dual regulators [197]. Transcription factors bind operator sequences within a promoter, allowing targeted transcription regulation of the specific gene [198]. In some cases, the same transcription factor can act as an activator or a repressor of different promoters [197]. In other cases, one transcription factor can activate or repress transcription on the same promoter depending on environmental clues [199].

Seven *E. coli* transcription factors (CRP, FNR, IHF, Fis, ArcA, NarL and Lrp), are considered as the global regulators, as they control 50% of all genes in *E. coli* [200, 201]. The other genes are regulated by specific transcription factors that regulate only a small set of promoters, or single-target gene regulators, that target just one gene [197, 202].

Based on sequence, function and structure, transcription factors are grouped into 63 families [203]. Common families include the CRP family, LuxR family, LacI family, OmpR family, LysR family, AraC/XylS family, and MerR family [197, 199]. Members of CRP family

are the best-studied transcription factors [139, 204], that became a model of transcriptional regulators in general. In further chapters, we will work with some members of AraC/XylS family [205], and will need a basic understanding of transcriptional activation by MerR family transcriptional factors [199].

CRP family: The best studied member of this family is CRP protein, which stands for cAMP Repressor Protein and regulates around 200 genes in *E. coli* [206]. CRP forms an inactive dimer, that has a C-terminal HTH DNA-binding motif and an N-terminal domain to bind to its effector molecule 3'-5'-cyclic AMP (cAMP) [207]. In its active form, bound to cAMP, CRP interacts with its cognate CRP-binding site (CBS) on promoter sequence of genes involved in catabolism.

CRP can up- or down-regulate gene expression depending on the position of its CBS with respect to RNAP binding site [208]. If the CBS locates upstream of the promoter, it interacts with α CTD, facilitating RNAP binding to the promoter [204]. When the CBS overlaps the -35 element, CRP recruits the RNAP through interactions with both α CTD and σ [206]. On other promoters CRP interacts with promoter DNA at multiple sites in combination with other proteins [209].

AraC/XylS family: Members of this family regulate genes involved in stress response, pathogenesis, and carbon metabolism [210]. Some of the best characterised members of this family are MarA, SoxS and Rob, which regulate genes involved in the intrinsic response to antibiotics [211]. Overall, members of AraC/XylS family are characterised by a conserved C-terminal DNA-binding domain, which is a 100 amino acid long, containing two HTH motifs that bind asymmetrical DNA sites [212, 213]. The N- terminal domain binds to ligands and is usually around 200 amino acids long. Members of this family recognise the same DNA target site, however with different affinity and specificity [212, 214, 215].

MerR family: The best-characterised members of this family are functionally and structurally similar to the founder of the family, mercury sensor MerR, a transcriptional activator [216, 217]. They include various bacterial cytoplasmic proteins that respond to a versatile range of signals, including metal ions, toxins, and endogenous metabolites [218]. Members of this family have similarity in the first 100 amino acids, including a DNA binding HTH motif, followed by a coiled-coil region. Binding sites for MerR-like regulators usually locate within sub-optimal σ^{70} -dependent promoters with unusually long spacing between the -35 and -10 elements [217, 199]. When ligands of MerR-like regulators are present, these proteins attach to the long spacer, on the side of the DNA opposite to the RNAP. They do not interact with the holoenzyme but cause a 90° bend in the middle of the spacer. This bend broadens the minor groove and narrows the major groove, reducing the distance between the -10 and -35 elements and bringing the spacer closer to the standard 17-base pair configuration, which is optimal for RNAP recognition.

1.3.5 Transcriptional activators

Transcriptional activators are the regulatory proteins that help an RNAP holoenzyme in promoter recognition and stabilisation of an open complex. Those proteins assist on promoters with low or no basal activity. Activators can directly enhance promoter recognition by RNAP via making contacts with its α CTD subunit, or both α CTD and σ_{D4} . The other mechanisms are indirect, such as remodelling of promoter DNA, and anti-repression (Figure 1.6) [138, 219].

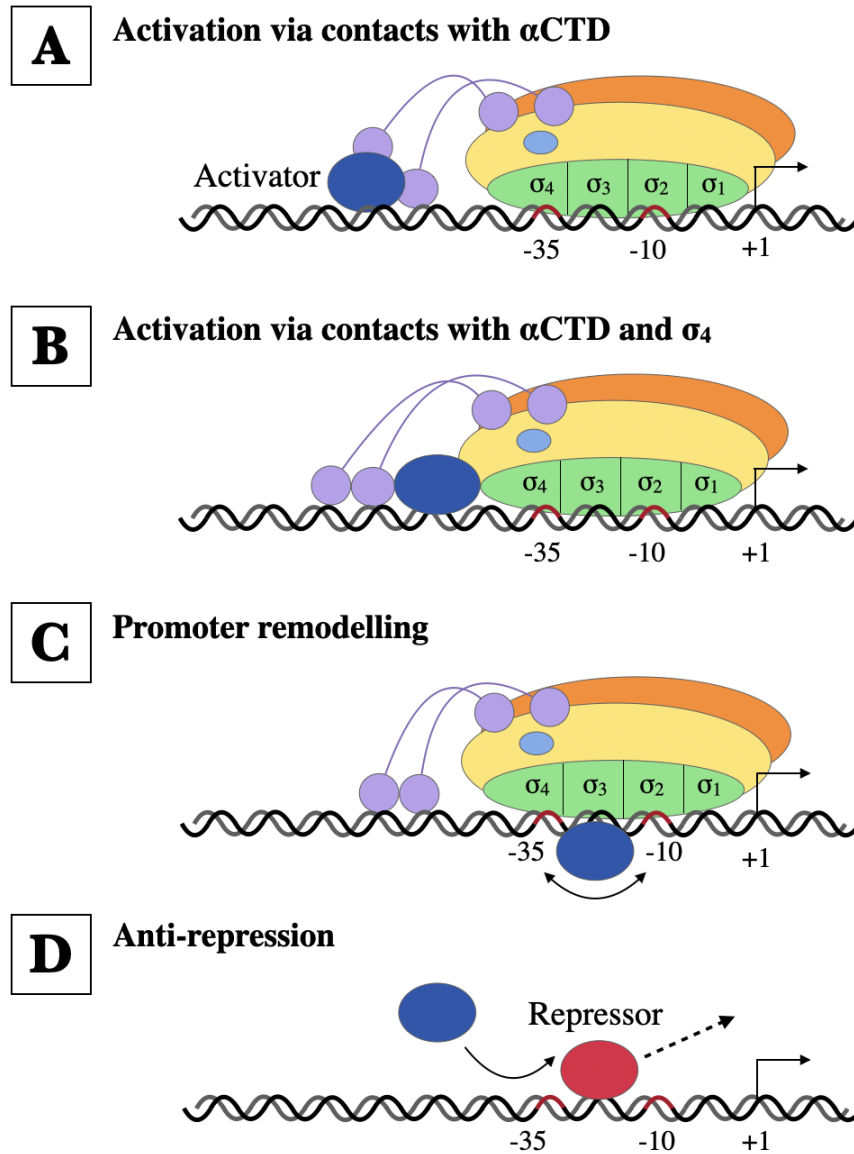


Figure 1.6: Schematic of different types of action of transcriptional activators.

A. Activation by enhancing the interaction of α CTD and promoter. The activator binds to a site upstream of the -35 element and contacts α CTD of RNAP.

B. Activation by enhancing the interaction of α CTD and σ_4 . The activator binds next to, or overlapping with, the -35 element and contacts σ_4 of RNAP.

C. Activation by DNA remodelling on sub-optimal promoter. The activator binds to the promoter and remodels the -10 and -35 elements for better positioning of those elements for RNAP binding.

D. Activation by removing a repressor. The activator binds to transcriptional repressors and inhibits their activity on the promoter.

Activator proteins are shown in blue, repressor is shown in red. Adapted from Busby and Browning [138].

1.3.5.1 Activation via contacts with α CTD

Activators that recruit RNAP via the α CTD domain, bind UP elements with 265-determinant upstream of the -35 region, and usually target promoters with one or two elements with sequences distinct to canonical binding motifs, and in literature referred as Class I activation (Figure 1.6A), [220, 221, 138]. Thus, activator- α CTD binding compensates for the interactions of RNAP with the missing element, positioning RNAP so that the domain 2 of the σ subunit can facilitate transcription bubble formation [222]. A classic example is CRP activation is the *lac* promoter [223, 224, 225]. Several AraC/XylS family activators, such as MarA, SoxS, and RamA, usually activate transcription through contacts with α CTD. Those activators contact an α CTD surface including 265-determinant before it is bound to DNA, 're-educating' α CTD to different DNA-binding specificity [138]. In this mechanism, called 'pre-recruitment' [226, 227], α CTD loses its ability to bind UP elements and instead targets specific activator binding sites.

1.3.5.2 Activation via contacts with σ_4

Binding sites for activators that target σ_4 of RNAP, usually are adjacent to, or overlap, the -35 promoter element. In literature, this activation is categorised as Class II [228, 229, 230]. Here, the activator assists σ_4 in binding to its cognate -35 element, or it can aid in repositioning and remodelling the σ_4 domain, targeting its 593-604 determinants through changes in its binding specificity (Figure 1.6B), [231, 138]. In some cases, the activator also additionally contacts α CTD, and in this case is referred as 'ambidextrous' [232, 233, 138]. SoxS activation of *micF* promoter can be an example [234]. Here, SoxS binds site overlaps -35 element and simultaneously interacts with one of the α CTD DNA-binding 265-determinant and conserved 596-determinant of σ_4^{70} .

1.3.5.3 Activation via promoter remodelling

To this group of activators can change promoter DNA confirmation (Figure 1.6C). As discussed above, the MerR-family transcriptional activators, such as MerR, SoxR and CueR, target sub-optimal promoters, which promoter elements locates 19-20 bp apart [217, 199]. Operator for those activators locates between the -10 and -35 regions, create kinks on promoter DNA upon binding, thus, shortening the distance between key elements into an optimal positioning [218]. In other cases, activators bind upstream of a -35 element, creating negative supercoiling in its promoter elements [235]. Activation of this type includes IHF at *ilvC* promoter.

1.3.5.4 Activation via anti-repression

Transcription initiation on some bacterial promoters is inhibited by repressors (Figure 1.6D). Such repression can be caused by NAPs, such as H-NS that forms filaments that block access of RNAP to promoter [236]. Repression by H-NS filaments can be relieved by insertion of H-NS paralogs, that form heterodimers with H-NS [237]. Interestingly, that repression caused by H-NS is relieved by PhoP transcriptional activator, that up-regulate promoters silenced by H-NS, thus, acting as a disruptive counter-silencer [238].

1.3.6 Transcriptional repressors

Transcriptional repressors can inhibit gene expression via one or a combination of the following mechanisms: steric hindrance, changing topology of promoter DNA, RNAP inhibition and anti-activation [239, 240], (Figure 1.7).

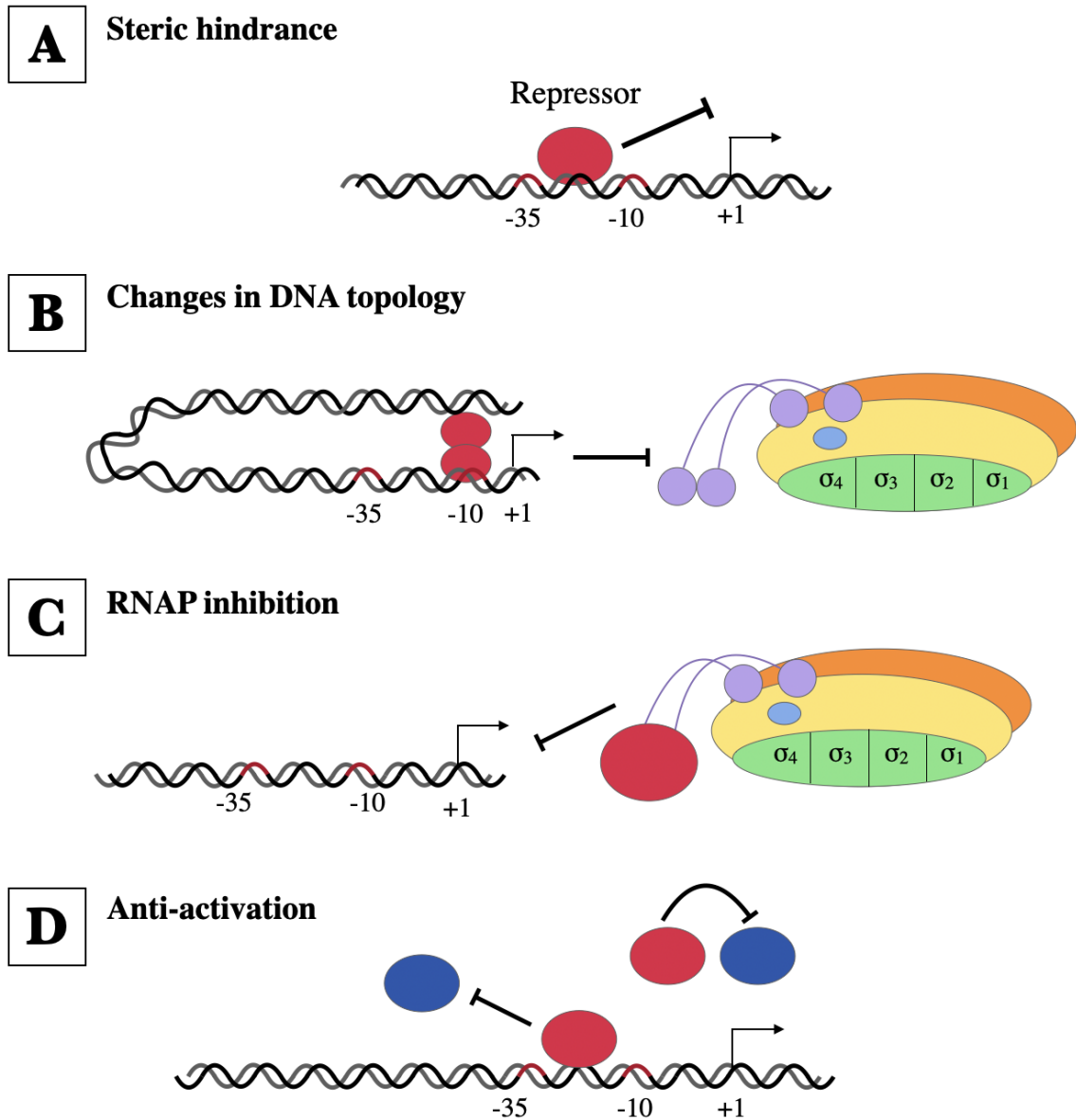


Figure 1.7: Schematic of different types of action of transcriptional repressors.

A. Repression by steric hindrance. Promoter key elements are blocked by the repressor.

B. Repression by changes in DNA conformation. DNA looping blocks the access of RNAP to the promoter.

C. Repression by a direct interaction with RNAP. Repressor directly binds RNAP to block its activity.

D. Repression by anti-activation. Repressor binds to promoter DNA, blocking the activator operator sequence, or binds to activators directly.

Activator proteins are shown in blue, repressor is shown in red. Adapted from Busby and Browning [138].

1.3.6.1 Steric hindrance

In steric hindrance, transcriptional repression occurs when a repressor binds over the RNAP -10 or -35 promoter elements [239], (Figure 1.7A). This binding physically blocks RNAP from accessing the promoter, preventing the initiation of transcription. In some cases, steric hindrance can modify a promoter geometry itself, which will prevent its recognition by RNAP [241]. On the *lac* promoter, LacI repressor arrests transcription initiation, via formation of a 'nonproductive complex' with RNAP, which allows the later to make abortive transcripts, but the promoter escape step is inhibited [242]. There also might be several adjacent binding sites for repressors on one specific promoter, such as *lac*, that can enhance its repression [243].

1.3.6.2 DNA micro-loops

In DNA looping, distal repressor binding sites interact with each other, generating a DNA loop that modifies promoter geometry, and blocks RNAP access (Figure 1.7B). For example, the *lac* promoter has three operators for LacI. Two of them locate upstream and downstream of the promoter sequence, and LacI can form a stable tetramer that binds both operators, to make a micro-loop. This prevents access of RNAP to the promoter, increasing repression by an extra 50-fold, compare to repression caused by only steric hindrance of LacI binding to a single site [244]. However, interplay of both DNA looping and steric hindrance together, can enhance the strength of transcription repression [245].

1.3.6.3 RNAP inhibition

Some repressors make direct contacts with RNAP to repress transcription (Figure 1.7C). In the case of the Gp2 repressor, encoded by phage T7, Gp2 binds its *E. coli* host's RNAP σ_{D1} and β' jaw subunits, blocking the active site channel, preventing formation of the open complex [246]. In addition, factors such as NusA and NusG also interact with RNAP,

inhibiting the later on the elongation step [113].

1.3.6.4 Anti-activation

Some repressors can block activity of transcriptional activators, that cause transcriptional repression (Figure 1.7D). For example, CytR repressor binds directly to CRP transcriptional activator, that blocks CRP from binding to α CTD subunit of RNAP on promoters dependent on it [247].

1.4 Resistance to toxic chemicals in Gram-negative bacteria

All Gram-negative bacteria respond to stress caused by exposure to toxic substances, which can cause cell damage or death in many different ways. Antibiotics are examples of such compounds. However, over the past century, extensive use of antimicrobial agents has led to bacteria becoming more tolerant of these treatments. For at least 440 million years, since the emergence of *Streptomyces* species, and other filamentous actinomycetes, bacteria have evolved in environments pressured by antibiotic-producing organisms [248]. This long exposure has driven bacteria to evolve and develop methods to avoid and resist the harmful effects of antibiotics through intrinsic mechanisms, spontaneous mutations, and the acquisition of new DNA.

Increased ability to resist antimicrobial substances allows bacteria to thrive despite antibiotics, and selection pressure promotes the proliferation of resistant strains, leading to widespread resistance. The overuse of antibiotics in healthcare and agriculture, coupled with a decline in new drug development by pharmaceutical companies, has accelerated the rise of multidrug resistance. It is predicted that, by 2050, antimicrobial resistance will cause approximately 10 million deaths annually [249]. Antibiotic resistance is a growing concern

in both community and hospital settings. Gram-negative bacteria represent the majority of multidrug-resistant organisms, that became resistant to all known antibiotics, which is a significant issue in healthcare worldwide [250, 251, 252, 253, 254].

1.4.1 Classes of antibiotics and their mechanism of action

Antimicrobial agents affect the bacterial cell by either stopping cell reproduction or by changing important processes inside the bacterial cell, leading to its destruction. Antibiotics are typically classified into two main groups based on their *in vitro* effects on bacteria: bactericidal, that reduces MBC to MIC ratio less than or equal to 4, and typically considered as 'killing bacteria' effect, and bacteriostatic, which reduces MBC to MIC ratio greater than 4, and typically considered as 'stop bacteria from growing' effect [255], Table 1.1. The effectiveness of antibiotics in a clinical setting depends on many factors, such as the particular bacteria, bacterial load, site of infection and other. In addition, some bacteriostatic antibiotics, such as linezolid can present bactericidal activity against particular bacteria, like *Streptococcus pneumoniae* [1].

Antimicrobial agents can also be categorised into groups based on the mechanism of antimicrobial activity, such as: drugs that block cell wall synthesis, disrupt the cell membrane, stop protein synthesis, inhibit nucleic acid synthesis, and interfere with bacterial metabolic pathways [256], Table 1.2.

Table 1.1: Types of antimicrobial agents, [1].

Antimicrobial agent	Drug Class and its <i>Specific Antibiotics</i>
Bacteriostatic	Glycylcyclines: <i>Tigecycline</i> Tetracyclines: <i>Doxycycline, Minocycline</i> Lincosamides: <i>Clindamycin</i> Macrolides: <i>Azithromycin, Clarithromycin, Erythromycin</i> Oxazolidinones: <i>Linezolid</i> Sulfonamides: <i>Sulfamethoxazole</i>
Bactericidal	Aminoglycosides: <i>Tobramycin, Gentamicin, Amikacin</i> Beta-lactams: <i>Amoxicillin, Cefazolin, Meropenem</i> Fluoroquinolones: <i>Ciprofloxacin, Levofloxacin, Moxifloxacin</i> Glycopeptides: <i>Vancomycin</i> Cyclic Lipopeptides: <i>Daptomycin</i> Nitroimidazoles: <i>Metronidazole</i>

Table 1.2: Antimicrobial agents grouped based on mechanism of action, [2].

Mechanism of action	Antimicrobial group
Inhibition of cell wall synthesis	β -Lactams, Carbapenems, Cephalosporins, Monobactams, Penicillins, Glycopeptides
Inhibition of protein synthesis	<i>Bind to 30S ribosomal subunit:</i> Aminoglycosides, Tetracyclines <i>Bind to 50S Ribosomal Subunit:</i> Chloramphenicol, Streptogramins, Macrolides, Lincosamides, Oxazolidinones
Inhibition of nucleic acid synthesis	Quinolones, Fluoroquinolones
Inhibition of metabolic pathways	Sulfonamides, Trimethoprim
Depolarisation of the cell membrane	Lipopeptides

1.4.2 The importance of antibiotic resistance in Gram-negative bacteria

Infections caused by multidrug-resistant Gram-negative bacteria are rapidly rising, which is a significant global healthcare and economic challenge for many decades and years to come [257]. Due to the shortage of new treatment options, the World Health Organization has designated the Enterobacteriaceae group of Gram-negative bacteria as one of the most critical groups of multidrug-resistant pathogens that pose a particular threat in hospitals and nursing homes [258, 259]. Colistin is one of the few antibiotics still effective against multidrug-resistant infections, but it already harbors a mobile resistance gene, *mcr-1*, and Enterobacteriaceae play a significant role in the dissemination of this gene [260]. Additionally, a growing concern is that these bacteria often co-locate this gene with other resistance genes (such as CTX-M, NDM, and IMP), resulting in resistance to cephalosporins and carbapenems, which exacerbates the harmful effects caused by these microorganisms.

1.4.3 Innate resistance

Natural resistance to toxic chemicals can be defined as a characteristic that is not associated with horizontal gene transfer and is universally present within a bacterial species, independently of antibiotic exposure. Natural resistance can be described as intrinsic, as resistance genes are naturally present in the bacteria [261, 262].

The primary bacterial mechanisms associated with intrinsic resistance include reduced permeability of the outer membrane, particularly via lipopolysaccharides (LPS) levels in Gram-negative bacteria, and activity of efflux pumps [261].

1.4.3.1 Reduced permeability

The outer membrane is the first defense system in Gram-negative bacteria, which helps to deal with antibiotics. It allows bacteria to slow down the intake of small molecules, including antimicrobial agents and is very important in intrinsic resistance to β -lactam antibiotics.

In *E. coli*, small porins OmpF and OmpC are the primary gates for antibiotic entry into the cell. Repression of their cognate genes reduces membrane permeability and antibiotic uptake [263, 264].

1.4.3.2 Active efflux

Active secretion of antibiotics out of the cell after entry, known as efflux, significantly contributes to antibiotic resistance in bacteria and is encoded within the chromosome [265]. For example, deletion of putative efflux system MexAB-OprM in multiple resistant *P. aeruginosa* transform bacteria into a strain hypersensitive to many antibiotics [266]. There are many types of efflux pumps, which might export different types of molecules or be specific to one. Typically, efflux pumps export multiple molecules and in normal conditions are needed to export toxic compounds out of the cell [267]. Another efflux system common in *P. aeruginosa*, *S. typhimurium* and *E. coli* is an AcrAB-TolC multidrug efflux pump, which is an RND-based tripartite efflux system, spanning all three bacterial membranes [268, 269].

1.4.4 Mechanisms of multidrug resistance

Several significant mechanisms exist through which bacteria are capable to resisting the effect of antibiotics. These mechanisms include drug inactivation, modification of the drugs target, reduced drug accumulation, protection of the drug target, and target bypass [270]. The combination of those mechanisms usually leads to development of multidrug resistance, resulting in high levels of resistance against various antibiotics.

1.4.4.1 Inactivation of antibiotic

Bacteria can produce enzymes that can inactivate an antibiotic by modification of its structure. Such drugs as ciprofloxacin, and other quinolone antibiotics, can be inactivated by acetylation by a plasmid-encoded acetyltransferase [271]. In addition to acetylation, the antibiotic chloramphenicol can be also inactivated by O-phosphorilation [272]. Aminoglycoside antibiotics including neomycin and streptomycin, can be inactivated by aminoglycoside-modifying enzymes, that are encoded in mobile genetic elements [273]. β -lactamase encoded in the chromosome and in the presence of inducer (for example, salicylic acid) helps bacteria to hydrolyse β -lactam ring if β -lactam antibiotics are passes into a periplasm [274].

1.4.4.2 Target protection

Some bacterial proteins physically bind with an antibiotic target to protect it from antibiotic-mediated inhibition [275]. Tetracyclines bind to the 30S ribosomal subunit, thus inhibiting bacterial translation [276]. Tetracycline ribosomal protection proteins, including plasmid and chromosomally coded Tet(O) and Tet(M), are closely related to GTPases, and protect the cell from the tetracycline mode of action. Tet(O) and Tet(M) bind to the ribosome, in a post-translocational state, and overlap the tetracycline-binding site on the ribosome, phisically displacing the drug [277]. However, they may come through the outer membrane and attack its targets in the cell.

Another group of target protectors, ABC-F proteins, mediate antibiotic resistance by physically displacing the antibiotic from the 50S subunit of the ribosome, to prevent translation inhibitors from inhibiting protein synthesis [275]. These protection proteins induce conformational changes within the ribosome that dissociate the drug from the target, thus restoring ribosome functionality [278].

1.4.4.3 Target modification

Modification of the target site is one of the most prevalent mechanisms of antibiotic resistance observed in bacterial pathogens, impacting a wide range of antimicrobial compound classes across various families [279].

Target changes may occur through point mutations in the genes encoding the target site. This is the common cause of rifampicin resistance [280]. Rifampicin binds the β subunit of RNAP and blocks the path of the nascent RNA to prevent transcription. Rifampicin resistance occurs by single-step point mutation, resulting in amino acid substitutions in *rpoB*, encoding the β subunit. These mutations spare the catalytic activity of the RNAP, although the affinity of the rifampicin for the β subunit is decreased, so the transcription is allowed.

Another type of target modification requires enzymatic alterations of the binding target. For example, addition of methyl groups to the ribosome, catalyzed by an erythromycin-resistance methyltransferases. Those enzymes mono- or dimethylating an adenine residue in position A2058 of the 23rRNA of the 50S ribosomal subunit, which prevent macrolides, lincosamides, and streptogramin B and other antibiotics with the same target from binding [281].

1.4.4.4 Target bypass

In the target bypass strategy, bacteria develop novel targets, that serve similar biochemical roles to the initial target, but remain unaffected by the antimicrobial agent. Methicillin resistance in *S. aureus* is an example. This bacterium acquires an exogenous penicillin-binding proteins (PBP), PBP2a, which leads to vancomycin resistance through modifications of the peptidoglycan structure mediated by *van* gene clusters [279]. PBP2a binding affinity towards β -lactam antibiotics is low, thus, PBP2a is not inhibited.

1.4.4.5 Decrease of intracellular antibiotic concentration

Gram-negative bacteria have an outer membrane (OM), which allows them acquire less antibiotics from the environment compared to Gram-positive bacteria. However, there are two additional mechanisms for reduction of intracellular concentration of antibiotics: reduced uptake of a drug or increased efflux.

Reduction of an uptake. OmpF and OmpC porins locate in the outer membrane, and are responsible for the non-specific diffusion of small hydrophilic molecules such as sugars, ions and amino acids into the cell cytoplasm [282]. β -lactam antibiotics such as penicillin enter the cell through those porins, thus down-regulation of *ompF* and *ompC* genes leads to reduction in membrane permeability, as well as uptake of the drug. Recently, it has been showed that this mechanism became more prevalent in clinical isolates [264, 283]. Down-regulation of *ompF* and *ompC* gene expression was also noted in some clinical isolates resistant to one of the last resort antibiotic carbapenem [284, 264].

Increased efflux. Efflux mechanisms play a significant role in the intrinsic resistance of Gram-negative bacteria. Resistance mediated by efflux systems primarily arise from the modulation of regulatory pathways controlling the expression of efflux pump genes, leading to increased efflux pump production [285]. Efflux occurs through membrane-spanning efflux pumps, that excrete small molecules, including antibacterial agents, out from the cell [286]. In addition to providing an escape from antibiotics, efflux pumps play a role in various bacterial processes such as stress adaptation, virulence mechanisms, the development of biofilms, and modulation of host physiological functions. Therefore, efflux pumps represent a promising target for the development of novel inhibitors.

Six major families of efflux pumps have been identified: the ATP-binding cassette (ABC) superfamily, the resistance nodulation division (RND) superfamily, the small multidrug resistance (SMR) superfamily, the multidrug and toxic compound extrusion (MATE) super-

family, the proteobacterial antimicrobial compound efflux (PACE) family, and the major facilitator superfamily (MFS) [287]. All bacterial efflux pumps are incorporated into the inner membrane, but only RND and ABC superfamilies are tripartite and span further into peptidoglycan layer and outer membrane [267, 288]. Some members of RND superfamily have a broad specificity, and remove multiple classes of antibiotics via active transport, even though they are not the natural substrates, but they evolved to do so due to the selective pressure.

1.4.5 Mobile genetic elements

Bacteria that lack resistance to antimicrobials are able to obtain pre-existing resistance determinants from the bacterial gene pool. This is possible due to the precise activities of mobile genetic elements (MGE) that can move between or within DNA molecules [289]. Namely, this includes insertion sequences (IS), transposons, gene cassettes/integrans, as well as those with the capacity to transmit between bacterial cells, for instance plasmids and integrative conjugative elements. Usually, there are multiple copies of MGE across one genome, and they also able to promote homologous recombination, which involves the interchange of sequences among identical or closely related DNA segments. Genetic exchange mechanisms between cells include: conjugation/mobilization, which is facilitated by plasmids and integrative conjugative elements [ICE], transduction, which is mediated by bacteriophages, and transformation.

1.4.5.1 Insertion sequences

IS elements are DNA segments that can move themselves to random locations in DNA molecules within one cell [289]. These mobile elements can cause mutations or alter the genome by inserting themselves into genes or regulatory regions. IS typically carry one or

two transposase (*tnp*) genes, that are flanked by short inverted repeat sequences, and are divided into groups based on Tnp active site motifs and the mode of transposition. The IS can be replicated in a cointegrate, via copy-and-paste mechanism, that once resolved gives a copy of the IS to both donor and recipient cell. The other mechanism for IS replication is copy-out-paste-in mechanism, in which IS is replicated into a double-stranded circular DNA intermediate, which is integrated into a recipient cell.

IS can also play a significant role in antibiotic resistance by facilitating genetic rearrangements and mobilising resistance genes. When an IS element inserts into the regulatory region of a resistance gene, it can provide strong promoter sequences that increase the expression of the resistance gene, leading to higher levels of antibiotic resistance [290, 291]. IS can also disrupt genes that are involved in the susceptibility to antibiotics, potentially leading to resistance [292]. However, this is less common as a direct resistance mechanism.

1.4.5.2 Transposons

When two IS elements insert on either side of a gene or a cluster of genes, for example, an antibiotic resistance gene, they form a composite transposon and can cause the transmission of antibiotic resistance genes to other bacterial species [293, 294]. These IS elements can be identical or different, but they must have functional transposase genes. Transposon elements can move to any location in a gene, however, they may have more preferable location for insertion within one genome [295]. Some transposons remain always kept in the same insertion site within the genome, others are inactivated and cannot move. Transposons are divided into two classes: retrotransposons and DNA transposons.

The non-composite transposons contain *tnp* gene and other genes in between terminal inverted repeats, however, they are not dependent on IS elements [291]. In this case, transposition is mediated by a transposase within transposon. In most Gram-negative bacteria (including Enterobacteriaceae), the antibiotic resistance by non-composite Tns [294]. Among

different species, transposons that can carry antibiotic resistance genes are spread by bacteriophages and plasmids.

1.4.5.3 Gene cassettes

Gene cassettes are small mobile elements, typically comprising a single promoterless gene and a recombination site [296]. There are inverted repeats on the ends of those recombination sites that form a stem-loop structure. Although these cassettes can temporarily exist in a free circular form, they do not possess all the functions necessary for their own movement and are generally associated with integrons. An integron is typically characterized by the presence of the *intI* gene, which encodes an integrase, and an *attI* recombination site. The integrase catalyzes recombination between *attI* and/or *attC* sites, leading to the insertion or excision of cassettes. Multiple cassettes can be sequentially inserted into a single integron, forming a tandem array. In many cases, the spread of antibiotic resistance is facilitated by the incorporation of resistance genes into integrons, first noticed in Tn21 transposon [297]. Tn21 contains multiple resistance gene cassettes, including those encoding aminoglycoside-modifying enzymes and a mercury resistance *mer* operon. Additionally, Tn21 carry an integron, In2 with *aadA1* gene cassette conferring resistance to streptomycin/spectinomycin, and the *sul1* gene encoding a modified dihydropteroate synthase resistant to sulfonamides, along with other insertion sequences.

1.4.5.4 Integrative Conjugative Elements

Integrative and conjugative elements (ICEs), also called conjugative transposons, have three main features: they are integrated in a host genome, encode a functional conjugation system and contain genes needed for their own integration and excision [298]. ICEs are usually mosaic and contain functional gene modules from different sources, where genes of similar function are grouped together. ICEs can also carry some additional cargo genes with func-

tions unrelated to the ICE life cycle itself, but advantageous for a host cell. Most ICEs are passed to other cell generations during chromosomal replication, segregation, and cell division, however some are capable of independent plasmid-like replication.

In Proteobacteria and Firmicutes, ICEs and plasmids are a key factors for the spreading of antibiotic resistance genes [299]. Such ICEs as Tn*916*, SXT and CTnDOT carry an antibiotic resistance phenotype [298]. It was shown that Tn*916* ICE with tetracycline resistance could be transferred between *E. faecalis* strains through conjugation even in the absence of F-plasmids [300]. Tn*916* encoded the necessary genes for its own conjugative transfer and could integrate at multiple sites within a plasmid or the host chromosome, similar to a transposon. Additionally, Tn*916* was functional in cells lacking homologous recombination, indicating that host recombination functions were not required for the integration of the element into other DNA.

1.5 The *E. coli* *mar* stress response

The *E. coli* species is genetically diverse, and includes human commensal strains, which inhabit the intestine as part of normal microbiome, as well as pathogenic *E. coli* strains [301]. Those pathogenic *E. coli* species are divided into virotypes, each encoding distinct virulence factors and causing different infections, and there are, for example, six known virotypes of InPEC and four known virotypes of ExPEC [302]. The *E. coli* core genome approximately contains 867 core genes, with only 243 essential genes, but the pan-genome may have up to 43,415 genes [303].

Pathogenic *E. coli* is a leading cause of gastroenteritis, urinary tract infections (UTIs), meningitis, septicemia, and various other infections [11]. These bacteria remain a significant threat due to their low infectious dose, widespread transmission through food and water, and their high rates of mortality. They are a primary cause of infant and traveler's diarrhea

in developing countries. Enterotoxigenic *E. coli* (ETEC) alone is estimated to cause 43.7 million cases and 88.685 deaths in 2010 in South Asia and Africa [304]

1.5.1 Innate antibiotic resistance (*mar*) phenotype and *marRAB* operon

The *mar* locus is usually associated with multiple antibiotic resistance in Gram-negative bacteria, including *E. coli*. The *mar* locus was identified by Stuart Levy [305] using a Tn5 transposon insertion that demonstrated a multiple drug resistance (MDR) phenotype in *E. coli* isolates selected by growth on a high level of tetracycline and chloramphenicol. The *mar* locus comprises two transcribed units: *marC* (encodes a putative integral inner membrane protein) and the *marRAB* operon that encodes for repressor MarR, transcriptional regulator MarA and a periplasmic protein MarB (Figure 1.8) [306, 307]. Between two central units locates the operator *marO*. MarR is the first encoded protein in the *marRAB* operon and in the absence of a signal, MarR forms dimers that bind two specific palindromic sites within the *marO* operator. This represses further transcription of *marRAB* operon. Binding of MarR to the *marO* promoter region is relieved when a ligand, such as tetracycline, salicylate, chloramphenicol, organic solvents or other antimicrobial compounds, interacts with MarR, inactivating it and allowing transcription of the *marRAB* operon. Hence, MarA protein, that belongs to AraC-XylS family of transcriptional activators, is expressed [308, 263, 309].

The *mar* locus is an important part of the intrinsic antimicrobial resistance system; many mutations within this locus (for example in MarR binding site or its cognate gene *marR* [310]) result in constitutive expression of the *marRAB* operon, which leads to clinically relevant antimicrobial resistance in *E. coli* [311, 312, 313]. This allows bacterial populations to resist exposure to antibiotics. However, even in wild-type cells, *marRAB* expression is stochastic, even in the absence of antibiotics [314]. The mechanism behind this dynamic is unclear, but

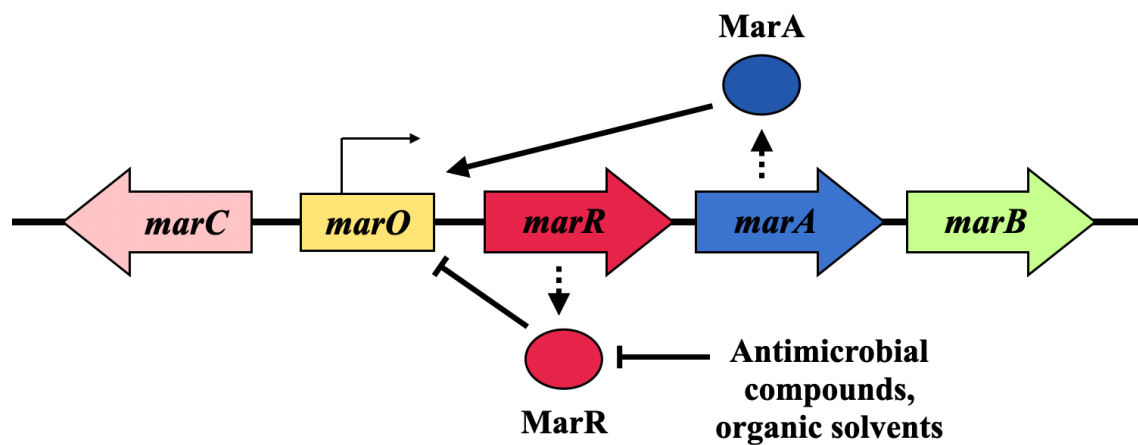


Figure 1.8: Schematic representation of *mar* locus.

The *mar* locus contain two divergent transcriptional units *marC* and *marRAB*, with *marO* being a central promoter with recognition sites. MarR is encoded by the first gene of the operon, and forms a dimer that in the absence of an inducing signal, binds to *marO* operator and repress transcription of *marRAB* operon. Some organic solvents and antimicrobial compounds can bind to MarR and inactivate it, allowing activation of the operon and expression of MarA.

it is assumed to allow populations to survive antibiotic treatment without requiring all cells to express costly genes involved in antibiotic resistance.

While the *mar* locus alone generally does not lead to clinical resistance (except for tetracycline, nalidixic acid, and rifampicin), it does reduce sensitivity to numerous antibiotic classes [312]. This reduction in sensitivity can accelerate the development of further antimicrobial resistance, or even enhance it when combined with existing mechanisms [315]. For example, *E. coli* strains with mutations in the *mar* locus acquire resistance to fluoroquinolones more rapidly than wildtype strains [263]. Therefore, the *mar* locus is crucial in the evolution of clinical antibiotic resistance. It regulates various defence mechanisms in *E. coli*, such as drug efflux and porin expression, which collectively reduce the intracellular accumulation of antimicrobial agents.

1.5.2 AraC-XylS family of transcriptional activators

Most of the positive regulators in bacteria belong to the AraC-XylS family of transcriptional regulators that are involved in antibiotic resistance and pathogenesis [316]. The family is characterised by two domains: a non-conserved domain that involved in dimerisation and signal/effecter recognition, and a conserved domain that consist of one homolog region that includes more than 100 residues containing the helix-turn-helix (HTH) DNA-binding motif, common for all family members [316, 317]. AraC-XylS family members usually assist σ^{70} -dependent transcription via binding upstream of the -35 element as monomers or dimers and recruiting RNAP through contacts with its α CTD domain [318, 319, 211].

MarA, SoxS and Rob proteins are the most important members of AraC-XylS family for control of the antibiotic resistance response in Enterobacteriaceae. They bind many sites in bacterial chromosome, activating genes responsible for intrinsic drug resistance. Encoded functions include efflux, biofilm formation, motility, lipid traffic and DNA repair [211, 215].

1.5.3 Transcriptional regulator MarA

MarA activates over 40 genes in its regulon involved in intrinsic response to exposure of toxic chemicals [320, 321]. One of the genes upregulated by MarA is *micF*. This gene encodes an antisense RNA, which represses expression of the outer membrane porin OmpF [263]. MarA targets many other genes involved in stress responses and multi-drug resistance, including those for the AcrAB-TolC multi-drug efflux pump system, which actively transport many antibiotics out of the cell [322]. Overexpressed efflux pumps can also confer high levels of resistance to previously clinically valuable antibiotics [323, 324, 325]. Although it was discovered as up-regulating the expression of efflux pumps, members of its regulon play a variety of roles. Regulon DB currently lists 57 genes and 29 operons under MarA regulation.

The half-life of MarA is just one minute, as it is intrinsically unstable [326]. This ensures that the response cascade is quickly eliminated once the stress signal dissipates. Also, additional regulation is provided by the Lon protease that rapidly degrades MarA [212]. MarA also activates its own transcription by binding as a monomer to the 15 bp *marbox* (Figure 1.9A), located upstream of the -35 hexamer in the *marO* region.

MarA is comprised of seven α -helices and forms two structurally similar sub-domains with an extended C-terminal loop, Figure 1.9B. The protein has sub-domains N and C (residues 8-59 and 60-108, respectively), each have an HTH DNA-binding domain (residues 29-50 and 77-100, respectively). Protruding side chains of the two HTH motifs fit into the two adjacent segments of the major groove on one face of the DNA. This causes DNA bending of approximately 35° [212]. Electrostatic interactions between the DNA backbone and MarA is mediated by conserved residues R46, T93, and R96 at the N-terminus. Additionally, amino acids Y39, S40, H43, and K49 are engaged in non-specific phosphate interactions with the DNA backbone, playing the major role in *marbox* affinity. Upon *marbox* recognition, MarA makes 15 contacts with the DNA, and its N-terminal domain may also participate in DNA

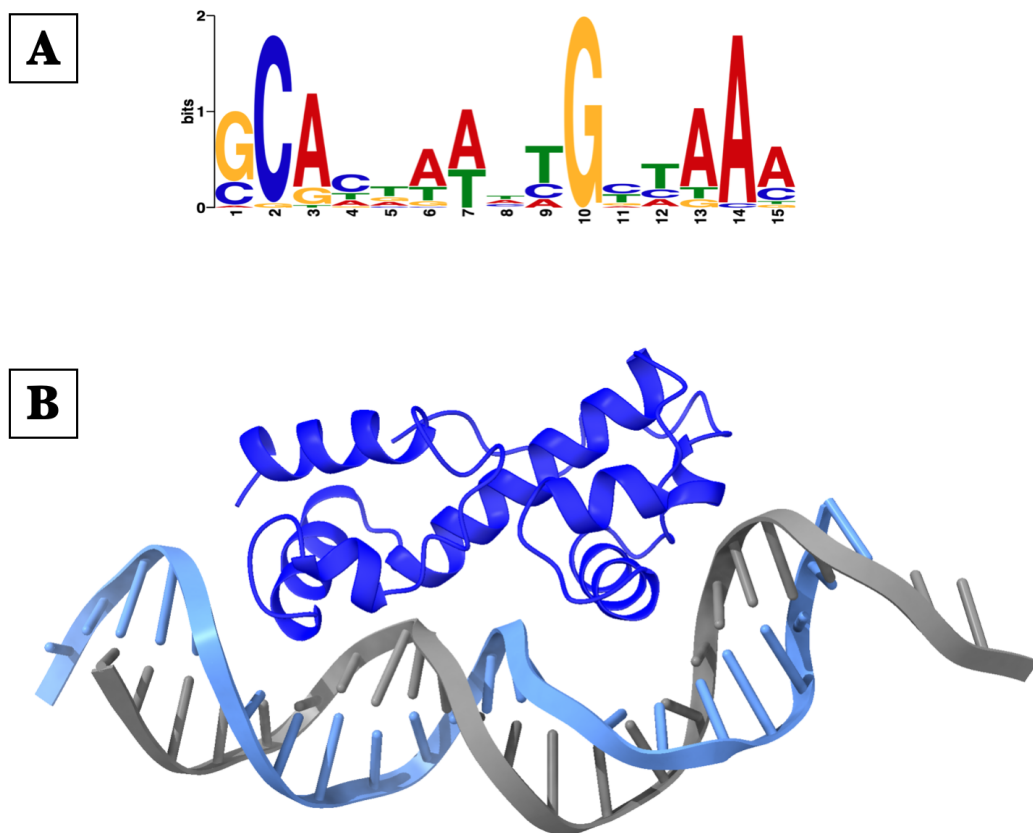


Figure 1.9: *Marbox* sequence and MarA crystal structure.

A. *Marbox* consensus sequence generated in a ChIP-seq experiment [215].

B. Model of a MarA crystal structure made in ChimeraX-1.7 [212]. MarA is shown in blue and DNA strand with the *marbox* shown in light blue and gray. MarA comprised of 7 α -helices. Helix 3 and helix 6 bind two adjacent major grooves and bend DNA 35°. Helices 2 and 3 comprise the N-terminal HTH motif, and helices 5 and 6 form C-terminal motif.

binding [327]. Due to these non-specific backbone interactions, MarA is capable of binding to a range of divergent sequences.

1.5.3.1 MarA pre-recruits RNAP

Usually, the activator first binds to an operator sequence, which then leads to RNAP recruitment [328]. In contrast, MarA employs a pre-recruitment mechanism, where the activator initially binds to RNAP [318]. This activator-RNAP complex then scans the DNA, binding to a *marbox* only when it is correctly aligned and positioned relative to the -10 and -35 elements. *Marboxes* are quite abundant in the *E. coli* genome, so the pre-recruitment model helps to explain why most of these operator sequences remain unbound by MarA, and why only a limited number of *marboxes* serve as true MarA targets.

1.5.3.2 MarA as a transcriptional activator

Functional MarA binding sites for transcription activation centered at positions -52 or -42, operate in the 'forward' orientation. This orientation positions the C-terminus of MarA in close proximity to RNAP, overlapping the -35 element [318, 318, 319, 211]. In this case, MarA recruits RNAP through contacts between the α CTD domain and glutamic acid residue 89 of MarA [319]. MarA also interacts with σ_4 domain [329, 228]. More distant *marboxes*, located upstream of the -35 element at positions -62 or -72, are oriented 'backward,' placing the N-terminus of MarA in proximity to RNAP [232, 330]. The RNAP interaction happens through MarA residues D18, W19, D22 and R36, that interacts with α CTD [331]. The same rules apply to SoxS and Rob, that also recognise *marboxes* and require the α CTD of RNAP, interacting with its DNA binding 265-determinant [232, 332, 331].

MarA is a well-known activator of the *acrAB* operon, which encodes an inner membrane transporter (AcrB) and a periplasmic adapter protein (AcrA), as well as the *tolC* gene that codes for an outer membrane channel (TolC) [333]. Together, these proteins form the

AcrAB-TolC efflux pump, a key mechanism of intrinsic antibiotic resistance in Gram-negative bacteria [334]. This efflux pump has broad substrate specificity, enabling the extraction of a variety of antibiotics, including fluoroquinolones and β -lactams, from the bacterial cytoplasm [335]. Overexpression of these genes is frequently detected in clinical strains of multidrug-resistant Gram-negative bacteria [336, 337, 283].

The *mar* phenotype also involves reduced uptake of antimicrobial agents through the OmpF and OmpC porins, that form pores in the outer membrane for the passive diffusion of small molecules [263, 338]. MarA indirectly down-regulates the expression of *ompF* and *ompC* by activating the transcription of the non-coding RNA *micF*, that binds to the target mRNA, inhibiting its translation and promoting degradation [339, 340]. This leads to reduced passive diffusion of molecules into the bacterial cell, decreasing effect of antibiotics [264, 283]. Additionally, MarA controls OM permeability to reduce antimicrobial uptake via activation of the *mlaFEDCB* operon [215], inhibits biofilm formation, and controls DNA repair through regulation of *ycgZ-ymgABC* operon and *xseA* gene, respectively [341]. Other genes, regulated by MarA include: *zwf* gene, that is involved in glucose metabolism, a stress response genes *inaA* and *ybjC*, that encode a putative IM protein [342, 332, 318].

1.5.3.3 MarA as a transcriptional repressor

Despite being a transcriptional activator of many genes, involved in antibiotic resistance in a cell, MarA also can act as a repressor, which can also increase resistance. At the *hdeA* and *purA* promoters, MarA binds in a reverse orientation to a site overlapping with the -35 element [343]. MarA also binds to the *rob* promoter in a reverse orientation, and overlaps both the -10 and -35 promoter elements, repressing transcription through steric hindrance [344]. It is suggested that repression in these cases may occur through DNA bending induced by MarA [345].

There is also evidence that MarA, and its paralogs SoxS and Rob, repress *flhDC* operon,

which product is involved in the regulation of flagella, thus, reducing the motility in *Salmonella* [346].

1.5.3.4 MarA as an antibiotics target

Taking in consideration clinical resistance of *mar* phenotype, it can be a promising target for new therapeutic strategies. Recent studies show that artesunate, that is used as an anti-malarial drug, also binds to MarA. Interestingly, artesunate inhibits MarA binding specifically to the *marA* promoter and prevents its self-transcriptional activation, meanwhile other *marboxes* are not affected [347]. Artesunate alone lacks direct antibacterial effects, it shows a synergistic effect when combined with β -lactam antibiotics, potentially extending the efficacy of existing treatments.

1.5.4 SoxS and Rob interplay on MarA regulon

MarA, SoxS and Rob multidrug resistance regulators function as monomers, which is unusual for transcription activators, as often they function in the dimer form [348, 230, 349]. MarA, SoxS and Rob bind to asymmetric DNA sites of a very high consensus similarity, recognise the *marbox* in the *mar* regulon, and have overlapping regulatory effects. However, Rob binds DNA with high affinity, but low specificity [350], whereas MarA and SoxS have a lower affinity but form ternary complexes with RNAP that enhance the recognition of promoters containing *marboxes* [318].

1.5.4.1 SoxS transcriptional activator

SoxS is the smallest member of AraC/XylS family of transcriptional regulators that is characterised by two conserved HTH DNA-binding motifs [351]. It is a component of the *soxRS* redox-sensing system of the superoxide regulon, where SoxS serves as the principal barrier

against superoxide anion (O_2^-), by activating genes that also confer tolerance to organic solvents and resistance to antibiotics [352, 353, 226, 211]. Both SoxS and Rob recognise *marboxes*, however, in literature their binding sites are called *soxbox* [351] and *robbox* [354], respectively. Here, we are referring to the SoxS and Rob binding sites as *marboxes*. SoxS establishes contacts with *marbox* via two recognition helices $\alpha 3$ and $\alpha 6$ in the conserved HTH DNA binding motifs [351]. However, it has been shown that SoxS alone does not establish strong contacts with the *marbox*, but in the presence of RNAP, a stable SoxS-transcription activation complex (TAC) complex is formed, allowing SoxS to remodel the enzyme and activate SoxS-dependent promoters [227, 234].

The best known SoxS-dependent promoters are *zwf*, *micF* and *fpr*, where SoxS stimulates transcription by binding upstream, or overlapping the -35 element [228, 234]. In case of *zwf* promoter, SoxS binding site locates 7 bp upstream of the -35 element. Here, SoxS contacts the 265-determinant on one of the α CTD subunits and the 287-determinant on the other α CTD subunit. On the *micF* promoter, the SoxS binding site overlaps the -35 element. Here, the N-terminal domain of SoxS binds to the 265-determinant of the α CTD. SoxS also interacts with the β flap tip helix and the 596-determinant of σ_4^{70} , which provides more stabilising interactions between RNAP and the activator. Interactions with SoxS occlude σ_4^{70} from binding the -35 element. Thus, on the *micF* promoter, SoxS pre-recruits RNAP and acts as a co- σ factor, directing RNAP from UP- and -35 element-containing promoters to SoxS-dependent promoters. On the *fpr* promoter, the SoxS binding site is in reverse orientation and is located 15 bp upstream of the -35 element. Here, the C-terminal domain of SoxS binds to the 265-determinant of one α CTD, while the 261-determinant of the other α CTD contacts the 596-determinant of σ_4^{70} . Consequently, both α CTDs interact to form a bridge that links SoxS and σ_4^{70} across long distances [234]. It also has been shown that SoxS enhances promoter escape of RNAP during transcription initiation depending on SoxS binding site location [355, 356, 234].

1.5.4.2 Rob transcriptional activator

The transcriptional activator Rob has about 45% sequence similarity to MarA and SoxS and features a GyrI-like domain, known for its interaction with various ligands such as organic solvents and superoxide-generating agents, causing antibiotics resistance in many clinically important pathogens [357, 358, 359, 360, 205].

Rob binds promoter DNA by two conserved HTH motifs, which possibly facilitates DNA bending, and promotes formation and stability of Rob-TAC. To make contacts with the DNA, Rob specifically interacts with two DNA major grooves of the Rob binding site and the acidic loop facilitates such interactions [354]. The GyrI-like domain locates within Rob CTD domain, and functions as a sensor for environmental stimuli, playing a role in structurally stabilising and promoting formation of a competent Rob-dependent TAC along with the DNA-binding Rob NTD [361]. Rob CTD also plays physiological roles in activating Rob through a mechanism involving the sequestration and dispersal triggered by an effector, also protecting Rob from cellular proteases such as Lon and ClpYQ [350].

When a Rob binding site overlaps a -35 region, Rob not only interacts with the α subunit of the RNAP, but also makes contacts with σ_4^{70} which occlude it from binding to the -35 element [362, 229]. In the 'sequestration' state, the residues participating in the Rob-RNAP α CTD and Rob- σ_4^{70} interfaces could be enclosed by the Rob CTD (and/or a co-factor), maintaining an inactive state. However, ligand binding alters the 'sequestration' conformation of Rob to a 'dispersal' state. This transition exposes the previously enclosed residues of Rob NTD, allowing them to interact with RNAP and initiate transcription.

1.6 Motility in *E. coli*

Motility is an ability of a free-living bacteria to move in the environment, which enables a cell not only to find new nutrition sources and colonise the host, but also avoid toxic

conditions (chemotaxis). In aqueous media, bacteria use rigid helical external filaments, such as flagella, or gyration of the whole cell. On solid surfaces bacteria twitch by assembling and disassembling type IV pili. Cells may also swarm collectively in a biofilm, powered by the rotation of flagella. Non-motile cells can use adhesion to move by sliding over surfaces in response to outward forces due to colony growth [363].

The most studied mode of motility in bacteria is swimming by rotation of flagellar filaments [364]. The energetic cost of synthesising the flagellum is significant for the growth rate of bacteria, which is about 2% [365]. However, flagellated bacteria compensate this energy loss by ability to compete for resources, avoid toxic compounds and detect signaling molecules by chemotaxis. Rotation of flagellum generate torque that pushes a cell body forward.

1.6.1 *E. coli* flagellum

The bacterial flagellum is a complex filamentous organelle responsible for motility, spanning both the inner and outer membranes, as well as peptidoglycan layer in Gram-negative bacteria. Overall, flagellum comprised of a basal body, hook and a filament, Figure 1.10. Usually, *E. coli* have 4-6 peritrichous flagella organised in a bundle behind the swimming cell [366]. Each flagellar filament is approximately 20 nm in diameter and several micrometers in length [367]. Rotation frequency of the filament is 100 Hz, which is generated by the flagellar rotary motor, located in the basal body which is powered by a proton motive force (PMF).

1.6.2 Flagellum composition

The flagellar basal body of *E. coli* composed of a rod and several rings (C, MS and LP), Figure 1.10. The C-ring is made of FliG, FliM and FliN, locates in the cytoplasmic side of the inner membrane, and responsible for generating torque and regulating the direction of

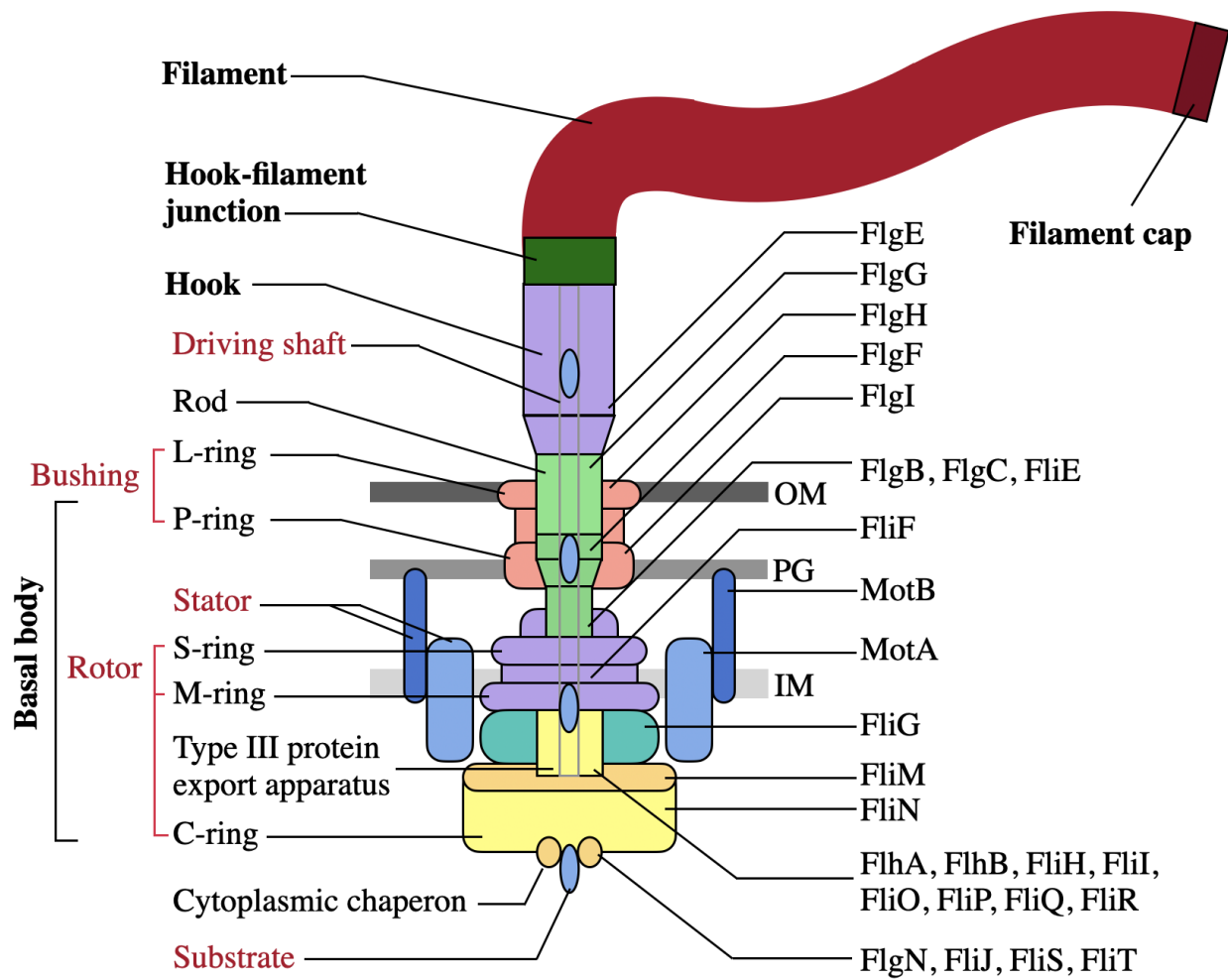


Figure 1.10: Structure of flagellum in *E. coli*.

OM - outer membrane; PG - peptidoglycan layer; IM - inner membrane. Adapted from J. Armitage and R. Berry [366].

rotation (clockwise (CW) or counterclockwise (CCW)) [368]. The MS ring is a transmembrane protein complex made of FliF, which is embedded in the IM, and together with C-ring comprise a flagellar rotor [369]. Torque is generated at the outer edge of the FliG ring by stator complexes composed of four MotA and two MotB proteins. These complexes span the IM and are anchored to the peptidoglycan layer through the C-terminus of MotB, and allow protons to move down an electrochemical gradient to generate a torque, causing rotation of the rotor [370, 371]. The chemotactic switching of the direction of the rotor is regulated by the signaling molecule CheY [372]. When phosphorylated, CheY (CheY-P) binds to FliM and subsequently to FliN, triggering a conformational change that propagates to FliG, thereby increasing the likelihood of the motor rotating in a clockwise (CW) direction. If the concentration of CheY-P is too low, causing the motors to rotate exclusively CCW, the C-ring compensates by recruiting additional FliM and FliN subunits, which create new binding sites for CheY-P [373]. This adaptation enables the motors to resume switching between CW and CCW rotations.

The export apparatus is a PMF-driven transmembrane export gate complex embedded in the IM and made of FlhA, FlhB, FliO, FliP, FliQ and FliR proteins. This is coupled with a cytoplasmic ATPase complex made up of FliH, FliI, and FliJ [374]. The flagellar export apparatus is evolutionarily related to the injectisome, a structure used by pathogenic bacteria to inject virulence proteins into eukaryotic host cells during invasion, so that both systems being classified as type III secretion systems (T3SS). FliH, FliI and FliJ are soluble, non-membrane proteins that deliver flagellum subunits to the export apparatus from the cytoplasm [375]. Substrate-specific chaperones FlgN, FliS, and FliT recognise proteins for filament formation such as FlgK/FlgL, FliC, and FliD, respectively, and also facilitate their export [376]. The subunits of the growing flagellum are transported through the narrow central channel inside the rod by the flagellar export apparatus, and once they reach the distal end, these proteins are incorporated into the growing flagellar structure [377].

The rod of the basal body comprises the drive shaft of the flagellum, with an approximate length of 30 nm that connects the rotor of the basal body to the hook [377]. The rod comprises of FlgB, FlgC, FlgF, and FlgG proteins [378]. FliE protein forms the base of the flagellar axial structure and acts as a structural adaptor that connects the MS ring to the rod [379]. FlgJ is a capping protein that possesses a muramidase activity. Its CTD domain digests the PG layer around the rod of the growing flagellum [380]. The NTD domain of FlgJ binds to the growing tip of the rod and assist in the assembly of the rod subunits through the PG layer. The rod penetrates PG layer and the OM through the LP ring.

The LP ring comprises a periplasmic rod, that functions as a bushing, providing support to the distal rod, enabling its stable rotation with minimal friction. The FlgH subunits polymerise in the OM into the L-ring, FlgI polymerises into the P-ring, which is embedded within the PG. The later ring is positively charged and is important in initial assembly of the LP complex around the negatively charged rod [381].

Hook subunits FlgE assemble directly onto the tip of the distal rod, and polymerise into curved tubular structure, approximately 55 nm long [379]. FlgD hook cap protein assists in the hook assembly, but is replaced by FlgK and FlgL proteins, that comprise the hook-filament junction, once the hook is completed. Further, a filament cap is assembled from five FliD subunits at the tip of the hook-filament junction, and then a long helical filament polymerises from FliC flagellin subunits.

1.6.3 Transcriptional regulation of flagellar biosynthesis

More than 50 genes requires for successful filament assembly [382]. These genes are organised in multiple operons which are arranged in a hierarchical cascade (Classes I-III), Table 1.3. During transcription of flagellar genes, the order of expression is very important, as it is coordinated with the assembly of the flagellar apparatus. There is only one *flhDC* operon,

Table 1.3: Classes of flagellar operons (based on D. Fitzgerald et al. [90]).

Class Name	Regulated by	Operon Name
Class I	RNAP σ^{70}	<i>flhDC</i>
Class II	RNAP σ^{70} and FlhDC	<i>flgAMN</i> , <i>flgBCDEFGHIJ/KL</i> , <i>flhBAE</i> , <i>fliAZY</i> , <i>fliE</i> , <i>fliFGHIJK</i> , <i>fliDST</i> , <i>fliLMNOPQR</i>
Class III	RNAP σ^{28}	<i>flgKL</i> , <i>fliDST</i> , <i>flgMN</i> , <i>fliC</i> , <i>tar-tap-cheRBYZ</i> , <i>motAB-cheAW</i> , <i>fliLMNOPQR</i>

that belong to Class I. This operon is RNAP σ^{70} -dependent and encodes for heterohexameric complex FlhDC, that turns on the further transcriptional cascade of flagellar biosynthesis.

Class II operons are activated by FlhDC and RNAP σ^{70} . At least eight operons are regulated in this manner, including: *flgAMN*, *flgBCDEFGHIJ/KL*, *flhBAE*, *fliAZY*, *fliE*, *fliFGHIJK*, *fliDST* and *fliLMNOPQR* [383, 145, 384, 385, 140, 90]. The Class II operons encode for structural components of the export apparatus, flagellar basal body, hook, as well as σ^{28} (FliA), and its cognate anti-sigma factor, anti- σ^{28} (FlgM) [145, 386]. FlgM interacts with σ^{28} promoter-binding domain, inhibiting its activity and preventing binding of σ^{28} to RNAP [387]. FlgM also protects σ^{28} from degradation by Lon protease [155]. Upon hook-basal body completion, FlgM is secreted out of the cell, which releases σ^{28} , that drives transcription of the last Class III genes.

Class III operons are activated by RNAP σ^{28} . There are at least 7 operons, that encode subunits, required for completion of flagellum, such as: *flgKL*, *fliDST*, *flgMN*, *fliC*, *tar-tap-cheRBYZ*, *motAB-cheAW*, and *fliLMNOPQR* [90]. Interestingly, RNAP σ^{28} can also drive transcription of Class II operons such as *fliAZY*, *fliDST* and *fliLMNOPQR* [388, 90]. There are other σ^{28} -binding sites found upstream of canonical Class II operons, such as *fliE* and *fliFGHIJK*, however, they were inactive in qRT-PCR experiments in the conditions tested [389, 90].

There are also σ^{28} -dependent sRNAs, that are expressed sequentially when the cell is transitioning from mid-exponential to stationary phase growth, assisting in flagellar gene expression through post-transcriptional regulation. These sRNAs are: UhpU (transcribed from its own promoter inside the coding sequence of *uhpT*, encoding a hexose phosphate transporter, as well as from its 3' UTR), MotR (originates from the 5' UTR of *motA*, encoding for the flagellar motor) and FliX (originates from the 3' UTR of *flhC*, encoding for flagellin) [90, 390].

Each of these sRNAs, expressed in different stages of bacterial growth, plays a different

role in *E.coli* motility. For example, MotR has 19 potential targets, expressed earlier in growth, and acts as an accelerator of flagellar synthesis by increasing expression of *fliC* gene and flagella number, likely by stabilising or promoting the translation of mRNAs involved in flagella assembly [391, 390]. FliX has 17 potential targets, and is expressed later in growth. This protein decreases both expression of the *fliC* gene, and flagella number, by destabilising or repressing the translation of *fliC* mRNAs. The UhpU sRNA has approximately 111 potential targets, including genes in the flagellar regulon, such as *fliC*. UhpU slightly increase flagella number and motility by repressing transcription of LrhA, a transcriptional repressor of *flhDC* operon. Overall, these sRNAs modulate the expression of flagellar components post-transcriptionally, either enhancing or repressing the synthesis of flagella by influencing mRNA stability and translation.

1.6.4 FlhDC transcription activator

FlhDC is an atypical hexameric transcription factor, encoded in the Class I *flhDC* operon, and playing a role as a flagellar master regulator in *E. coli* [392]. FlhDC binding sites usually locate in the -40 to -80 regions of cognate promoters, overlapping -35 elements and activating Class II operons in σ^{70} -dependent manner [100]. FlhDC-dependent activation also requires contacts with α CTD domain of RNAP [393]. FlhDC is comprised of four FlhD subunits, that undergoes a major structural changes upon FlhC binding, and two FlhC subunits, each have a zinc binding domain, that consists of four conserved cysteine residues [394], Figure 1.11A. The FlhD dimer, and FlhC monomer alone, can also bind DNA, but regulate different promoters, that are not regulated by FlhDC [395, 90]. FlhDC recognises a degenerate DNA consensus (Figure 1.11B) and binds to at least 11 sites on *E. coli* chromosome. Promoter DNA is bent by approximately 111° upon FlhDC binding [90, 394].

1.6.5 Molecules, that regulate flagellar biosynthesis

It is important to highlight, that the flagella construction cost for the *E. coli* varies from 0.1% to 5% of the total cell energy, and the operating cost varies from 2% to 3.4% (taking into account 5 flagella, a 100 Hz rotation rate, and a 1 hr cell division time) [396, 397]. Thus, precise regulation of flagella gene expression is important.

Activators and enhancers of motility. Activation of flagellar biosynthesis may not only increase the range of nutrients that *E. coli* can access, but also prevent bacteria from being exposed to toxic chemicals that might be present in the environment. For example, flagellar genes are activated at different stages of infection, as well as under oxygen-limited conditions [398, 399].

The *flhDC* operon, encoding FlhDC flagellar master regulator, that stands on top of flagellar biosynthesis cascade, is itself controlled by at least 13 transcription factors, each of them is active under different conditions [400]. The best known activators of this operon are histone-like nucleoid structuring protein H-NS [401, 402], quorum-sensing regulator QseB [403], cAMP receptor protein cAMP-CRP [404] and chromosomal replication initiator protein DnaA [405]. The two-component system AtoSC, facilitates *E. coli* response to acetate, spermidine, and histamine via enhancing transcription of both *flhD* and *flhA* [406].

Small regulatory RNA McaS positively regulates FlhDC on the post-transcriptional level by pairing with two regions of the *flhDC* mRNA, relieving the secondary structure around the ribosome binding site, thus, increasing its translation [407]. Interestingly, McaS represses *csgD*, the transcription regulator of curli biogenesis by enhancing degradation of *csgD* mRNA. Another RNA-binding protein CsrA, also known as a repressor of stationary-phase genes in *E. coli*, activates *flhDC* on the posttranscriptional level as well [408].

IS elements and transposons also play an important role in regulating flagellar biosynthesis. In *E. coli*, IS1, IS3 and IS5 elements can insert into the upstream region of the *flhDC*

operon, enhancing motility [409]. Interestingly, the insertion of those IS elements happens only when environment permits motility (on soft-agar plates), and no insertion upstream *flhD* is detected on solid or liquid media.

Repressors and inhibitors of motility. Overall, there are more inhibitors of flagellar biosynthesis, then activators, due to high energy cost of building and utilising it. The expression of flagellar genes is reduced when D-glucose is present, as well as under conditions of high temperature, elevated salt levels, extreme pH, or exposure to DNA gyrase inhibitors [410, 411, 412].

There are many other regulatory proteins that represses *flhDC* transcription. Such regulators as LysR homologue LrhA, HNS-dependent *flhDC* regulator HdfR, integration host factor IHF, catabolite repressor Hha, fimbriae activator MatA, iron response regulator Fur, acriflavine resistance regulator AcrR, regulator of curli expression and the motility FlhZ, and the inhibitor of motility and biofilm formation YjjQ, all downregulate transcription of *flhDC* operon by binding upstream [400, 413].

The EnvZ/OmpR osmolarity system also regulates *flhDC* transcription [400]. When osmolarity changes, EnvZ phosphorylates OmpR, enhancing its binding to the *flhD* promoter, thus repressing *flhDC* transcription. Acetyl phosphate further enhances this repression by also phosphorylating OmpR. RcsAB complex, which is a part of RcsCDB system responsible for activation of colanic acid, plays a role not only in biofilm formation, also found to be a downregulator of *flhD* promoter [414].

There are several small RNAs that inhibit *flhDC* mRNA stability at the post-transcriptional level: ArcZ, OmrA, OmrB, and OxyS [415]. AsfHd is a small antisense regulatory RNA that is transcribed from the coding sequence of *flhD*, affects genes related to motility through the repression of the transcription elongation of *flhD* gene via transcriptional attenuation [416]. AsfHd also represses *flhD* transcription via transcriptional interference, as both promoters locates in close proximity to each other.

Phosphodiesterase YdiV regulates FlhDC complex on the posttranslational level, by binding to the FlhDC, when the concentration of the latter is high, thus, inhibiting its activity as transcriptional regulator [417].

The transcription of *fliA* gene, encoding for σ^{28} , that triggers transcription of late genes involved in completion of flagellar, can be repressed by several regulators, including CsgD and CpxR [418]. Additionally, both the *fliAZY* and *flhDC* operons are repressed at high temperatures by DnaK, DnaJ, and GrpE [411, 405]. In strains lacking Hha and YbaJ, biofilm formation decreases, while the expression of FliA and FlhDC increases, enhancing motility [419].

1.7 MarA as a motility regulator

The relationship between motility and antibiotic resistance in *E. coli* is complex, as both traits are influenced by various genetic and environmental factors. The same mechanisms that allow *E. coli* to develop resistance to antibiotics may also affect its motility. For example, it has been shown that MarA activates the *ycgZ-ymgABC* operon, which regulate the formation of curli fibers and colanic acid production. Cognate proteins of this operon YcgZ, YmgA, and YmgB form a complex, that interacts with RcsC, a histidine kinase component of the phosphorelay system, leading to the inhibition of biofilm formation [341]. Interestingly, products of *ycgZ-ymgABC* operon also indirectly repress *csgD* gene. CsgD is a transcriptional activator of genes encoding for curli fibres. In *S. typhimurium* MarA and its homologous repress *csgD*, causing inhibition of the biofilm formation [420]. Those findings are specifically interesting as biofilms considered as most adaptive feature of bacterial community against antibiotics, as bacteria in a biofilm are approximately 15-times more resistant compare to a free-swimming planktonic cells [421].

Additionally, MarA activates transcription of the anti-sense RNA MicF, which in turn

inhibits the translation of the OmpF porin [422, 423]. In *C. werkmanii* OmpF is required for cell aggregation and biofilm formation, swimming motility and regulation of flagellar assembly-related genes [424].

In the *E. coli* O157:H7 strain, a MarA binding site was identified in the promoter region of the prophage-encoded transcription factor PchE. In *E. coli*, PchE is an inhibitor of biofilm formation and attachment to cultured epithelial cells within a host [425, 426]. In these studies it has been shown that PchE also acts as a transcriptional repressor of *csgD* and *csgA*, curli subunit gene, is activated by MarA. PchE production was increased in a presence of sodium salicylate (MarA inducer), and PchE have a small positive effect for flagellar motility [426].

However, in another study, MarA, SoxS and Rob directly inhibit flagellar gene expression, production of flagella, and motility in *S. typhimurium* [346].

Taken together, those studies show that motility is an important feature in bacterium, which allows them to modify their survival strategy depending on environmental stimuli, including toxic chemicals and antibiotics. It is important to notice that different species of bacteria use different approach when encounter antibiotics.

1.8 Objectives of this project

Induction of the MarA operon or the formation of flagella assists *E. coli* in surviving better under stress. Note, that the expression of the MarA regulon permits bacterium to cope with toxic substances, while the expression of the flagellar regulon allows *E. coli* to escape from them. Thus, the former system enables a ‘fight’ mode, while the latter mediates a ‘flight’ mode. The expression of both systems is tightly regulated, and, to date, it has been believed that the two regulatory systems are distinct and orthogonal to each other.

In this study, my aim was to understand if there is any crosstalk between the MarA stress response system and motility due to flagellum biosynthesis. Hence, my first objective

in Chapter 3 was to investigate MarA binding at the putative targets in the promoter regions of flagellar genes. My second objective in Chapter 4 was to investigate whether MarA binding at these targets affects transcription. This led to the identification of additional promoter regions, one of which is activated by MarA. This promoter had an unusual architecture for a MarA-regulated promoter. Thus, in Chapter 5, I explored a novel mechanism of transcription initiation for MarA, working via a pre-opening of the first base of the transcription bubble.

Overall, my research links two separate processes: fight (MarA activating genes involved in antibiotic resistance) and flight (MarA activating genes involved in flagellar biosynthesis). Both modes are regulated by one *mar* regulon and involve complex regulation by many other factors, which in turn might contribute to the fine-tuned expression of the multi-drug resistance phenotype.

Chapter 2

Materials and methods

2.1 Buffers and generic reagents

All solutions were prepared using deionised and distilled water (ddH₂O) unless otherwise specified. The solutions underwent autoclaving at 121°C for 15 minutes at 5 psi. Enzymes were purchased from New England Biolabs (NEB) unless stated otherwise. Deoxy-oligonucleotides were ordered from Merck Life Science UK Limited. Solutions used for each protocol are listed in Table 2.1.

2.2 Solid growth media

LB agar:

Thirty-five grams of powdered agar (supplier: Sigma) was dissolved in ddH₂O and made up to 1 L, then autoclaving.

MacConkey lactose agar:

Fifty-two g of MacConkey agar (supplier: Oxoid) powder containing lactose (10 g/l) was dissolved in ddH₂O and made up to 1 L, then autoclaving.

2.3 Liquid growth media

LB broth:

Twenty g of powdered LB broth (supplier: Sigma) was dissolved in ddH₂O and made up to 1 L, then autoclaving.

Table 2.1: Solutions and reagents used in this study

Procedure	Solution Details
Polymerase chain reaction	1 mM Deoxynucleotide (dNTP) Solution Mix (10 mM stock diluted 1 in 10 with ddH ₂ O); Q5 High-Fidelity DNA Polymerase; 5x Q5 Reaction Buffer (contains 2 mM Mg ²⁺)
Restriction digests	10x rCutSmart buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 100 µg/ml rBSA, pH 7.9); EcoRI, Hind III, AatII, XhoI or NdeI restriction enzymes were used in 1:20 proportion for each reaction.
Ligation of DNA fragments	10x T4 DNA ligase buffer (50 mM Tris-HCl, 10 mM MgCl ₂ , 1 mM ATP, 10 mM DTT, pH 7.5); T4 DNA ligase
Agarose gel electrophoresis	Powdered agarose (Bioline); 6x Loading dye (Sigma): 10 mM Tris pH 7.5, 1 mM EDTA, 20% (<i>v/v</i>) glycerol, 0.025% (<i>v/v</i>) bromophenol blue, 0.025% (<i>v/v</i>) xylene cyanol FF; 5x TBE (Fisher Scientific) diluted to 1x with dH ₂ O
Phenol/chloroform extraction	Phenol/chloroform/isoamyl alcohol pH 8 (25:25:1)
Ethanol precipitation	100% (<i>v/v</i>) Ethanol; 70% (<i>v/v</i>) Ethanol; 3 M Sodium acetate pH 5.2; 20 mg/ml Glycogen
Preparation of competent cells	100 mM Calcium chloride; 50% (<i>v/v</i>) Glycerol

Procedure	Solution Details
Beta-galactosidase assays	Z-buffer: 8.53 g Na ₂ HPO ₄ , 4.87 g NaH ₂ PO ₄ ·2H ₂ O, 0.75 g KCl, 0.25 g MgSO ₄ per 1 L ddH ₂ O, then autoclaved; 13 mM ONPG (Sigma-Aldrich); β-mercaptoethanol (Sigma); 1% (v/w) Sodium deoxycholate (Sigma-Aldrich); 100% (v/v) Toluene; 1 M Sodium carbonate
Radiolabelling of DNA fragments	Micro Bio-Spin P-30 Column (Bio-Rad); T4 polynucleotide kinase; 10x T4 polynucleotide kinase buffer; P ³² -labelled γ-dATP (MP Biomedicals); TE buffer: 10 mM Tris-HCl, 1 mM EDTA (pH 8.0)
Electrophoretic mobility shift assays	10x TNSC buffer: 400 mM Tris acetate pH 7.9, 10 mM MgCl ₂ , 1 M KCl, 10 mM DTT (supplier: Roche); 50% glycerol; 250 ng/μl Herring Sperm DNA (Thermo Fisher Scientific)
<i>In vitro</i> transcription	10x TNSC buffer; STOP solution: made up of 97.5% (v/v) deionised formamide, 10 mM EDTA, 0.3% (v/v) bromphenol blue and 0.3% xylene cyanol; P ³² -labelled α-UTP (Perkin-Elmer); NTP mix: 1 mM ATP/GTP/CTP and 50 μM UTP (Thermo Fisher Scientific); 1 mg/ml BSA (Sigma-Aldrich); <i>E. coli</i> RNA Polymerase Holoenzyme and Core Enzyme; <i>E. coli</i> σ ²⁸ factor
G+A ladder generation	DNase I blue: 5 M urea, 20 mM NaOH, 1 mM EDTA, 0.025% (v/v) bromophenol blue, 0.025% (v/v) xylene cyanol FF; 3 M CH ₃ COONa; 10 M Piperidine; 100% Formic acid; 100% Ethanol; 70% Ethanol

Procedure	Solution Details
DNase I footprints	Recombinant DNase I (Roche); DNase I blue; DNase I STOP solution: 0.3 M sodium acetate, 10 mM EDTA, 0.025% (<i>v/v</i>) bromophenol blue, 0.025% (<i>v/v</i>) xylene cyanol FF; 10x TNSC buffer; 250 ng/μl Herring Sperm DNA (Thermo Fisher Scientific); 100% and 70% Ethanol
Potassium Permanganate footprinting	DNase I blue; 10 M Piperidine; 10x TNSC buffer; Stop solution: 3 M NH ₄ CH ₃ CO ₂ , 0.1 mM EDTA, 1.5 M 2-mercaptoethanol; 100% and 70% Ethanol; 200 mM KMnO ₄ ; <i>E. coli</i> RNA Polymerase Holoenzyme and Core Enzyme; <i>E. coli</i> σ ²⁸ factor
Denaturing sequencing gel 6% and Acrylamide gel 7.5%	UreaGel Concentrate, UreaGel Diluent, and UreaGel Buffer (National Diagnostics); 10% APS solution (Sigma-Aldrich); TEMED; ProtoGel (National Diagnostics)
SDS-PAGE	Protein ladder (New England Biolabs); 5x SDS loading dye; NuPAGE 4-12% Bis-Tris gel (Invitrogen); 10x NuPAGE MES SDS Running Buffer (Invitrogen); Staining solution: 50% (<i>v/v</i>) methanol, 10% (<i>v/v</i>) acetic acid, 2 g Brilliant Blue R in 1 L ddH ₂ O; Fast Destain solution: 40% (<i>v/v</i>) methanol, 10% (<i>v/v</i>) acetic acid, made to 1 litre with ddH ₂ O.

Procedure	Solution Details
Purification of MarA	1 M IPTG solution (supplier: Sigma-Aldrich); Lysis buffer: 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 M NaCl; Wash buffer: 4 M urea, 50 mM Tris-HCl (pH 8.5); Denaturing buffer: 50 mM Tris-HCl (pH 8.5), 6 M guanidinium-HCl; Buffer A: 1 M NaCl, 50 mM Tris-HCl (pH 8.5); Buffer B: Buffer A + 1 M imidazole; Buffer X: 1 M NaCl, 50 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid, pH 8.0], 1 mM DTT, 5 mM EDTA, 0.1 mM Triton X-100.
Purification of FlhDC	1 M IPTG solution; Buffer A: 0.05 M NaCl, 20 mM Tris-HCl (pH 7.9); Buffer B: 1 M NaCl, 20 mM Tris-HCl (pH 7.9); Buffer C: 0.1 M NaCl, 20 mM Tris-HCl (pH 7.9); Storage buffer: 0.1 M NaCl, 20 mM Tris-HCl (pH 7.9), 50% (<i>v/v</i>) glycerol, 0.1 mM DTT (supplier: Roche), 0.1 mM EDTA; Lysis buffer: Buffer A + PIC (supplier: Roche) + 0.1 mM DTT.
Purification of FlhA	1 M IPTG solution; Buffer A: 20 mM Tris-HCl (pH 7.9), 5% (<i>v/v</i>) glycerol, 600 mM NaCl, 8 M Urea; Buffer B: 20 mM Tris-HCl (pH 7.9), 5% (<i>v/v</i>) glycerol, 600 mM NaCl, 8 M Urea, 200 mM Imidazole; Reconstitution buffer: 20 mM Tris-HCl (pH 7.9), 200 mM NaCl, 20% glycerol, 1 mM DTT, 1 mM EDTA, 10 mM MgCl ₂ , 1 μ M ZnCl ₂ ; Lysis buffer: 40 mM Tris(pH 7.9), 300 mM NaCl, 10 mM EDTA, PIC (supplier: Roche) 1 per 50 ml.

2.4 Antibiotics

Antibiotic stocks were made up and used as follows:

- Ampicillin: 100 mg/ml (dissolved in water) stored at -4°C . The final concentration used in agar and broth was 100 $\mu\text{g/ml}$.
- Kanamycin: 50 mg/ml (dissolved in water) stored at -4°C . The final concentration used in agar and broth was 50 $\mu\text{g/ml}$.
- Chloramphenicol: 50 mg/ml (dissolved in ethanol) stored at -20°C . The final concentration used in agar and broth was 50 $\mu\text{g/ml}$.
- Tetracycline: 35 mg/ml (dissolved in methanol) stored at -20°C . The final concentration used in agar and broth was 35 $\mu\text{g/ml}$.

2.5 Preparation of competent cells

Fifty ml of fresh LB broth was inoculated with 1 ml of overnight culture and incubated at 37°C with shaking until the culture reached the $\text{OD}_{650}=0.3-0.6$ (measured using a Jenway 6300 spectrophotometer). The culture was chilled on ice for 10 minutes, and then were harvested by centrifuging at 4000 rpm/min for 5 minutes at 4°C , supernatant was discarded. The pellet was resuspended in 25 ml 100 mM of ice-cold Cl_2 , incubated on ice for 20 minutes, reharvested by centrifuging at 4000 rpm/min and resuspended in 3.3 ml ice-cold 100 mM ice-cold CaCl_2 . The suspension was left on ice overnight to maximise competency. Afterwards, 650 μl of competent cells was aliquoted into microfuge tubes with 350 μl of ice-cold 50% (*v/v*) glycerol for storage at -80°C . Cells were thawed on ice before use.

2.6 Transformation of bacterial cells

One hundred ng (1-5 μ l) of plasmid DNA (or 20 μ l of ligation reaction) was incubated with 100 μ l of competent cells for 1 hour on ice. Cells were then heat shocked at 42°C for 2 minutes, and returned to ice for 2-3 minutes. After, cells were recovered with 500 μ l of LB medium at 37°C for 1 hour. Then cells were harvested by centrifugation at 4000 rpm/min for 5 minutes, and cell pellet was resuspended in 100 μ l of LB medium and plated out on an appropriate agar.

2.7 PCR reactions

Each PCR reaction was prepared in a total volume of 50 μ l. The reaction mix contained 0.5 mM each of ATP, GTP, CTP, and TTP, 200 pmol of each primer (Table 2.4), 50-100 ng of template DNA, 0.5 μ l of Q5 polymerase, and 5 μ l of 10x Q5 Buffer. The annealing temperature during PCR varied from 50°C to 72°C depending on the oligonucleotide sequence and was typically 3°C lower than the lowest primer T_m used. Elongation time was 30 seconds per 1000 bp of amplified DNA.

A standard PCR cycle consisted of the following stages:

1. 98°C 2 minutes (initial melting of DNA).
2. 98°C 30 seconds (DNA melting).
3. 50-65°C 30 seconds (primer annealing).
4. 72°C 30 seconds (elongation).
5. 72°C 5 minutes (final elongation).

Steps 2-4 were repeated for 35 cycles. For each primer pair used in PCR, the annealing temperature for Step 3 was chosen individually.

2.8 Megaprimer PCR reactions

To introduce point mutations into large DNA fragments lacking appropriately positioned restriction sites, the megaprimer PCR technique was used. The first round of PCR reaction contains forward primer that confer a mutation required, and a reverse primer. This generates a "megaprimer" carrying the point mutation. This megaprimer was used as a reverse primer in a second round of PCR, along with a second forward primer, to produce the final product containing the desired point mutation. (Figure 2.1).

2.9 Plasmid extraction using Qiagen miniprep extraction and ZymoPURE extraction kits

Small-scale extractions of DNA for creating plasmid stocks and cloning vectors were made using the Qiagen miniprep kit, following the manufacturer's instructions, from a 5 ml overnight culture. Briefly, the DNA is bound to a silica column and, after being washed in a high salt solution, the DNA was eluted in water or elution buffer and released into an eppendorf via centrifugation. For large-scale *in vitro* transcription, DNA extraction was done from a 50 ml overnight culture using the ZymoPURE II Plasmid Midiprep Kit.

2.10 Agarose gel electrophoresis

Agarose gel (1% (*w/v*)) was prepared by dissolving agarose in 1x TBE. The suspension was heated using a microwave for approximately 1 minute, until the agarose is dissolved, and then SYBR Safe DNA gel stain (1%) was added and well mixed. Gels were run at 100 V for 30-40 minutes in 1x TBE running buffer.

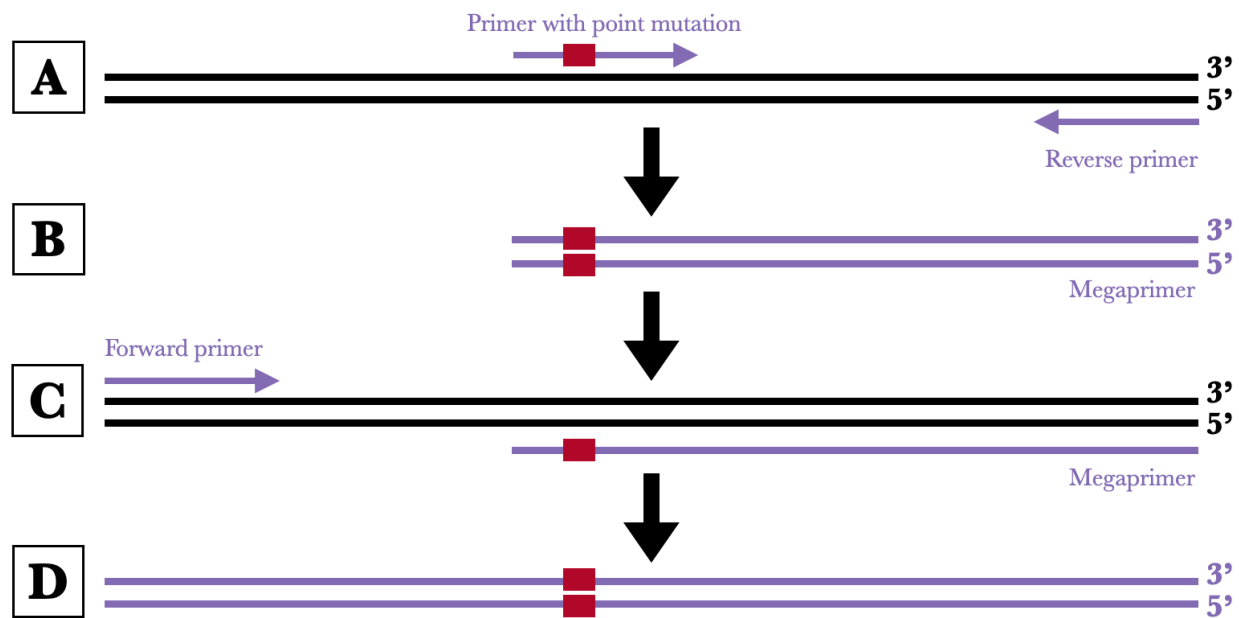


Figure 2.1: Mega-primer PCR.

A. First round PCR to generate megaprimer.

B. Megaprimer.

C. Second round PCR.

D. Final PCR product.

Black lines show template DNA, purple lines show PCR products and primers, red squares show point mutations.

2.11 Gel extraction

To purify PCR DNA fragments gel extraction was used. DNA samples were first separated on a 1% agarose gel by electrophoresis. After electrophoresis, the desired band was cut with a razor blade, and DNA was isolated from the gel using the Qiagen Gel Extraction Kit per the manufacturer's instructions.

2.12 Phenol-chloroform extraction/ethanol precipitation of DNA

To purify plasmid DNA phenol-chloroform extraction was used. Two hundred μl of the phenol-chloroform mix was added to 200 μl of DNA solution, transferred to a Phase Lock Gel Heavy 2ml tubes (supplier: Quantabio), vortexed for 10 seconds and centrifuged at 2600x g for 3 minutes. Two hundred μl of the upper aqueous phase was transferred to a new 1.5 ml eppendorf. Then 600 μl of ice-cold 100% (*v/v*) ethanol, 20 μl of 3 M sodium acetate pH 5.2 and 1 μl of 20 mg/ml glycogen were added. DNA was precipitated at -80°C for 30 minutes and collected by centrifugation at 17000x g and the pellet was washed in ice-cold 70% (*v/v*) ethanol, dried in a SpeedVac concentrator for 10 minutes, and resuspended in ddH₂O.

2.13 Restriction digest

For one digest reaction in total volume of 50 μl , 1.5 μl of a restriction enzyme was added along with 5 μl of 10x rCutSmart Buffer and 43.5 μl of 100-200 ng/ μl plasmid DNA solution. Double digestions were done using 42 μl of plasmid DNA and 1.5 μl of each enzyme, again in a 50 μl total volume. All restriction digests were incubated for 3 hours at 37°C. For plasmid vectors, 5 μl of Calf Intestinal Alkaline Phosphatase (CIP; supplier: New England Biolabs)

was added for the last 30 minutes, to cleave 5' hydroxyl groups and prevent self-ligation of the plasmid.

2.14 Ligation of PCR products into plasmid vectors

PCR products were purified after restriction digest using a Qiagen PCR purification columns according to the manufacturer's instructions; this step allows to remove oligonucleotides under 40 bp. Vectors smaller than 10 kb were gel extracted using Qiagen Gel Extraction kit after restriction digest. Vectors larger than 10 kb were purified using phenol-chloroform extraction, followed by ethanol precipitation. Purified PCR products and plasmids were used in ligation reactions. Ligation reactions were made in 20 µl of total volume, containing 3/1 ratio of a plasmid vector to PCR product (about 100 ng and 300-400 ng respectively), 2 µl of T4 DNA ligase and 2 µl of 10x T4 Ligase Reaction Buffer.

2.15 Sanger sequencing of plasmid constructs

Sanger sequencing was done at the University of Birmingham by the Functional Genomics and Proteomics Facility or by Eurofins Genomics service. Sequencing reactions were made up in a 10 µl total volume, with 1 µl of 10 µM primer, and at least 200 ng of DNA.

2.16 Genomic DNA extraction from bacterial cells using Monarch Genomic DNA Purification Kit

For gDNA extraction, 1 ml of o/n bacterial culture was harvested by centrifugation and treated according to the manufacturer's instructions. In short, the pellet was resuspended in 10 mM Tris-HCl pH 8 with 10 µl of lysozyme and 100 µl Tissue lysis buffer. This mix

was incubated at 37°C for 30 minutes. After addition of 10 µl of Proteinase K, the sample underwent further incubation in a thermal mixer at 56°C for another 45 minutes. Further, 3 µl of RNaseA was added and the lysate was incubated for 5 minutes more at 56°. Next, the reaction with gDNA was resuspended in gDNA binding buffer, bound to a silica column after centrifugation and washed with a Wash Buffer. Genomic DNA was eluted in 100 µl of ddH₂O.

2.17 β-galactosidase assay

β-galactosidase assays were done using a protocol adapted from Miller et al, 1972 [427]. Overnight cultures were set up from fresh colonies in 2 ml LB media with antibiotics required. The next day, 100 µl from each overnight culture was sub-cultured into 5 ml of fresh LB media with antibiotics. Cultures were grown to mid-log phase ($OD_{650} = 0.3-0.6$, measured using a Jenway 6300 spectrophotometer). Cells were lysed with 1 drop of 100% (*v/v*) toluene and 1 drop of 1% (*v/w*) sodium deoxycholate, and then vortexed for 10 seconds. Lysates were left with lids open at 37°C for 20 minutes, to allow the removal of toluene. Before doing the next part of experiment, 2-Nitrophenyl β-D-galactopyranoside (ONPG) was made up to a concentration of 13 mM (8 mg/ml) in 100 ml of "Z-buffer" with 271 µl of β-mercaptoethanol. One hundred µl of lysate was transferred into a new tube, and the reaction was initiated in a timed manner by adding 2.5 ml of 13 mM ONPG solution. After the reaction turned yellow, it was stopped with 1 ml of 1 M Na₂CO₃. The OD_{420} of the reaction was then measured, and β-galactosidase activity was then calculated using the following formula:

$$\text{Activity (Miller units)} = \frac{1000 * 2.5 * A * OD_{420}}{4.5 * T * V * OD_{650}} = \frac{20,000 * OD_{420}}{T * OD_{650}}$$

Where T= Time (mins), A= Final assay volume (3.6 ml), V= Volume of lysed culture used (0.1 ml); 2.5 = Conversion factor to convert OD_{650} into dry protein mass (mg) – assumes

that OD₆₅₀ of 1 is equivalent to 0.4 mg/ml bacterial dry mass, $1000/4.5 =$ Conversion factor to convert A₄₂₀ into moles of O-nitrophenol-assumes that 1 mole/ml O-nitrophenol has an absorption of 0.0045 at OD₄₂₀.

Data shown is from three independent replicates, featuring the average activity along with one standard deviation presented on both sides of the mean.

2.18 End labelling DNA with [$\gamma^{32}\text{P}$]-ATP

Purified with ZymoPURE II Plasmid Midiprep Kit pSR plasmid (Table 2.3) containing the promoter insert of interest was used for radiolabelling. Plasmid was first digested using HindIII and treated with alkaline phosphatase (CIP) to remove a phosphate from the 5'-end. The product was purified with the Qiagen PCR purification kit, and then digested with AatII. After that the fragment was purified again, and gel extracted. Following that, 16 μl of digested fragment was labelled at the 5'-HindIII restricted end, using 1 μl of [$\gamma^{32}\text{P}$]-ATP, 1 μl T4 polynucleotide kinase, 2 μl of 10x T4 polynucleotide kinase buffer, and then incubated at 37°C for 30 minutes. The labelled DNA was passed through a Micro Bio-Spin P-30 Chromatography Columns (supplier: Bio-Rad) for 4 minutes at 1000x g to clean up all unincorporated radioactivity. The flow through was collected and used in further protocols.

2.19 Electrophoretic mobility shift assay (EMSA)

Approximately 30 counts of [$\gamma^{32}\text{P}$]-ATP radiolabelled DNA fragment were mixed with 1x TNSC buffer and 12.5 $\mu\text{g/ml}$ Herring sperm DNA, in a 10 μl final volume. This mixture was incubated with 10 μl of a MarA(and/or RNAP σ^{28} or FlhDC)/1x TNSC solution for 20 minutes at 37°C. Samples were then loaded onto a 7.5% polyacrylamide gel, run at 150 V for 1-2 hours. The gel was vacuum dried, exposed overnight on a phosphor screen, and scanned

using an Amersham TYPHOON phosphoimager.

2.20 G+A ladder preparation

[$\gamma^{32}\text{P}$]-ATP radiolabelled DNA fragment was used for G+A ladder preparation. About 12 μl (100-200 ng) of this fragment was mixed with formic acid and incubated at room temperature for 2.5 minutes. The reaction was stopped with 700 μl of 100% (*v/v*) ethanol, 200 μl of 0.3 M CH_3COONa and 1 μl of GlycoBlue, precipitated at -70°C for 15 minutes and centrifuged at 13000x g for 30 minutes at 6°C . After centrifugation, supernatant was removed and pellets were dried in a SpeedVac concentrator, following resuspension in 1 M piperidine (10 M stock diluted with ddH_2O) and incubation at 90°C for 30 minutes. The reactions were stopped with 10 μl of 3M sodium acetate, 300 μl of 100% (*v/v*) ethanol and 1 μl of GlycoBlue following precipitation at -70°C for 15 minutes. The DNA was collected through centrifugation at 15000x g for 15 minutes at 6°C , followed by double wash in 70% (*v/v*) ethanol. Supernatant was removed and the pellets were dried in a SpeedVac concentrator and then resuspended in 20 μl of DNaseI blue.

2.21 *In vitro* transcription

DNA templates (pSR or pLSR, Table 2.3) were purified with ZymoPURE II Plasmid Midiprep Kit and contained promoter fragments upstream of the λ oop transcriptional terminator. For each reaction, 300-350 ng of DNA miniprep was mixed with 1x TNSC buffer, 100 $\mu\text{g}/\text{ml}^{-1}$ BSA, NTP mix (200 μM ATP/ GTP/ CTP and 10 mM UTP), and 4 μCi [$\alpha^{32}\text{P}$]-UTP in a final volume of 20 μl . This mix was then incubated with MarA, SoxS, FlhDC at indicated concentrations at 37°C for 10 minutes. *E. coli* RNA Polymerase holoenzyme, or *E. coli* RNA Polymerase core enzyme premixed with the σ^{28} -factor, were preheated at 37°C for 5 min-

utes; subsequently, 2 μ l or 4 μ l of the Polymerase, respectively, was added at timed intervals of 20 seconds to each DNA-protein mix. The reactions were stopped by adding 20 μ l of STOP solution at the same time intervals the reactions were started. Then, reactions were loaded onto a 6% denaturing sequencing gel. Electrophoresis was done in 1x TBE running buffer at 80 W for approximately 1-2 hours. The gel was dried by vacuum and exposed to a phosphor screen overnight. Images were then scanned using an Amersham TYPHOON phosphoimager.

2.22 DNaseI footprinting

[$\gamma^{32}\text{P}$]-ATP radiolabelled DNA fragments were used for DNaseI footprinting. All reactions were done at 37°C in 1x TNSC buffer. Ten to forty ng of DNA was used per footprint with 12.5 $\mu\text{g}/\text{ml}^{-1}$ of Herring Sperm DNA as a competitive inhibitor in a final volume of 20 μ l. Following this, different concentrations of proteins of interest were added and incubated with DNA for 10 minutes at 37°C. Three μ l of diluted DNase I was added at timed intervals and incubated at 37°C for 7 minutes. Reactions were stopped using 200 μ l of a DNaseI STOP solution, containing 0.3 M sodium acetate pH 7 and 10 mM EDTA. Digested DNA was then recovered by phenol-chloroform extraction and precipitated by 70% (*v/v*) ethanol and 1 μ l of GlycoBlue at -70°C, followed by centrifugation at 6°C for 45 minutes. After ethanol had been removed, reactions were dried in a SpeedVac concentrator, and 4 μ l of DNase I blue was added to each pellet.

Before loading, all samples and the G+A ladder were heated to 80°C for 2 minutes. Reactions were loaded onto a 6% denaturing sequencing gel. Electrophoresis was done in 1x TBE running buffer at 80 W for approximately 1-2 hours. The gel was dried by vacuum and exposed to a phosphor screen overnight. Images were then scanned using an Amersham TYPHOON phosphoimager.

2.23 Potassium Permanganate footprinting

Potassium permanganate (KMnO_4) is an oxidative agent that reacts with many organic molecules, including nucleic acids. In the case of nucleic acids, KMnO_4 attack only pyrimidines in single stranded regions, and the primary products of oxidation are the 5, 6-dihydro-5,6-diolpyrimidine derivatives. When low concentration of KMnO_4 is used, thymidilic acids become more sensitive to the oxidation among other pyrimidines [428]. Purine deoxyribonucleotides stays unaffected.

$[\gamma^{32}\text{P}]$ -ATP radiolabelled DNA fragments were used for KMnO_4 footprinting. All reactions for the footprint were done at 37°C in 1x TNSC buffer. For each reaction, 4-10 nM of the radiolabelled DNA were mixed with 1x TNSC buffer, $100\text{ }\mu\text{g}/\text{ml}^{-1}$ BSA, and proteins of interest in $20\text{ }\mu\text{l}$ total volume, then incubated at 37°C for 30 minutes. After, $1\text{ }\mu\text{l}$ of 200mM KMnO_4 solution was added to the sample and incubated at 37°C for 4 minutes. The reactions were stopped by adding $50\text{ }\mu\text{l}$ of STOP solution, containing 3 M $\text{NH}_4\text{CH}_3\text{CO}_2$, 0.1 mM EDTA, 1.5 M 2-mercaptoethanol, and then well mixed. One hundred and thirty μl of TE buffer was added to each reaction and extracted with $200\text{ }\mu\text{l}$ of phenol-chlorophorm. The DNA was then precipitated with 70% (v/v) ethanol and $1\text{ }\mu\text{l}$ of GlycoBlue at -70° for 15 minutes before being centrifuged for 30 minutes at maximum speed at 6° . After the removal of ethanol, the pellets were dried using a SpeedVac concentrator, and then resuspended in 1 M piperidine (10 M stock diluted with ddH_2O) and incubated at 90°C for 30 minutes. The reactions were stopped with $10\text{ }\mu\text{l}$ of 3M sodium acetate, $300\text{ }\mu\text{l}$ of 100% (v/v) ethanol and $1\text{ }\mu\text{l}$ GlycoBlue before precipitation at -70°C for 15 minutes. The DNA was collected by centrifugation at $17000\times g$ for 15 minutes at 6°C , followed by a double wash in 70% (v/v) ethanol. Supernatant was removed and the pellet was dried in a SpeedVac concentrator and after resuspended in $10\text{ }\mu\text{l}$ of DNaseI blue.

Prior to loading, all samples and the G+A ladder were heated to 80°C for 2 minutes.

Reactions were loaded onto a 6% denaturing sequencing gel. Electrophoresis was done in 1x TBE running buffer at 80 W for approximately 1-2 hours. The gel was dried by vacuum and exposed to a phosphor screen overnight. Images were then scanned using an Amersham TYPHOON phosphoimager.

2.24 MarA overexpression

MarA was purified following a protocol from Kettles et al., 2019 [341], using pET28a plasmid (Table 2.3) with *marA* cloned downstream of the IPTG-inducible T7lac promoter. The construct was used to transform *Escherichia coli* K-12 strain T7 Express (Table 2.2) for further MarA purification by liquid chromatography.

To overexpress recombinant protein, 20 ml of overnight culture was incubated at 37°C in 1 litre of LB containing 50 ug/ml of kanamycin + 1% (*v/v*) glucose with shaking. When the culture reached OD₆₀₀ of 0.6, 1 ml of 1 M IPTG was added and the culture grown for a further 3 hours. The culture was harvested by centrifugation at 2600x g for 15 minutes at 4°C. The pellet was washed with 25 ml of lysis buffer and frozen overnight at -80°C.

2.25 Purification of MarA

All subsequent steps were done at 4°C. The frozen pellet was resuspended in 40 ml of the lysis buffer; cells were sonicated using MiSonix XL2020 tip sonicator. The insoluble fraction was then collected by centrifugation at 15000x g for 1 hour; the supernatant was discarded and the pellet resuspended in 40 ml of wash buffer and centrifuged at same settings.

The pellet from a previous step was resuspended in 40 ml of denaturing buffer and centrifuged at 75000x g for 1 hour. The supernatant from the final centrifugation step was loaded onto a HisTrap 1 ml precharged Ni Sepharose High Performance column. Unbound

protein was washed from the column with Buffer A. Bound protein was then eluted with a linear gradient of Buffer B. An elution peak was seen at approximately 0.22 M imidazole; fractions were checked by SDS-PAGE and desired fractions dialysed against Buffer X + 20% (*v/v*) glycerol overnight. After dialysis, protein was concentrated to approximately 1 mg/ml using a 5,000 MWCO (molecular weight cut-off) Vivaspin 20 column. Purified protein was stored at -80 °C until required.

2.26 FlhDC overexpression

The *flhDC* operon was amplified by PCR and digested with NdeI and XhoI before ligation with pET26b plasmid (Table 2.3) downstream of the IPTG-inducible T7lac promoter. After verification by sequencing, constructs were used to transform the *Escherichia coli* K-12 strain T7 Express (Table 2.2).

To overexpress recombinant FlhDC protein complex, 20 ml of overnight were incubated at 37°C in 1 litre of LB containing 50 ug/ml of kanamycin. When the culture reached OD₆₀₀ of 0.6, 1 ml of 1 m IPTG was added and the culture grown for a further 3 hours. The culture was harvested by centrifugation at 2600x g for 15 minutes at 4°C. The pellet was washed with 25 ml of lysis buffer and frozen overnight at -80°C.

2.27 Purification of FlhDC

All subsequent steps were done at 4°C. The frozen pellet was resuspended in 40 ml of the lysis buffer; cells were sonicated using an MiSonix XL2020 tip sonicator, and then collected by centrifugation at 15000x g for 1 hour. The supernatant was separated from the solid precipitate and filtrated through 0.45 µl filter. The filtrated supernatant was injected into the AKTA prime machine and loaded onto a HiTrap 5 ml Heparin High Performance column.

The column was washed out with linear gradient of 0.05 M NaCl (Buffer A) to 1 M NaCl (Buffer B). Fractions were checked by SDS-PAGE, desired fractions were combined, diluted 4x fold in Buffer A and loaded onto pre-equilibrated HiTrap 5 ml Q High Performance anion-exchange column. The column was washed in 100 ml of Buffer A, so that FlhDC washed out in the flow-through and most of the other proteins bound to the column. Then 100 ml of FlhDC were concentrated to 4 ml and loaded onto the pre-equilibrated in Buffer C size-exclusion column (Sephacryl S-200) and let run through overnight. Fractions were checked by SDS-PAGE, desired fractions were combined and dialysed in 4L of Storage buffer.

2.28 FliA overexpression

FliA was purified following a protocol from Hollands et al., 2010 [429], using pET21a plasmid (Table 2.3) with *fliA* cloned downstream of the IPTG-inducible T7lac promoter. The construct was used to transform the *Escherichia coli* K-12 strain T7 Express (Table 2.2).

To overexpress recombinant protein, 20 ml of overnight were incubated at 37°C in 1 litre of LB with 1 ml of ampicillin + 1% glucose with shaking. When the culture reached OD₆₀₀ of 0.6, 1 ml of 1 M IPTG was added and the culture grown for a further 3 hours. The culture was harvested by centrifugation at 2600x g for 15 minutes at 4°C. The pellet was washed with 25 ml of lysis buffer and frozen overnight at -80°C. Further, FliA was purified using an adapted protocol based on Borukhov and Goldfarb prior work [430].

2.29 Purification of FliA

All subsequent steps were done at 4°C, unless specified otherwise. The frozen pellet was resuspended into 40 ml of the lysis buffer; cells were sonicated using MiSonix XL2020 tip sonicator. The cells were then collected by centrifugation at 15000x g for 1 hour; the su-

pernatant was discarded and the pellet resuspended in 40 ml of wash buffer prior to further centrifugation.

The supernatant from the previous step was discarded, and the pellet resuspended in 40 ml of denaturing buffer and centrifuged at 15000x g for 1 hour. The supernatant from the final centrifugation step was loaded onto a His Trap FF 1 ml High Performance column. Unbound protein was washed from the column with Buffer A. Bound protein was then eluted with a linear gradient of imidazole. The FliA protein has been eluted at 0 mM imidazole; fractions were checked by SDS-PAGE and 0 mM imidazole fraction has been kept and dialysed overnight in reconstruction buffer. After dialysis, protein was concentrated to 1 mg/ml using a 10,000 MWCO (molecular weight cut-off) Vivaspin 20 column. Purified protein was stored at -80 °C until required.

2.30 Statistical analysis

The statistical significance of β -galactosidase activity was tested using a one-way two-sample equal T-test analysis to understand whether there is a significant difference between the means of two groups. Significance for each time point was defined as a p-value. P-values less than or equal to 0.05 are indicated by one asterisk, p-values less than or equal to 0.01 are indicated by two asterisks, p-values less than or equal to 0.001 are indicated by three asterisks, and p-values less than or equal to 0.0001 are indicated by four asterisks. All statistical analysis was carried out using Excel and significance was defined as $p < 0.05$.

2.31 Bacterial strains and plasmid vectors

All bacterial strains used in this study are shown in Table 2.2. All plasmid vectors used in this study are shown in Table 2.3.

2.32 Oligonucleotides

Oligonucleotides were obtained from Life Technologies. All oligonucleotides used in this study are shown in Table 2.4.

2.33 List of fragments

All fragments used in this study were generated via PCR, and are shown in Table 2.5.

Table 2.2: *Escherichia coli* K-12 strains used in this study

Strain	Description	Source
JCB387	Δ nirB Δ lac	Typas and Hengge (2006)[431]
T7 Express	$fhuA2$ $lacZ::T7$ $gene1$ $[lon]$ $ompT$ gal $sulA11$ $R(mcr73::miniTn10-Tet^S)_2$ $[dcm]$ $R(zgb-210::Tn10-Tet^S)$ $endA1 \Delta(mcrCmrr)114::IS10$	New England Biolabs

Table 2.3: Plasmids vectors used in this study

Plasmid	Size	Description	Source
pRW50	16.9 kb	Encodes Tet ^R ; RK2 origin. Features <i>EcoRI-HindIII</i> restriction sites sandwiching a cloning site upstream of a <i>lacZ</i> fusion. Used for β -galactosidase assays	Lodge et al. (1992), [432].
pSR	2.6 kb	Encodes Amp ^R , derived from pBR322; ColE1 origin. Features <i>EcoRI-HindIII</i> restriction sites sandwiching a cloning site upstream of a λ oop terminator site. Used for <i>in vitro</i> transcription.	Kolb et al. (1995), [139].
pLSR	2.6 kb	Encodes Amp ^R , derived from pSR; ColE1 origin. Derivative of pSR carrying two λ oop transcription terminators. Used for <i>in vitro</i> transcription.	Busby and Elrobb (2002), [433].
pUC19	2.7 kb	Encodes Amp ^R . Protein overexpression vector using the T7lac promoter; used for FlhDC overexpression.	New England Biolabs.
pJ203	2.9 kb	Encodes Chlor ^R ; pUC origin. Protein overexpression vector; used for MarA overexpression for β -galactosidase assays.	ATUM.
pET21a	5.4 kb	Encodes Amp ^R . Protein overexpression vector using the T7lac promoter; features both C-terminal and N-terminal His tags. Used for purification of FliA.	Novagen.

Plasmid	Size	Description	Source
pET26b	5.4 kb	Encodes Kan ^R . Protein overexpression vector using the T7lac promoter; features C-terminal His tag. Used for purification of FlhDC.	Novagen.
pET28a	5.4 kb	Encodes Kan ^R . Protein overexpression vector using the T7lac promoter; features both C-terminal and N-terminal His tags, an internal T7 tag, and a thrombin cleavage site. Used for purification of MarA.	Novagen.

Table 2.4: Oligonucleotides used in this study

Name of a primer (F-forward; R-reverse)	Sequence (5' → 3'); bold text indicates restriction sites
Oligonucleotides used for sequencing of candidate plasmid constructs	
pRW50_F	GTTCTCGCAAGGACGAGAATTTC
pRW50_R	AATCTTCACGCTTGAGATAC
pSR_F	CCATATATCAGGGTTATTGTCTC
pSR_R	CATCACCGAAACGCGCGAGG
pET_F	GATTATGCGGCCGTGTAC
pET_R	ATGCGTCCGGCGTAG
Oligonucleotides used to generate a control for EMSA	
<i>estA</i> _F	GGCTGCG GAATTC TAACATGATGCAACTCACAAAAAAAT AAAAAAATTGCAAAATCCGTTTAACTAATCT
<i>estA</i> _R	GCCCG AAGCTT CATGTTACCTCCCGTCATGTTGTTTCAC GGATATTTGAGATTAGTTAAACGGATTTTG
Oligonucleotides used for amplification of the flagellar promoter regions	
<i>flgA</i> _F	GGCTG GAATTC CTGCCATCAGCTTAAATGCC
<i>flgA</i> _R	GCCCG AAGCTT CATTTTCGCCCCCAGCCATTTC
<i>flgA</i> _F(truncated)	GGCTG GAATTC ATCGGCGGAATAAACGC
<i>flgB</i> _F	GGCTG GAATTC CGCCCCCAGCCATTTC
<i>flgB</i> _R	GCCCG AAGCTT CATATCTCCTCCGCAG
<i>flgB</i> _F(truncated)	GGCTG GAATTC GCATAAATAGCGACCC
<i>flgJ</i> _F	GGCTG GAATTC CAGACTGTGGTTACTCCACAAAC
<i>flgJ</i> _R	GCCCG AAGCTT CATCAGATGATTTCCAG
<i>flgK</i> _F	GGCTG GAATTC CGCAAACCTACCAACATG

Name of a primer (F-forward; R-reverse)	Sequence (5' → 3'); bold text indicates restriction sites
<i>flgK</i> _R	GCCCGA AGCTT CATGGAGGTTCCCTTATAAGCC
<i>flgM</i> _F	GGCTG GAA TT CGG CGTTGTTGATGCAGATG
<i>flgM</i> _R	GCCCGA AGCTT CATGGTTTATTCCTCATTGAGGGC
Megaprimers with mutated -10 regions in the flagellar promoters (point mutations are underlined)	
<i>flgA</i> ::(-10) σ^{28} mut_F	GGTCGCTATTTATGCG <u>CT</u> TGATGGTCATTGCG
<i>flgA</i> ::(-10) σ^{70} mut_F	GTCATTGCGGACAGG <u>CC</u> CAATTCACGTTGTAG
<i>flgB</i> ::(-10) σ^{28} mut_F	TTGCGTTTATTCCGCG <u>C</u> ATAACGCGCGCGTAA
<i>flgB</i> ::(-10) σ^{70} _F	GGCTG GAA TT CCG CCCCCAGCCATTTCTACAACGTGA ATTGTACCTGTCCGCAATGACCATCAACGGCATAAATA GCGACCCATTTTGCGTTTATTCCGCCGATAACGCCGCG TAAAGG
<i>flgB</i> ::(-10) σ^{70} mut_R	GCCCGA AGCTT CATATCTCCTCCGCAGGTATCAAAAT TCTGCCATCAGCTG <u>G</u> AATGCCTTTACGCGCGCGTTATC GGCGGAATAAACGCAAAATGGGTC
Oligonucleotides used for generation of constructs for FlhDC overexpression and purification	
<i>flhD</i> _F	GGCTG CA TATGGGAATAATGCATACCTCCG
<i>flhC</i> _R	GCCCG CTCGAG TTAAACAGCCTGTACTCTCTG

Table 2.5: Sequences of DNA fragments used in this study

Name of a DNA fragment	Sequence (5' → 3'); bold text indicates restriction sites, mutations are underlined
<i>flgA</i>	GGCTG GAATTC CCTGCCATCAGCTTAAATGCCTTTACG CGCGCGTTATCGGCGGAATAAACGCAAAATGGGTCGC TATTTATGCCGTTGATGGTCATTGCGGACAGGTACAAT TCACGTTGTAGAAATGGCTGGGGGCGAAAATGA AAGC TT CGGGC
<i>flgA</i> truncated	GGCTG GAATTC ATCGGCGGAATAAACGCAAAATGGG TCGCTATTTATGCCGTTGATGGTCATTGCGGACAGGT ACAATTCACGTTGTAGAAATGGCTGGGGGCGAAAATG AAGCTT CGGGC
<i>flgA::(-10)σ²⁸mut</i>	GGCTG GAATTC CCTGCCATCAGCTTAAATGCCTTTACG CGCGCGTTATCGGCGGAATAAACGCAAAATGGGTCGC TATTTATGCG <u>CT</u> TGATGGTCATTGCGGACAGGTACAA TTCACGTTGTAGAAATGGCTGGGGGCGAAAATGA AAG CTT CGGGC
<i>flgA::(-10)σ⁷⁰mut</i>	GGCTG GAATTC CCTGCCATCAGCTTAAATGCCTTTACG CGCGCGTTATCGGCGGAATAAACGCAAAATGGGTCGC TATTTATGCCGTTGATGGTCATTGCGGACAGG <u>CC</u> CAA TTCACGTTGTAGAAATGGCTGGGGGCGAAAATGA AAG CTT CGGGC

Name of a DNA fragment	Sequence (5' → 3'); bold text indicates restriction sites, mutations are underlined
<i>flgB</i>	GGCTG GAATT CCGCCCCCAGCCATTTCTACAACGTG AATTGTACCTGTCCGCAATGACCATCAACGGCATAAA TAGCGACCCATTTTGC GTTTATTCCGCCGATAACGCG CGCGTAAAGGCATTTAAGCTGATGGCAGAATTTTGAT ACCTGCGGAGGAGATATGA AGCTT CGGGC
<i>flgB</i> truncated	GGCTG GAATT CGCAATGACCATCAACGGCATAAA TAGCGACCCATTTTGC GTTTATTCCGCCGATAACGCG CGCGTAAAGGCATTTAAGCTGATGGCAGAATTTTGAT ACCTGCGGAGGAGATATGA AGCTT CGGGC
<i>flgB::(-10)σ²⁸mut_F</i>	GGCTG GAATT CCGCCCCCAGCCATTTCTACAACGTG AATTGTACCTGTCCGCAATGACCATCAACGGCATAAA TAGCGACCCATTTTGC GTTTATTCCGCGCATAACGCG CGCGTAAAGGCATTTAAGCTGATGGCAGAATTTTGAT ACCTGCGGAGGAGATATGA AGCTT CGGGC
<i>flgB::(-10)σ⁷⁰mut_F</i>	GGCTG GAATT CCGCCCCCAGCCATTTCTACAACGTG AATTGTACCTGTCCGCAATGACCATCAACGGCATAAA TAGCGACCCATTTTGC GTTTATTCCGCCGATAACGCG CGCGCCAAGGCATTTAAGCTGATGGCAGAATTTTGAT ACCTGCGGAGGAGATATGA AGCTT CGGGC

Name of a DNA fragment	Sequence (5' → 3'); bold text indicates restriction sites, mutations are underlined
<i>flgJ</i>	GGCTG GAA TTCC AGACTGTGGT TACTCC ACAAACGC AGATCGATT TACGCC AGAGCGGCGG TTCGCTG CAAAG CGTACG TTCCAGCGCC AGCCTCAATAACGTGGTGCGC GCGCTCAATGCGCTGGGCGCTACGCCGATGGATCTGA TGTCCATACTGCAATCAATGCAAAGTGCGGGATGTCT GCGGGCAA AACTGGAA ATCATCTGATGA AAGCTT CGGGC
<i>flgK</i>	GGCTG GAA TTCC GCAA ACTCACCA ACATGATTCAGC AGATGAAATCGATAAGCGACAAGGTGAGCAAAACCT ACAGTATGAACATTGATAATCTGTTCTGAATAACTC AAGTCCGGCGGGTCGCTGCCGATAATACTCTGTAAT TGAAGGCTTATAAGGAACCTCCATGA AAGCTT CGGGC
<i>flgM</i>	GGCTG GAA TTCC GGCGTTGTTGATGCAGATGGGAATA TTCTTATAAACCTGTAAGCTGT TAAAGATTACCCGT CCCTTGCCGATAAATAAGCAACACATGATAAAAGCG CCCTCAATGAGGAATAAACCATGA AAGCTT CGGGC

Chapter 3

Targets for MarA binding in flagellar gene cluster

3.1 Introduction

Since MarA is a repressor of curli and biofilm formation, the previous search for potential *marboxes* was reexamined for other genes related to biofilm or motility [434]. Computer-based screen for possible MarA targets in the *E. coli* chromosome suggested targets in the regulatory regions of genes encoding flagellar components. Surprisingly, we found MarA binding sites (*marbox* consensus represented on Figure 3.1A) in the promoter region of the *flgKLO* operon, and the sheared intergenic region of the divergent *flgAMN* and *flgBCDEFGHIJ* operons (a schematic of those operons is shown on Figure 3.1B). We also found *marboxes* within these operons upstream of the *flgJ* and *flgM* genes.

Within the flagellar cascade, the *flgAMN* is a Class II operon, regulated by FlhDC and RNAP σ^{70} . The encoded proteins are: FlgA a chaperon required for the assembly of the flagellar P ring [435], FlgN cytoplasmic, substrate-specific chaperone of the flagellar export system [436]), and flagellar anti- σ^{28} factor FlgM (FlgM prevents transcription of Class III operons that are required for the completion of flagellar [155, 90]).

The *flgBCDEFGHIJ* operon also belongs to Class II and encodes structural subunits for the hook and upper part of the flagellar basal body. For instance, FlgB, FlgC, FlgF and FlgG comprise the rod of the flagellar basal body [378], FlgD is a chaperon for FlgE hook assembly [437, 438], FlgH and FlgI make L- and P-rings, respectively [439, 440]. FlgJ is a peptidoglycan hydrolase required for flagella rod assembly [441]. The *flgKLO* operon belongs to Class III and encodes hook-filament junction proteins FlgK and FlgL [442] and small RNA *flgO* [390]. It is also important to notice that *flgBCDEFGHIJ* and *flgKLO* operons can be transcribed as a unit *flgBCDEFGHIJKLO* from FlhDC-dependent *flgB* σ^{70} promoter. Thus, together, operons *flgAMN*, *flgBCDEFGHIJKLO* encode 25% of the genes involved in flagellar biosynthesis [90]. Thus, we hypothesised that MarA may be involved in some stages of flagellar biosynthesis.

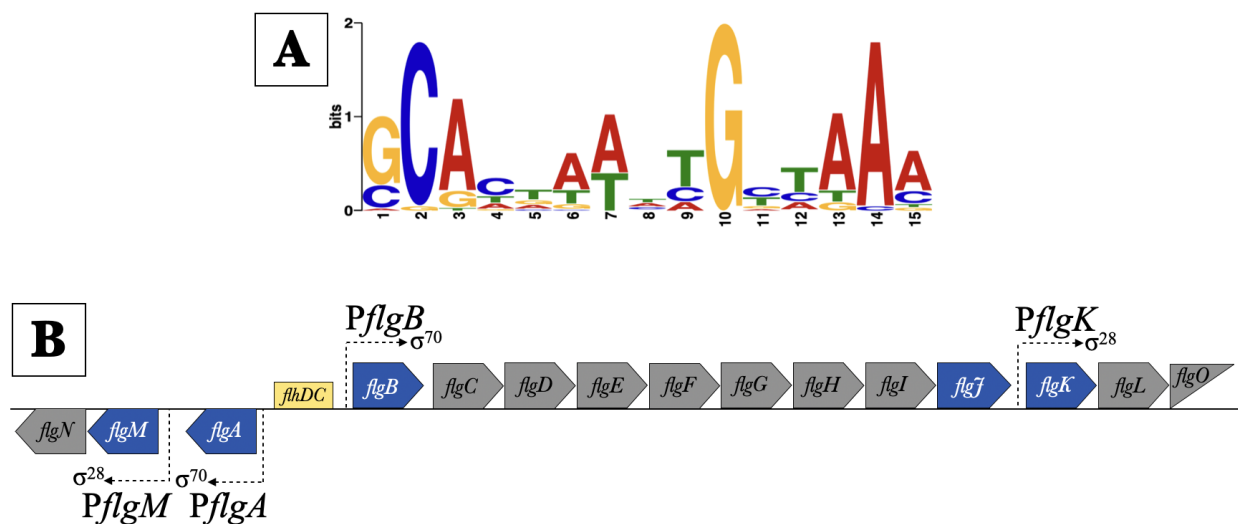


Figure 3.1: Putative marboxes in *flgAMN*, *flgBCDEFGHIJ* and *flgKLO* flagellum gene clusters.

A. The *marbox* consensus sequence generated by a ChIP-seq experiment [443].

B. The organisation of flagellar operons *flgAMN*, *flgBCDEFGHIJ* and *flgKLO* based on the EcoCyc database [19]. Genes with promoter regions containing a putative *marbox* are highlighted in blue. Binding site for FlhDC is shown as a yellow rectangle.

3.2 In silico analysis of MarA binding in flagellar gene cluster

According to the EcoCyc database, both *flgA* and *flgB* promoters are σ^{70} -dependent, and activated by FlhDC. The activator binds between -76 and -32 bp upstream of *flgA* TSS. This corresponds to -75 and -33 bp upstream of the divergent *flgB* TSS. The putative *marbox* sequence locates upstream of the *flgA* and *flgB* -35 elements, at position -68 and -54 in forward orientation, and -53 and -39 in reverse orientation, respectively. The *flgK* and *flgM* promoters are σ^{28} -dependent. Their putative *marboxes* locates between -72 and -86 bp upstream of *flgK* TSS, and between -25 and -35 bp upstream of *flgM* ATG. The *flgJ* gene doesn't have an identified promoter, its putative *marbox* locates between -38 and -51 bp upstream of *flgJ* ATG. Sequences of the promoter regions with putative *marboxes* are shown on Figure 3.2.

3.3 EMSA analysis of MarA binding in flagellar gene cluster

We made 133 bp, 154 bp, 200 bp, 158 bp and 120 bp fragments in length carrying the DNA sequence upstream of *flgA*, *flgB*, *flgJ*, *flgK* and *flgM*, respectively (Table 2.5). The ... fragment was used as a negative control. Fragments were first used for EMSA (electrophoretic mobility shift assay) with increasing concentrations of MarA (from 0.25 to 1 μ M), Figure 3.3. With increased concentrations of MarA, MarA:DNA complexes are formed with all five sequences tested, and less free DNA was observed. No binding of MarA to a control DNA fragment was detected. This confirms that MarA binds to the putative *marboxes* within *flgA/B*, *flgJ*, *flgK* and *flgM* regulatory regions.

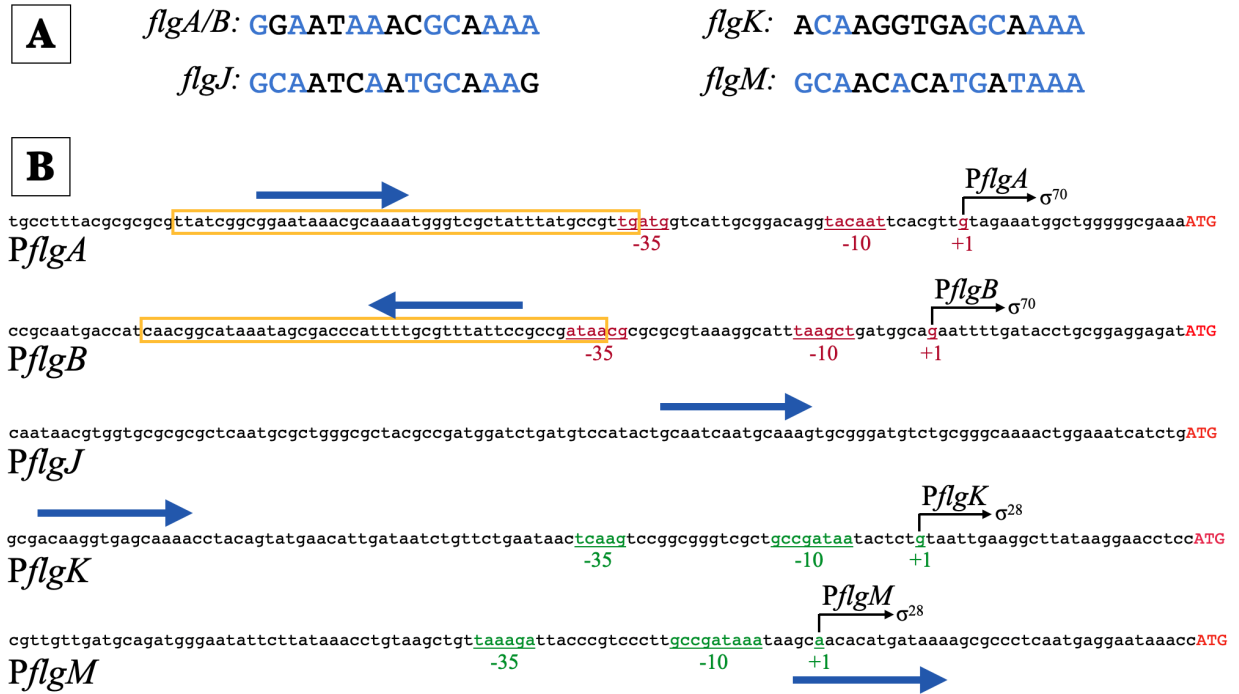


Figure 3.2: Annotations of putative *marboxes* in the DNA sequence of flagellar genes.
A. *Marbox* sequences of *flgA/flgB* intergenic region and *flgJ*, *flgK*, *flgM* promoters. Matches to the *marbox* consensus are in blue.
B. Annotations of the *PflgA*, *PflgB*, *PflgJ*, *PflgK*, *PflgM* regulatory regions. FlhDC binding sites are shown in yellow boxes, *marboxes* and their directions are shown in blue arrows, σ^{70} -binding sites are shown in red and underlined, σ^{28} -binding sites are shown in green and underlined. Transcription start sites are labeled as +1, in red or green and underlined for σ^{70} or σ^{28} , respectively. Start codons are shown in red capital letters

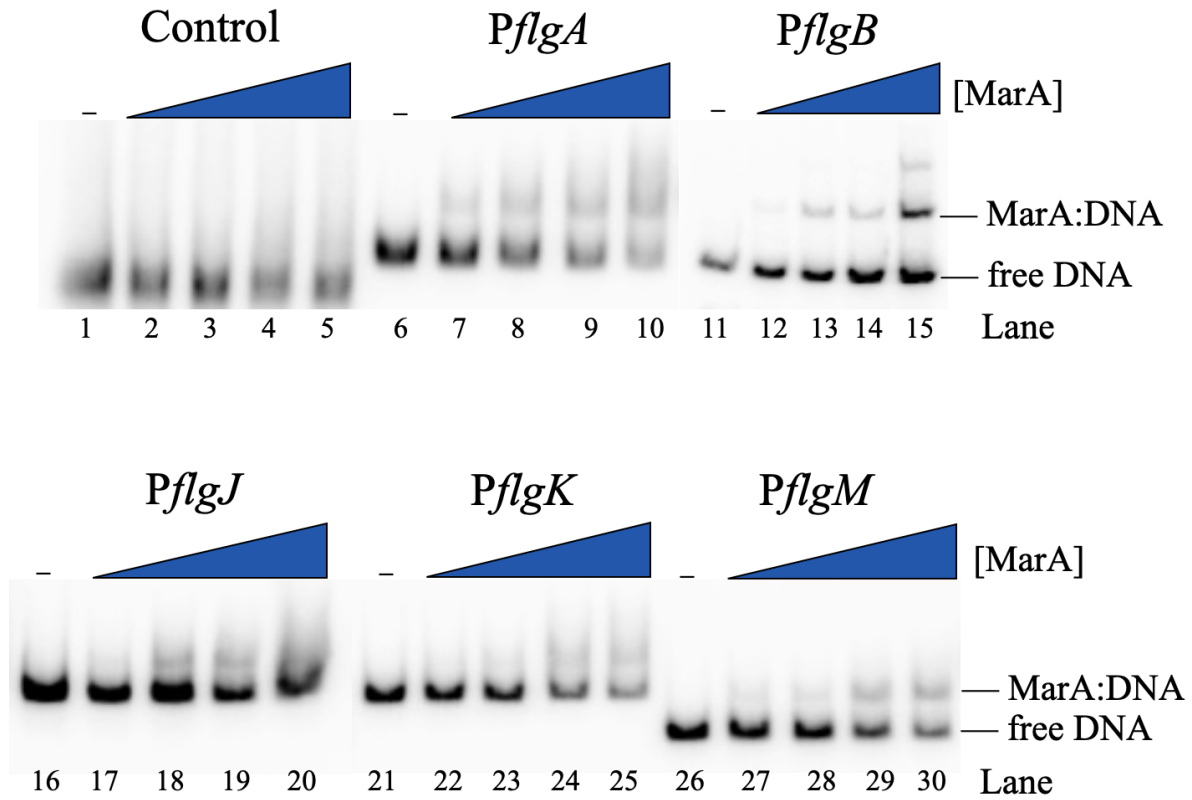


Figure 3.3: Binding of MarA to the *PflgA*, *PflgB*, *PflgJ*, *PflgK*, *PflgM* putative *marboxes* in EMSA.

The EMSA assays were carried out with the *PflgA*, *PflgB*, *PflgJ*, *PflgK*, *PflgM* derivatives. Images show the separation of the MarA:DNA complexes on the 6% polyacrylamide gels. Increasing concentrations of MarA (0.25 μ M – 0.5 μ M – 0.75 μ M - 1 μ M) represented by blue triangles. MarA:DNA complexes and free DNA are indicated.

PestA is used as a negative control fragment that contains no *marbox*.

3.4 EMSA analysis of MarA and FlhDC binding at *flgA/B* intergenic region

According to the literature, FlhDC binding is crucial for the activation of the *flgA* and *flgB* σ^{70} -dependent promoters [90]. As mentioned in the previous section, the FlhDC binding site between *flgA* and *flgB* overlaps the *marbox*. We wondered if MarA may impact FlhDC binding to DNA.

To investigate the effect of MarA on FlhDC binding, the 154 bp *flgB* promoter fragment was used in a further EMSA. The fragment has both the FlhDC binding site and the *marbox*. The *flgB* fragment was incubated with increasing concentrations of FlhDC (from 0.05 to 0.25 μ M) and increasing concentrations of MarA (ranging from 0.5 to 2 μ M) separately and together (Figure 3.4). As MarA concentrations increased, more MarA:DNA (lanes 1-5) complexes were observed, along with a decrease in free DNA. Note that MarA has a tendency to form non-specific protein-DNA aggregates that get stuck in the wells of the gel. FlhDC forms a single complex (lanes 6-10). However, when MarA concentrations are increased, in the presence of FlhDC, the amount of the FlhDC:DNA complex decreases and the increasing amount of MarA:DNA complex is observed (lanes 11-13). This indicates competitive binding between MarA and FlhDC for the overlapping binding sites.

3.5 Measurement of *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* promoter activity with MarA and FlhDC

To understand MarA impact on the expression of *flgA*, *flgB*, *flgJ*, *flgK* and *flgM*, the DNA fragments were cloned upstream of the *lacZ* gene in reporter plasmid pRW50. These constructs, along with pJ203 plasmid, expressing MarA, or empty pJ203, were used to transform *E. coli* JCB387 (Figure 3.5A). Activity of β -galactosidase was then measured in each strain

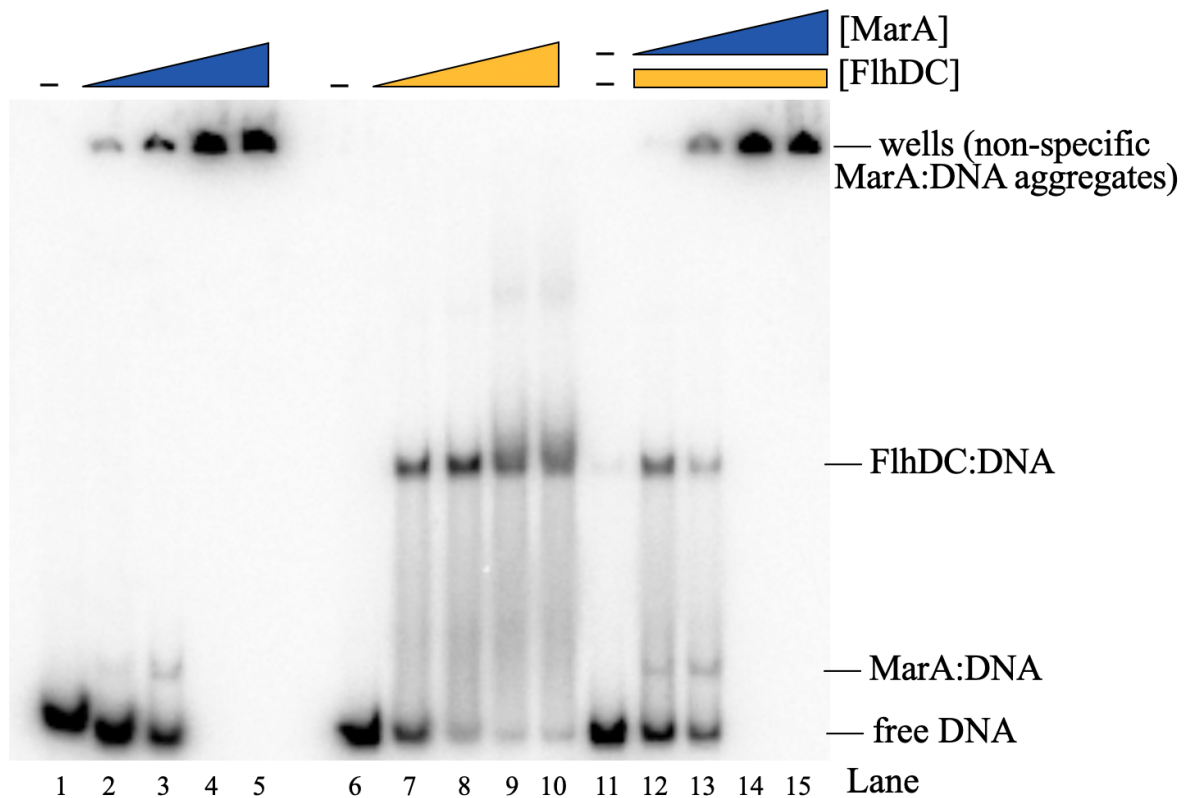


Figure 3.4: Binding of MarA and FlhDC to the *flgA/B* intergenic region in EMSA. The EMSA assays were carried out with the P*flgB* derivative. Images show the separation of the MarA:DNA and FlhDC:DNA complexes on the 6% polyacrylamide gels. Increasing concentrations of MarA (0.5 μ M – 1 μ M – 1.5 μ M - 2 μ M) are represented by blue triangles. Increasing concentrations of FlhDC (0.05 μ M – 0.1 μ M – 0.2 μ M - 0.25 μ M) are represented by yellow triangles. MarA:DNA complexes and free DNA are indicated. *PestA* is used as a negative control fragment that contains no *marbox*. Experiment was repeated more than three times with consistent results.

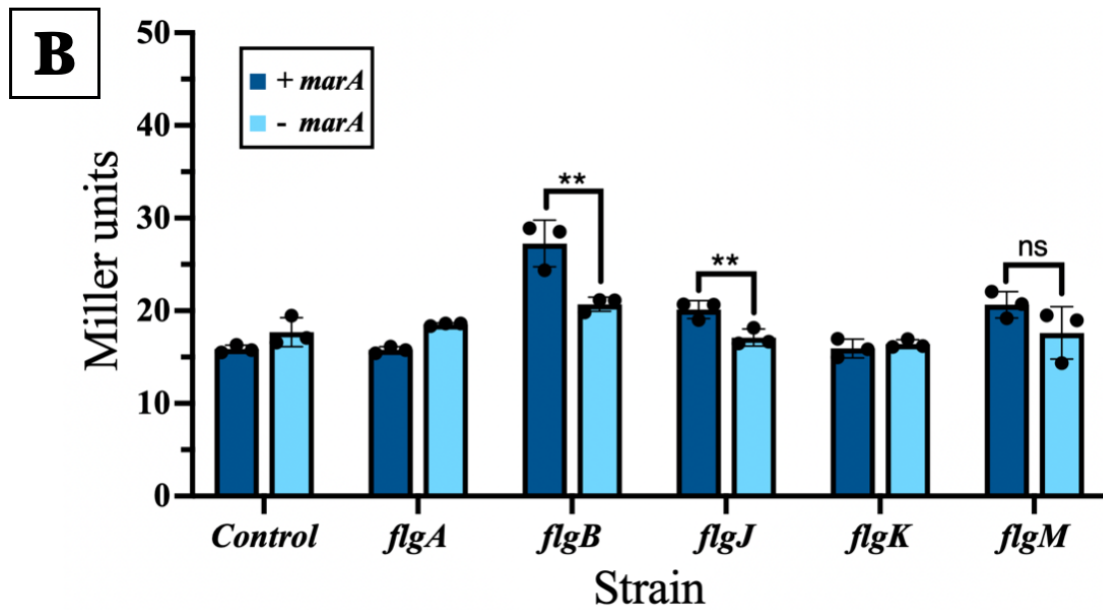
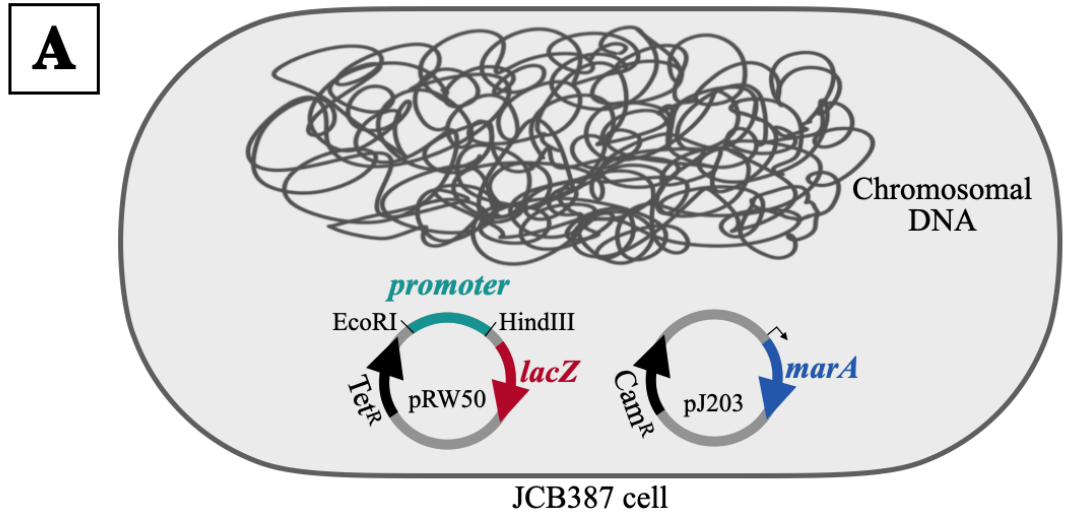


Figure 3.5: Regulation of the *flgA*, *flgB*, *flgJ*, *flgK*, and *flgM* regulatory regions *in vivo* by MarA.

A. Schematic of *E. coli* JCB387 cell and plasmids used in LacZ assay.

B. Expression of *lacZ* gene driven by *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* promoters derivatives assessed by β -galactosidase assay with MarA expressed from pJ203 plasmid.

Assays were done using lysates of *E. coli* JCB387 grown to mid-log phase ($OD_{650}=0.3-0.6$). Standard deviation is shown for three biological replicates. A two-tailed two sample equal T-test was calculated in excel using the promoter activities, showing a statistically significant results (for *flgB* and *flgJ* $p \leq 0.01$).

strain (Figure 3.5B). There was no significant difference detected upon MarA expression between strains carrying *flgA* and *flgK* promoter derivatives ($p=0.77891$, $p=0.92056$, comparing MarA and no MarA controls against each strain respectively). However, there was a statistically significant difference, due to MarA expression in β -galactosidase activity from *flgB*, *flgJ*, *flgM* ($p = 0.00151$, 0.00213 , 0.00499 , respectively). Activity of the *flgB* and *flgJ* promoters are slightly increased in the presence of MarA ($p = 0.00628$ and 0.00855 , respectively).

According to literature [90], the *flgA* σ^{70} and *flgB* σ^{70} promoters require the FlhDC transcriptional activator. Consequently, we constructed a pUC19 plasmid, with the *flhDC* operon, that was also used to transform *E. coli* JCB387 strains from previous LacZ assay (Figure 3.6A). Activity of β -galactosidase was then measured in both the presence and absence of MarA with FlhDC present in all strains (Figure 3.6B).

MarA significantly increases the activity of the *flgA* and *flgB* promoters ($p=0.00003$, $p=0.00032$, respectively), suggesting that MarA can play a role in enhancement of their activation by FlhDC even though, in the previous section, was shown that both transcriptional activators compete for the same binding site *in vitro*. This implies MarA may activate promoters other than the FlhDC dependent *flgA* and *flgB* promoters. Additionally, MarA has a slight activation effect on the *flgK* ($p=0.04157$) and *flgM* ($p=0.00208$) promoters, while it does not affect the regulatory region of *flgJ* ($p=0.06696$) in the presence of FlhDC.

3.6 Discussion

In this chapter, we aimed to understand if the promoter regions of the flagellar genes *flgA*, *flgB*, *flgJ*, *flgK*, and *flgM* are targets of MarA, following the discovery of putative *marboxes* within these regions. Through EMSA analysis, we confirmed that MarA binds to all the targets (Figure 3.3). Using *in vivo* experiments, we demonstrated that MarA alone can

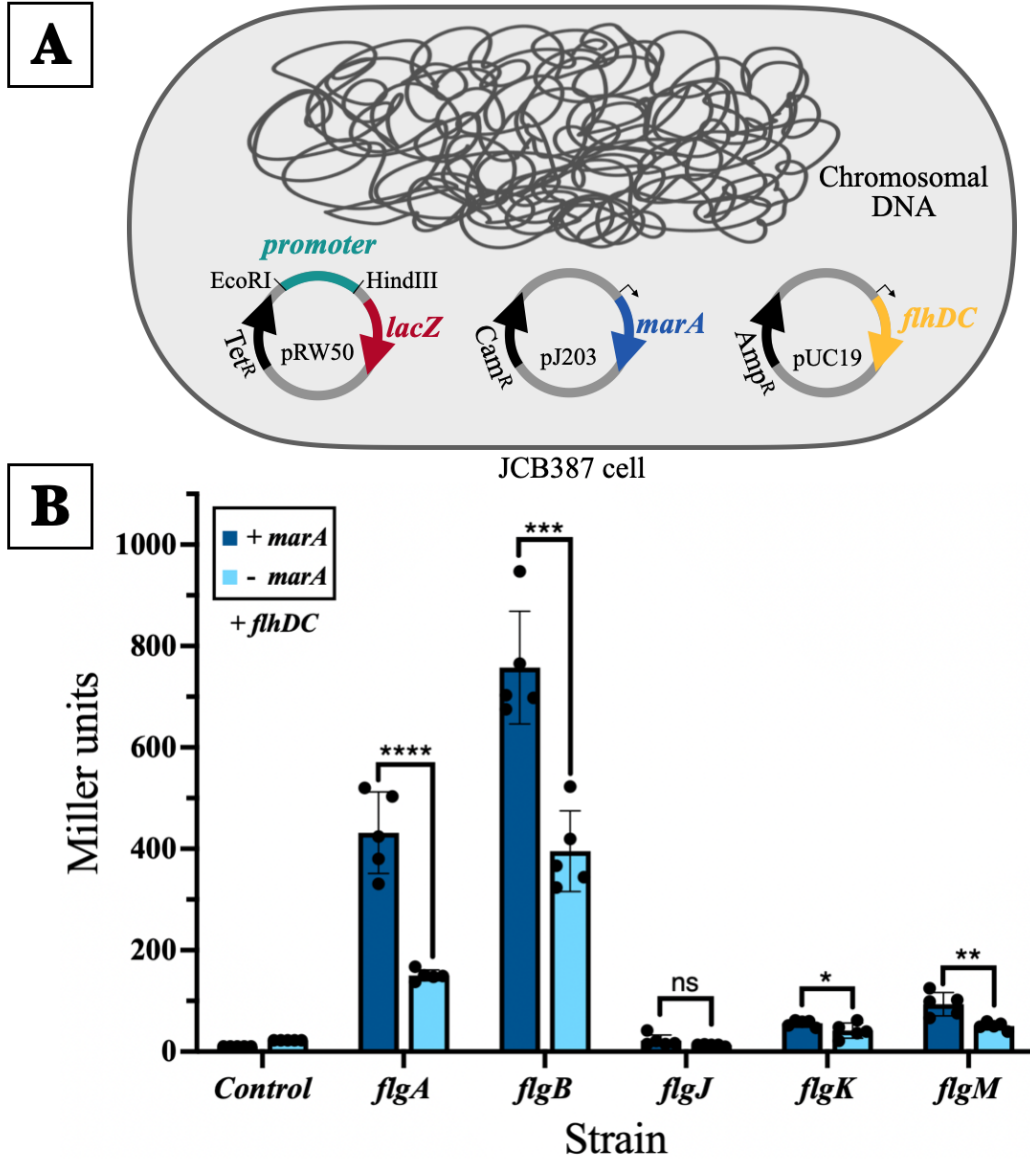


Figure 3.6: Regulation of the *flgA*, *flgB*, *flgJ*, *flgK*, and *flgM* regulatory regions *in vivo* by MarA and FlhDC.

A. Schematic of *E. coli* JCB387 cell and plasmids used in LacZ assay.

B. A. Expression of *lacZ* gene driven by *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* promoters derivatives assessed by β -galactosidase assay with MarA expressed from pJ203 plasmid and FlhDC expressed from pUC19 (FlhDC expressed in all strains).

Assays were done using lysates of *E. coli* JCB387 grown to mid-log phase (OD₆₅₀=0.3-0.6). Standard deviation is shown for three biological replicates. A one-tailed two sample equal T-test was calculated in excel using the promoter activities, showing a statistically significant results (for *flgA* $p \leq 0.0001$, for *flgB* $p \leq 0.001$, for *flgK* $p \leq 0.05$ and for *flgM* $p \leq 0.01$).

activate *flgB* and *flgJ* promoters, albeit only by a small amount. (Figure 3.6A). This occurs indirectly of FlhDC expression. Conversely, MarA substantially increased the activity of the *flgA* and *flgB* promoters in the presence of FlhDC activator (Figure 3.6B), despite their competitive interaction for the binding site observed *in vitro* (Figure 3.4).

Transcriptional factor MarA has an extensive regulon that includes genes involved in the expression of efflux pumps, porins, DNA repair, lipid biosynthesis, and other processes critical for the bacterial cell to survive under high antibiotic stress [288, 423, 443]. Additionally, evidence supports MarA's role in motility and biofilm formation.

The role of MarA as a regulator of biofilm formation is enhanced by the effects of sodium salicylate, a well-known inducer of MarA [308, 444, 446, 426]. Sodium salicylate cause a variety of morphological and physiological changes in bacteria by primarily inducing an intrinsic multiple antibiotic resistance phenotype through the activation of the *marRAB* operon, which in turn stimulates MarA production [445, 446]. Salicylic acid also acts as a strong biofilm inhibitor, affecting biofilm formation through multiple pathways [426]. Notably, while sodium salicylate does not affect the planktonic growth of *E. coli*, it inhibits cell adhesion, affects oxidative stress and increase indole production - the quorum sensing molecule, that inhibits the biofilm growth in many bacterial species [447, 448]. Biofilms treated with salicylate appear thicker, featuring a monolayer of dispersed cells and a reduced amount of polysaccharide matrix [447]. This layer of dispersed cells could potentially be caused by the acquisition of motility in bacterial cells, possibly through salicylate-induced activation of flagellar biosynthesis mediated by MarA.

The transcription factor MarA itself acts as a repressor of many genes involved in biofilm formation. MarA represses the *fimA* and *fimB* genes, crucial for the expression of type 1 fimbriae, essential for adhesion to surfaces which is the initial step in biofilm formation in UPEC [446]. In other *E. coli* strains, MarA suppresses transcription of the outer membrane protein OmpA, which plays a role in biofilm formation and cellulose production [444, 419].

Taking into account all these evidence of salicylate and its upregulated factor MarA acting as repressors of biofilms, it was surprisingly to find that in *S. Typhimurium*, MarA acts as a repressor of flagellar formation [346]. Here, MarA and its homologous directly represses the transcription of the *flhDC* operon and also inhibits its expression post-transcriptionally. This operon encodes the FlhDC master regulator, which stands on top in the flagellar biosynthesis cascade. Furthermore, MarA along with its homologous AraC-family transcription factor SoxS, Rob, and RamA, reduce the production of the *fliC* gene, which encodes the flagellin subunits - building blocks of flagellar filament [346].

Chapter 4

Identification of new promoters in and
upstream of the *flgAMN* and
flgBCDEFGHIJ/KL operons and the
impact of MarA

4.1 Introduction

Flagellum biosynthesis is a highly regulated process that requires the activation of flagellar genes in a timed manner, involving interplay between RNAP σ^{70} , RNAP σ^{28} and FlhDC. The *flhDC* operon is activated first and this is influenced by environmental conditions. The best known activators of this operon are histone-like nucleoid structuring protein H-NS [402], quorum-sensing regulator QseB [403], cAMP receptor protein cAMP-CRP [404] as well as IS-elements and transposons [90, 449]. The cognate product of this operon is the FlhDC flagellar master regulator, which activates the whole downstream cascade of flagellar biosynthesis. Together with RNAP σ^{70} , FlhDC directly activates Class II flagellar operons: *flgAMN*, *flgBCDEFGHIJ/KL*, *flgBAE*, *fliAZY*, *fliE*, *fliFGHIJK*, *fliDST* and *fliLMNOPQR* [383, 145, 384, 385, 140, 90]. These operons encode structural components of the flagellar basal body as well as regulatory factors and export apparatus. The *fliA* and *flgM* genes encode σ^{28} (FliA), and the anti- σ^{28} (FlgM) factors, respectively [145, 386]. In complex with RNAP core, the σ^{28} factor allows transcription initiation from the Class III promoters controlling *flgKL*, *fliDST*, *flgMN*, *fliC*, *tar-tap-cheRBYZ*, *motAB-cheAW*, and *fliLMNOPQR*.

Several observations have challenged the view of clean separation between Class II and Class III promoters in the flagellar cascade. Some regulatory regions of flagellar operons, such as those of *fliDST* and *fliLMNOPQR*, contain both Class II and Class III promoters regulated by FlhDC and RNAP σ^{70} or σ^{28} [90]. The σ^{28} factor can also direct RNAP to initiate transcription from its own operon, *fliAZY*. Additional σ^{28} -dependent promoters were found by mathematical predictions (quantitative blueprint), and ChIP-seq, upstream of the *fliE* and *fliFGHIJK* operons, that are known to be regulated by σ^{70} [389, 90]. However, in qRT-PCR experiments, in the conditions tested, all transcription came from FlhDC-dependent σ^{70} -dependent promoters in the same regulatory regions [90].

Thus, there are only 5 operons (*flgAMN*, *flgBCDEFGHIJ/KL*, *flgBAE*, *fliE*, *fliFGHIJK*)

that are solely regulated in a Class II manner by σ^{70} and FlhDC. Similarly, only 3 operons (*fliC*, *tar-tap-cheRBYZ* and *motAB-cheAW*) are regulated in a Class III manner by σ^{28} . Most Class III operons encode subunits for the completion of the flagellum, such as motor subunits, regulatory factors for chemotaxis, and the structural component of the filament, flagellin. Thus, it has been accepted that the division between Class II and Class III operons is divided in space and time by FlhDC, σ^{28} (FliA) and the cognate anti- σ^{28} (FlgM) [450].

In some cases, Class III promoters have been found within operons regulated from an upstream Class II promoter. For example, the Class II *flgBCDEFGHIJ/KL* operon contains a σ^{28} -dependent promoter upstream *flgKL*. Hence, in addition to being transcribed from the σ^{70} -dependent *flgB* promoter, *flgKL* can be transcribed from its own σ^{28} -dependent Class III promoter. Similarly, the Class III *flgMN* operon, can also be transcribed from a σ^{70} -dependent promoter upstream of *flgA*, as a part of the *flgAMN* operon [90]. Dual regulation of *flgMN* can also fine-tune the activity of σ^{28} to coordinate flagellar biosynthesis via the anti σ^{28} factor FlgM. Also, the FlgN chaperone can assist folding and transport of the FlgK junction protein during flagellar repair. In studies on *Salmonella*, Class III operons, *flgKL* and *fliDST*, are required for repair of shredded flagella [451]. So that, it has been suggested that additional transcription of these genes from Class II promoters is utilised for building a new flagellum, whilst Class III promoters are used for repairing damaged ones.

In Chapter 3, we found that MarA activates transcription from the *flgB* and *flgJ* regulatory region in the absence of FlhDC *in vivo*. Upon FlhDC expression, MarA could activate *flgA* and *flgB* to much higher extend compared to activity of those promoters in the absence of FlhDC. However, EMSA experiments showed that FlhDC and MarA compete for overlapping binding sites upstream of *flgA* and *flgB*, indicating no synergy between these two activators *in vitro*. Hence, MarA may act independently of FlhDC. Despite binding MarA in EMSA, *flgJ*, *flgK* and *flgM* regulatory regions were not subjected to regulation by MarA when FlhDC was expressed. In the experiments described in this chapter, new promoters

were identified in the intergenic regulatory regions in and upstream of the *flgAMN* and *flgBCDEFGHIJ/KL* operons. Regulation of these promoters by MarA is also tested.

4.2 Identification of new σ^{28} -dependent promoters regions upstream and within *flgAMN* and *flgBCDEFGHIJ/KL* operons

To further investigate the regulatory regions of the *flgA*, *flgB*, *flgJ*, *flgK*, and *flgM* genes, *in vitro* transcription was done using either RNAP σ^{70} or RNAP σ^{28} . The regulatory regions of *flgA*, *flgB*, *flgJ*, *flgK*, or *flgM* were cloned in plasmid pSR, upstream of the phage-derived transcription terminator *loop*. Increasing concentrations of MarA were added to each pSR plasmid derivative. Either RNAP σ^{70} or RNAP σ^{28} was then added to initiate transcription. The [α - ^{32} P]-UTP labelled transcripts generated were analysed on an 8% denaturing acrylamide gel. Note that *flgA*, *flgB* and *flgM* are the only regulatory regions known to have a σ^{70} -dependent promoters of those tested. RNAs made in the presence of RNAP σ^{70} are shown in Figure 4.1A. The RNAI transcript, generated from the pSR replication origin, serves as a control for σ^{70} -dependent transcription, and is approximately 100 nt long (note that σ^{28} associated RNAP does not make RNAI). Transcription of *flgA* and *flgB* is σ^{70} -dependent and known to be activated by FlhDC, which is not present in this assay. Regardless of FlhDC, we detected a 108 nt long transcript from the partially active *flgA* σ^{70} -dependent promoter. This transcription is repressed by increasing concentrations of MarA (Figure 4.1A, lanes 1-5). No transcript was detected for *flgB*, presumably because its σ^{70} -dependent promoter is completely FlhDC dependent (lanes 6-10). For *flgJ* and *flgK* no transcript was detected (lanes 11-15 and 16-20, respectively). Hence, these regulatory regions are unlikely to contain σ^{70} -dependent promoters, unless they are activated by a transcriptional activator of which

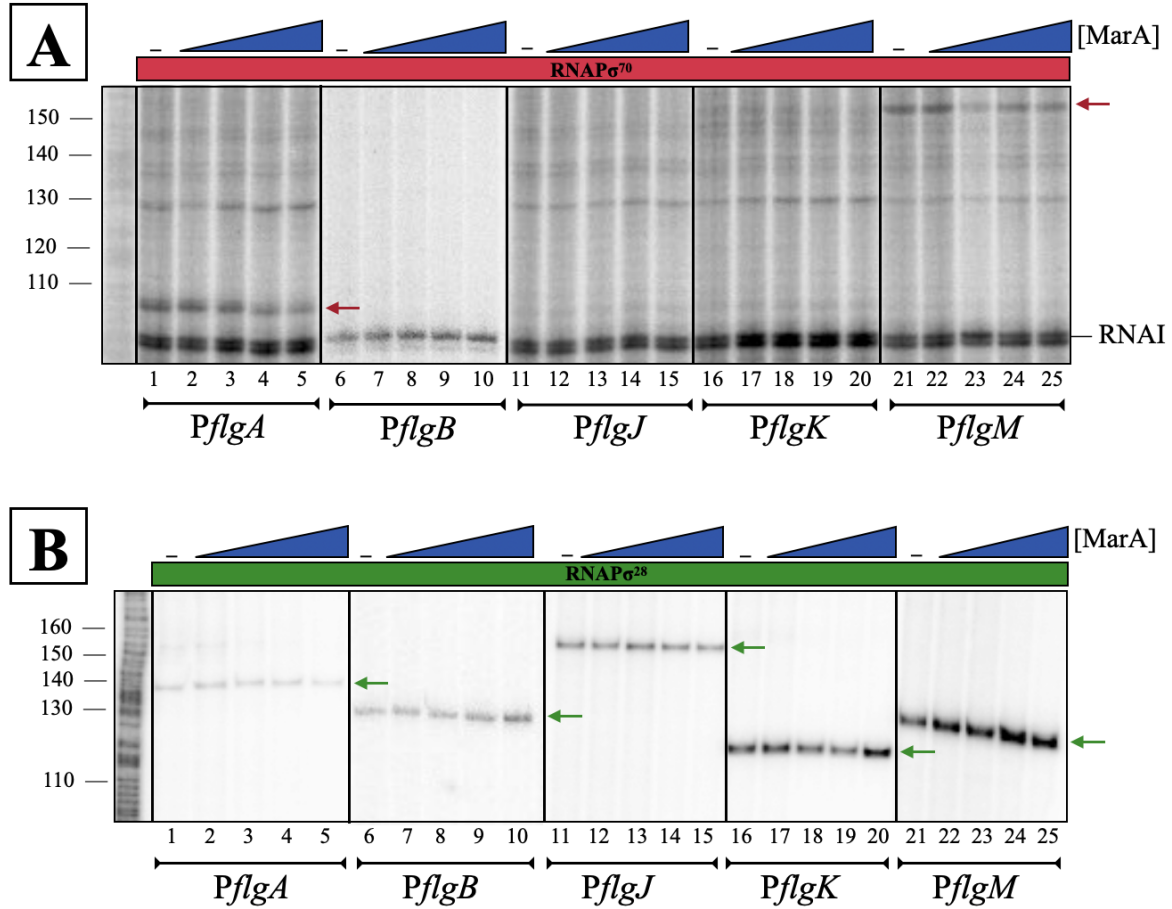


Figure 4.1: *In vitro* transcription of the *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* regulatory regions with RNAP σ^{70} and RNAP σ^{28} .

A. *In vitro* transcription from the *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* regulatory regions done with RNAP σ^{70} . Increasing concentrations of MarA (1 μ M – 2 μ M – 4 μ M – 5 μ M) represented by blue triangles. RNAP σ^{70} is indicated by red rectangles and used at a concentration of 0.15 μ M.

B. *In vitro* transcription from the *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* regulatory regions done with RNAP σ^{28} . Increasing concentrations of MarA (1 μ M – 2 μ M – 4 μ M – 5 μ M) represented as blue triangles. RNAP σ^{28} is indicated as green rectangles; 1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme and used to initiate transcription. Gel with *flgB* fragment (lanes 6-7) is merged.

Images of 8 % polyacrylamide gels used to separate transcripts generated *in vitro* using *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* derivatives, respectively. Bands formed by transcripts originating from *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* regulatory regions are indicated by red or green arrows when RNAP σ^{70} or RNAP σ^{28} were used, respectively. Control RNAI band is indicated by black dash. Experiments were repeated three times with consistent results.

we are not aware. For the *flgM* regulatory region, we detected a 158 nt long transcript, which was not described in any previous study (lanes 21-25). Increasing concentrations of MarA repress this *flgM* σ^{70} -dependent promoter.

In the experiment with RNAP σ^{28} , 135 nt, 132 nt, 153 nt, 117 nt and 127 nt transcripts were generated from pSR with the *flgA*, *flgB*, *flgJ*, *flgK*, and *flgM* regulatory regions, respectively (Figure 4.1B). Transcription of *flgA*, *flgB* and *flgJ*, from σ^{28} -dependent promoters, has not been described previously. Increasing concentrations of MarA activate the *flgB* and *flgM* σ^{28} -dependent promoters (lanes 6-10 and 21-25, respectively), whilst repressing the σ^{28} -dependent transcript of *flgA* (lanes 1-5). MarA had no effect on the *flgJ* and *flgK* promoters (lanes 11-15 and 16-20, respectively). Thus, in our experiments, we identified three new σ^{28} -dependent promoters in regulatory regions of *flgA*, *flgB* and *flgJ*. A new σ^{70} -dependent promoter was also found upstream *flgM*.

Transcript length was used to determine TSS location, and associated RNAP σ^{70}/σ^{28} binding sites for the *flgA*, *flgB*, *flgJ*, *flgK* and *flgM* regulatory regions (Figure 4.2). Importantly, transcripts from the *flgA* σ^{70} , *flgK* σ^{28} and *flgM* σ^{28} -dependent promoters exactly match the size predicted based on known promoter annotations [384, 385, 90]. As expected, we identified σ^{28} promoters elements upstream of *flgA*, *flgB* and *flgJ* that matched our predicted σ^{28} binding sites. The existence of a σ^{28} -dependent *flgJ* promoter indicates that this gene is not always transcribed as part of the *flgBCDEFGHIJ/KL* operon, from the σ^{70} -dependent *flgB* promoter. We also found a σ^{70} -dependent promoter upstream of *flgM*, suggesting that this regulatory region can be used by both σ^{28} and σ^{70} . For such regulatory regions, where our analysis identified a second promoter, we have numbered the upstream promoter as P1 and the downstream promoter as P2. Thus, for *flgA* and *flgB*, which are the focus of subsequent work in this chapter, the upstream σ^{28} -dependent promoters are designated as *flgAP1* and *flgBP1*. The downstream σ^{70} -dependent promoters are named *flgAP2* and *flgBP2*.

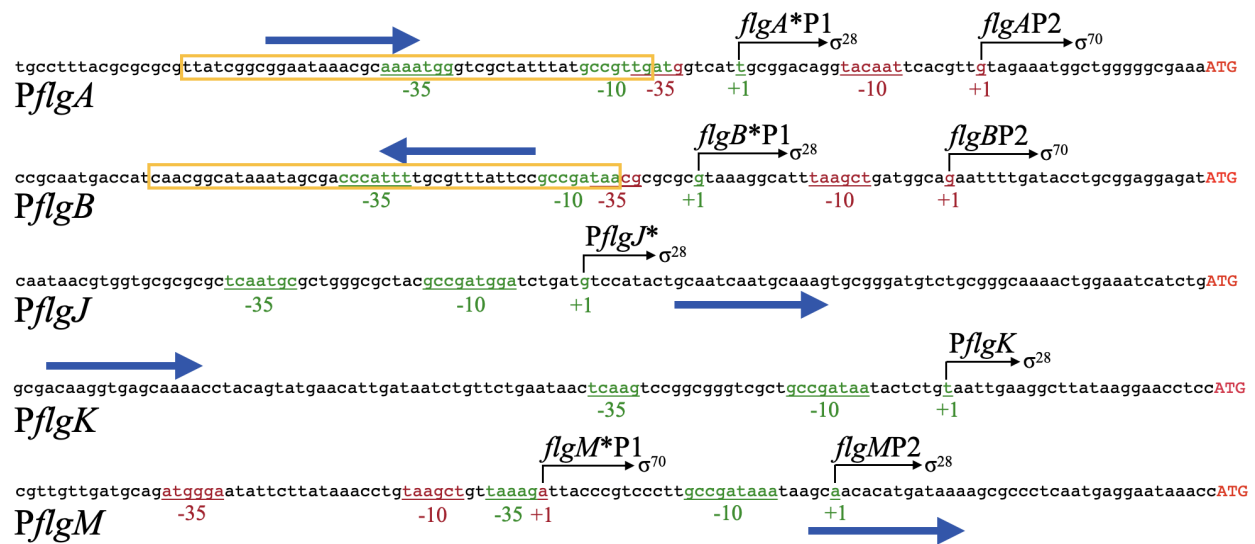


Figure 4.2: Annotation of DNA sequences for new promoter regions upstream of flagellar genes.

Annotation of the *flgA*, *flgB*, *flgJ*, *flgK*, *flgM* regulatory regions with new promoter regions. New transcription start sites found via *in vitro* transcription are indicated by an asterisk. FlhDC binding sites are shown in yellow boxes, marboxes and their directions are shown in blue arrows, σ^{70} -binding sites are shown in red and underlined, σ^{28} -binding sites are shown in green and underlined. Transcription start sites are labeled as +1, in red or green and underlined for σ^{70} or σ^{28} , respectively. Start codons are shown in red capital letters.

The newly identified *flgAP1* and *flgBP1* promoters for RNAP σ^{28} , overlap the FlhDC binding site needed to activate the overlapping σ^{70} -dependent *flgAP2* and *flgBP2* promoters (Figure 4.2). The marbox in the *flgAB* intergenic region is positioned in the forward orientation with respect to *flgA*, and overlaps the 4 last bases of the *flgAP1* -35 element. The same marbox is in the reverse orientation with respect to *flgBP1*, and locates between the -10 and -35 elements of this promoter. Thus, for *flgAP1*, the marbox is in a position usually associated with class II MarA dependent transcription activation, albeit upstream of a σ^{28} -dependent promoter in this case. The location of the marbox between the *flgBP1* -10 and -35 elements, is unusual. Indeed, there is no evidence in the literature of MarA assisting transcription dependent on any σ -factor other than σ^{70} .

4.3 *In vitro* transcription from *flgA* σ^{70}/σ^{28} promoters

The two new σ^{28} -dependent promoters, *flgAP1* and *flgBP1*, are located upstream of *flgAP2* and *flgBP2*, respectively. In the previous chapter, activation of *flgA* and *flgB* by MarA *in vivo* was dependent on the presence of FlhDC (Figure 3.6B). However, in these experiments, the presence of the additional σ^{28} -dependent promoter was not known. Hence, we do not know which promoter is regulated by MarA.

To investigate effects of MarA and FlhDC on the *flgA* regulatory region, we used the wild type *flgA1.1* fragment, that has both promoters, for *in vitro* transcription with RNAP σ^{28} and RNAP σ^{70} (Figure 4.3B). Here, the σ^{70} -dependent *flgAP2* promoter is repressed by MarA (lanes 1-5). As expected, FlhDC activates *flgAP2* (lanes 6-10). Increasing concentrations of MarA repressed transcription from *flgAP2* in the presence of FlhDC (lanes 11-15). When MarA and FlhDC concentrations are increased with RNAP σ^{28} , both regulators repress transcription from *flgAP1* (lanes 16-20 and 21-25, respectively). Increasing concentrations of MarA in the presence of FlhDC, also lead to *flgAP1* repression (lanes 26-30). When RNAP σ^{70}

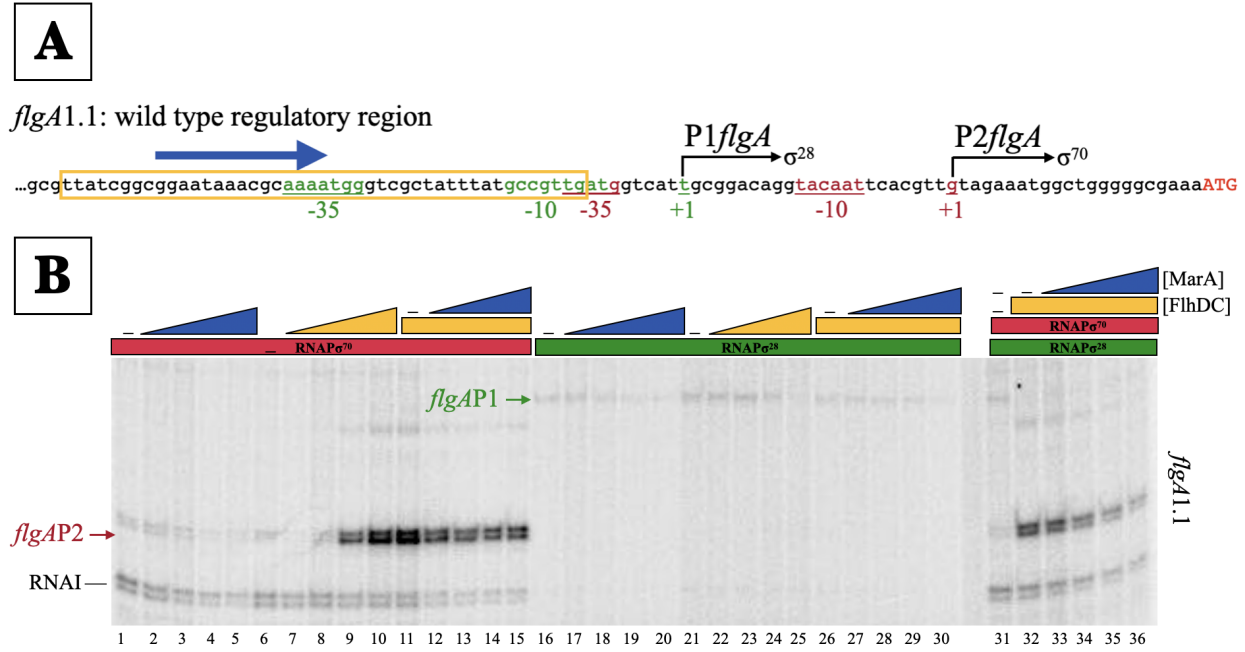


Figure 4.3: *In vitro* transcription from the *flgA1.1* regulatory region and its schematic.

A. On the schematic overview of the *flgA1.1* fragment, FlhDC binding site is shown as a yellow box, marbox and its direction is shown in blue arrow, σ^{70} -binding and σ^{28} -binding sites are in red and green and underlined, respectively. Transcription start sites are labeled as +1, in red or green and underlined for σ^{70} or σ^{28} , respectively. Start codons are shown in red capital letters.

B. Images of 8 % polyacrylamide gels used to separate transcripts generated *in vitro* using *flgA1.1* fragment. Increasing concentrations of MarA (1 μ M – 2 μ M – 4 μ M – 5 μ M) represented as blue triangles. Increasing concentrations of FlhDC (0.05 μ M – 0.1 μ M – 0.2 μ M – 0.25 μ M) represented as yellow triangles. Yellow rectangles represent 0.2 μ M concentration of FlhDC. RNAP σ^{28} is indicated as green rectangles; 1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme and used to initiate transcription. RNAP σ^{70} is indicated as red rectangles and used at a concentration of 0.15 μ M. Bands formed by transcripts originating from *flgA* σ^{28} and *flgA* σ^{70} are indicated by green and red arrows, respectively. RNAI control is indicated by black dashes. Experiment was repeated three times with consistent results.

and RNAP σ^{28} are added together, both *flgAP1* and *flgAP2* transcripts are produced (lane 31). However, when FlhDC is also added, only the *flgAP2* transcript is present (lane 32). Subsequent addition of MarA represses both promoters (lanes 33-36). Thus, *in vitro*, MarA represses *flgA* transcription alone or in the presence of FlhDC. Our models for control of the two promoters, by MarA and FlhDC *in vitro*, are represented in Figure 4.4. Clearly, these observations are not consistent with observations made *in vivo*, where MarA activated the *flgA1.1* regulatory region in the presence of FlhDC.

To understand if we correctly identified the *flgAP1* and *flgAP2* promoters, we made mutations in key residues of their cognate -10 elements and constructed the *flgA1.2* and *flgA1.3* fragments, that are derivatives from the wild type *flgA1.1* fragment (Figures 4.5A and 4.6A). Briefly, the CG motif at positions -11 and -12, with respect to the σ^{28} -dependent TSS, were mutated to GC. The TA at positions -12 and -13 of the σ^{70} -10 element, were mutated to CC. Hence, *flgA1.2* fragment lacks the σ^{28} -dependent promoter, and *flgA1.3* fragment lacks the σ^{70} -dependent promoter.

In vitro transcription from *flgA1.2* and *flgA1.3* was done with or without MarA, FlhDC and RNAP σ^{70} or RNAP σ^{28} . Results with *flgA1.2* are shown in Figure 4.5B. As expected, we could detect no transcription with RNAP σ^{28} (lanes 16-30) due to mutation of the *flgAP1* σ^{28} -10 element. The σ^{70} -dependent *flgAP2* transcript remains repressed by MarA (lanes 1-5), but FlhDC no longer activates transcription (lanes 6-15). This is likely because mutation of the *flgAP2* -10 element also damages the overlapping FlhDC binding site (Figure 4.5A).

Results with *flgA1.3*, lacking the σ^{70} -dependent *flgAP2* promoter, are shown in Figure 4.6B. As expected, in the presence of RNAP σ^{70} , with or without MarA or FlhDC, no transcript is generated from *flgAP2*. This confirms that the introduced mutations eliminated this promoter. However, two other unknown σ^{70} -dependent promoters upstream *flgAP2* become more active, and are considered as artefacts (lanes 9-15). Those artefacts might be created by absence of σ^{70} -binding site on *flgAP2*, so the excess of FlhDC and RNAP σ^{70} bound to

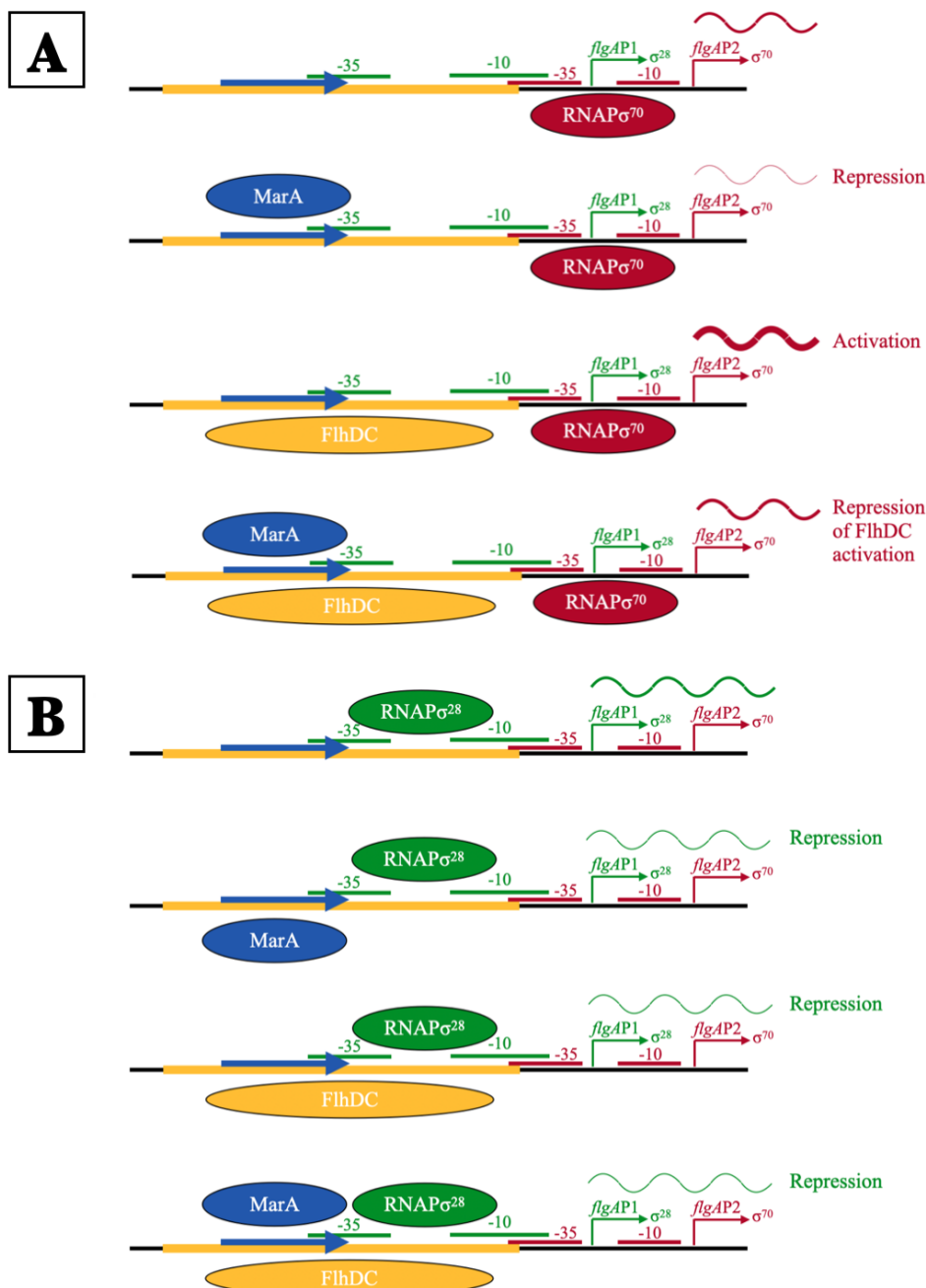


Figure 4.4: Schematic of *in vitro* transcripts from the *flgA1.1* regulatory region in the presence of FlhDC and/or MarA.

A. Model of transcripts generated in the presence of $\text{RNAP}\sigma^{70}$ and transcriptional activators. B. Model of transcripts generated in the presence of $\text{RNAP}\sigma^{28}$ and transcriptional activators. On the schematics FlhDC binding site is shown in yellow, marbox and its direction is shown in blue arrow, σ^{70} -binding and σ^{28} -binding sites are in red and green and underlined, respectively. Transcription start sites are shown in blended arrows, in red or green and underlined for σ^{70} or σ^{28} , respectively.

A

flgA1.1: wild type regulatory region

...gcgttatcggcggaataaacgc⁻³⁵aaaatgggtcgctatttatg⁻¹⁰cggttgatggtcattg⁻³⁵cgggacagg⁺¹tacaattcacgtt⁻¹⁰gtagaaatggctggggcgaaaATG

P1*flgA* σ^{28} P2*flgA* σ^{70}

flgA1.2: σ^{28} -10 element mutant

...gcgttatcggcggaataaacgc⁻³⁵aaaatgggtcgctatttatgc^{-12C}^{-11G}GCTtgatggtcattg⁻³⁵cgggacagg⁺¹tacaattcacgtt⁻¹⁰gtagaaatggctggggcgaaaATG

P2*flgA* σ^{70}

B

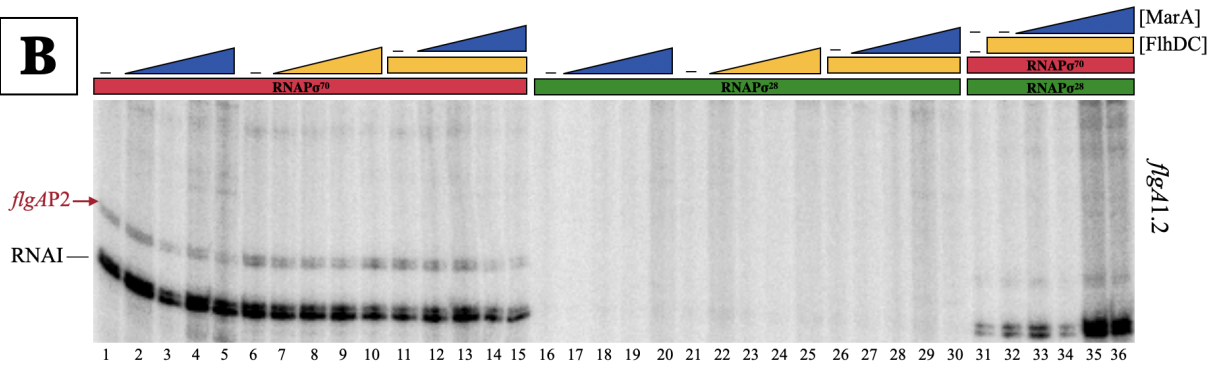


Figure 4.5: *In vitro* transcription from the *flgA1.2* regulatory region and its schematic.

A. On the schematic overview of the *flgA1.2* fragment, FlhDC binding site is shown as yellow box, marbox and its direction is shown by blue arrow, σ^{70} -binding and σ^{28} -binding sites are in red and green and underlined, respectively. Transcription start sites are labeled as +1, in red or green and underlined for σ^{70} or σ^{28} , respectively. Start codons are shown in red capital letters. Mutated bases in -10 element are shown in bold red. Positions of mutations are shown in green above the mutated sequence. Sequence of the wild type *flgA1.1* fragment is shown above for a comparison.

B. Images of 8 % polyacrylamide gels used to separate transcripts generated *in vitro* using *flgA1.1* fragment. Increasing concentrations of MarA (1 μ M – 2 μ M – 4 μ M – 5 μ M) represented as blue triangles. Increasing concentrations of FlhDC (0.05 μ M – 0.1 μ M – 0.2 μ M – 0.25 μ M) represented as yellow triangles. Yellow rectangles represent 0.2 μ M concentration of FlhDC. RNAP σ^{28} is indicated as green rectangles; 1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme and used to initiate transcription. RNAP σ^{70} is indicated as red rectangles and used at a concentration of 0.15 μ M. Bands formed by transcripts originating from *flgA* σ^{28} and *flgA* σ^{70} are indicated by green and red arrows, respectively. RNAI control is indicated by black dashes. Experiment was repeated three times with consistent results.

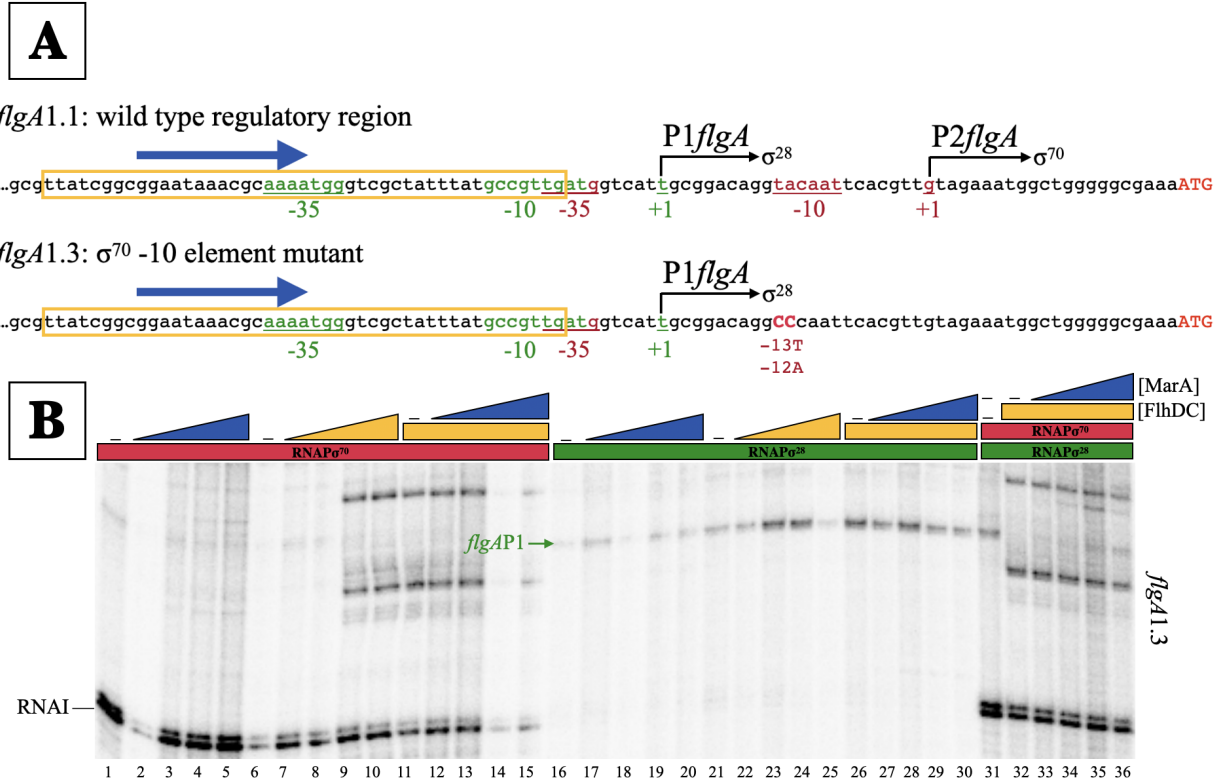


Figure 4.6: *In vitro* transcription from the *flgA1.3* regulatory region and its schematic.

A. On the schematic overview of the *flgA1.3* fragment, FlhDC binding site is shown in yellow box, marbox and its direction is shown by blue arrow, σ^{70} -binding and σ^{28} -binding sites are in red and green and underlined, respectively. Transcription start sites are labeled as +1, in red or green and underlined for σ^{70} or σ^{28} , respectively. Start codons are shown in red capital letters. Mutated bases in -10 element are shown in bold red. Positions of mutations are shown in red underneath the mutated sequence. Sequence of the wild type *flgA1.1* fragment is shown above for a comparison.

B. Images of 8 % polyacrylamide gels used to separate transcripts generated *in vitro* using *flgA1.1* fragment. Increasing concentrations of MarA (1 μ M – 2 μ M – 4 μ M – 5 μ M) represented as blue triangles. Increasing concentrations of FlhDC (0.05 μ M – 0.1 μ M – 0.2 μ M – 0.25 μ M) represented as yellow triangles. Yellow rectangles represent 0.2 μ M concentration of FlhDC. RNAP σ^{28} is indicated as green rectangles; 1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme and used to initiate transcription. RNAP σ^{70} is indicated as red rectangles and used at a concentration of 0.15 μ M. Bands formed by transcripts originating from *flgA* σ^{28} and *flgA* σ^{70} are indicated by green and red arrows, respectively. RNAI control is indicated by black dashes. Experiment was repeated three times with consistent results.

other weak consensus upstream, activating the artefacts. In the presence of RNAP σ^{28} , a very weak transcription from *flgAP1* is detected (lanes 16-30). Overall, both *flgAP1* and *flgAP2* are repressed by MarA alone or in the presence of FlhDC *in vitro*, meaning that MarA cannot activate those promoters directly.

4.4 MarA activates expression of the *flgAP2* promoter indirectly in the presence of FlhDC *in vivo*

Our *in vitro* data suggest that MarA must activate *flgA* indirectly *in vivo*. However, it is still not clear if this is mediated by *flgAP1*, *flgAP2*, or both promoters. To understand which promoter is affected by MarA *in vivo*, the three DNA fragments described above were cloned upstream of *lacZ* in reporter plasmid pRW50. These constructs were used to transform to *E. coli* JCB387 carrying pUC19flhDC and pJ203marA, or empty pJ203. The β -galactosidase activity was then measured in each strain (Figure 4.7).

Overall, the β -galactosidase activity driven by DNA fragment *flgA1.2*, having the σ^{70} -dependent *flgAP2* promoter only, decreased compared to the wild type DNA fragment *flgA1.1*. However, the decrease was small and not statistically significant in the absence of MarA expression (p=0.443328). In strains where MarA was expressed, *flgA1.2* activity was significantly lower (p=0.047762), but the size of the reduction remained minimal. Taken together, activity of the *flgA1.2* fragment dropped compared to *flgA1.1* wild type fragment, in the presence and absence of MarA. This indicates only a small contribution of the σ^{28} -dependent *flgAP1* promoter to the overall activity of the *flgA1.1* fragment. This is consistent with our measurements *in vivo*, where FlhDC activated transcription from *flgAP2* was much higher than transcription from the σ^{28} -dependent *flgAP1* promoter. No β -galactosidase activity was detected from the *flgA1.3* fragment with only *flgAP1*, with or without MarA expression. Overall, data generated *in vivo* and *in vitro*, are consistent with MarA activating

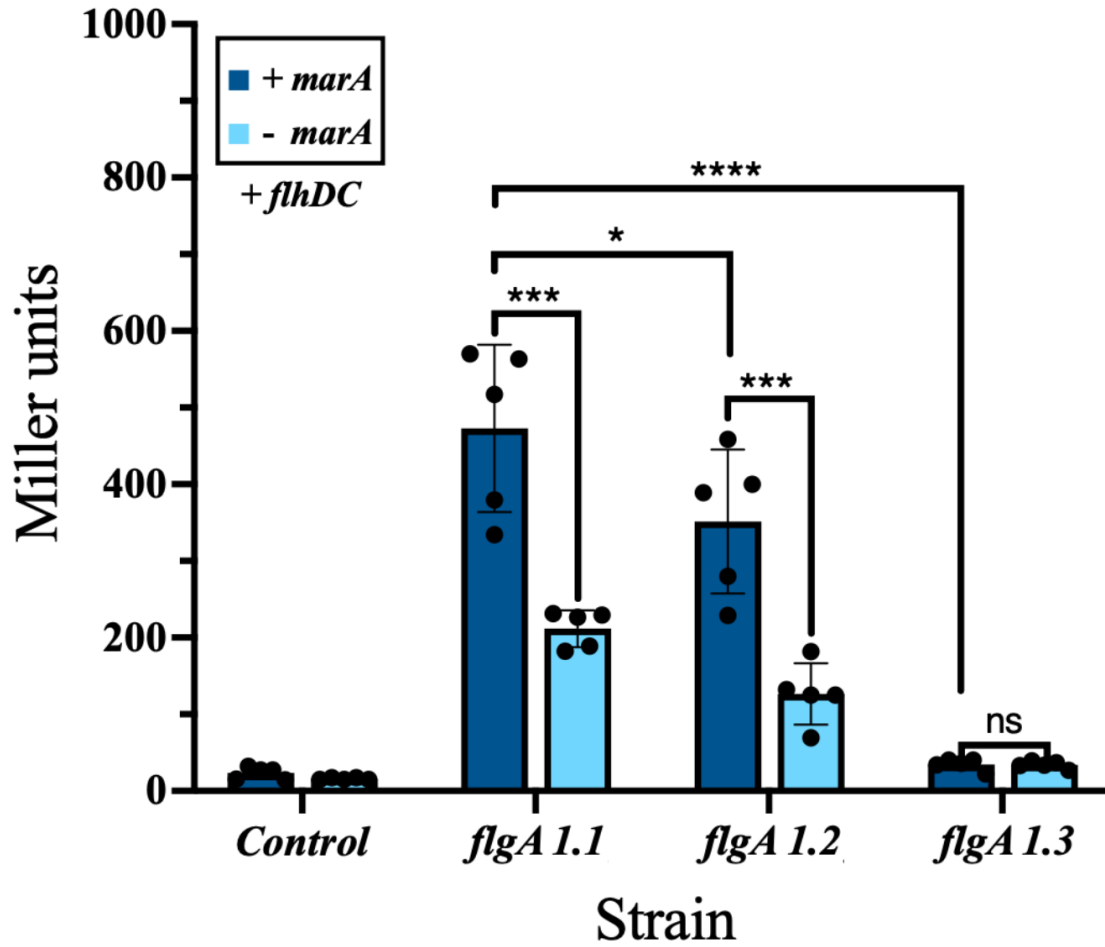


Figure 4.7: The β -galactosidase activity of the *flgA1.1*, *flgA1.2* and *flgA1.3* regulatory regions. Expression of *lacZ* gene driven by the *flgA1.1*, *flgA1.2* and *flgA1.3* promoters derivatives assessed by β -galactosidase assay with MarA expressed from pJ203 plasmid. Assays were done using lysates of *E. coli* JCB387 grown to mid-log phase (OD₆₅₀=0.3-0.6). Standard deviation is shown for five biological replicates. A one-tailed two sample equal T-test was calculated in excel using the promoter activities, showing a statistically significant results (for *flgA1.1* and *flgA1.2* $p \leq 0.0001$; for *flgA1.2* $p \leq 0.05$ against *flgA1.1*; for *flgA1.3* $p \leq 0.0001$ against *flgA1.1*).

flgAP2 promoter indirectly.

4.5 *In vitro* transcription from *flgB* σ^{70} / σ^{28} promoters

In LacZ assays in Chapter 3, the *flgB* regulatory region, containing both promoters, was activated by MarA in the presence and absence of FlhDC (Figure 3.6B). However, we were not aware of the σ^{28} -dependent *flgBP1* promoter, and it is not known if *flgBP1* or *flgBP2* was responsible for this activity.

To investigate effects of MarA and FlhDC on the *flgB* regulatory region, and understand which promoter is regulated by MarA, we used the wild type *flgB2.1* fragment, that contains both the *flgBP1* and *flgBP2* promoters *in vitro* (Figure 4.8A). There was no transcription with RNAP σ^{70} alone (Figure 4.8B, lane 1). This was expected, as the σ^{70} -dependent *flgBP2* promoter is activated by FlhDC. Consequently, there was no effect when MarA was added (lanes 2-5). When we added FlhDC, a transcript from *flgBP2* was generated (lanes 6-10). The same transcript was detected when both FlhDC and MarA were added in the presence of RNAP σ^{70} , but there is no effect of MarA (lanes 11-15). We then did the same analysis with RNAP σ^{28} . The σ^{28} -dependent promoter is not FlhDC dependent, and so the transcript from *flgBP1* was generated in absence of any transcription factors (lane 16). Addition of MarA led to an increase in transcription (lanes 17-20). The FlhDC binding site overlaps the σ^{28} -dependent promoter and FlhDC addition led to a decrease in transcription (lanes 21-25). This did not occur when both MarA and FlhDC were added together (lanes 26-30). Presumably, this is because MarA blocks FlhDC binding. In the presence of both RNAP σ^{28} and RNAP σ^{70} , only the σ^{28} -dependent transcript from *flgBP1* was generated (lane 31). When FlhDC was added, transcripts from both *flgBP1* and *flgBP2* appear (lane 32). Addition of MarA led to a decrease in transcription from *flgBP2* (lanes 33-36), when MarA was at the highest concentration. We note that, in lanes 31-36, loads of *flgBP1* and

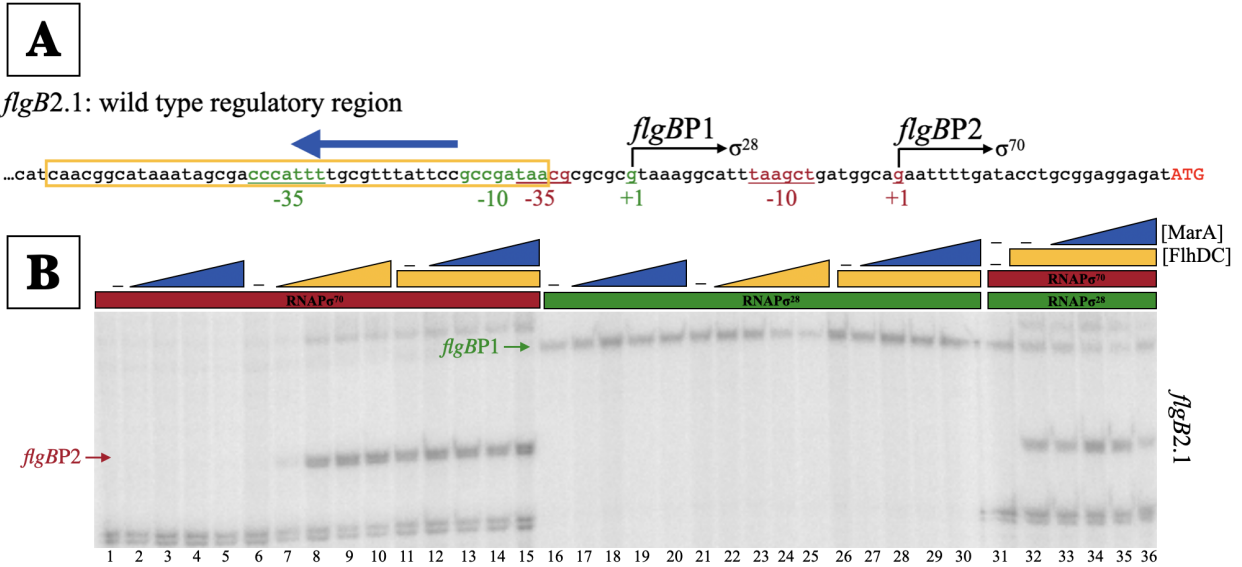


Figure 4.8: *In vitro* transcription from the *flgB2.1* regulatory region and its schematic. A. On the schematic overview of the *flgB2.1* fragment, FlhDC binding site is shown in yellow box, marbox and its direction is shown in blue arrow, σ^{70} -binding and σ^{28} -binding sites are in red and green and underlined, respectively. Transcription start sites are labeled as +1, in red or green and underlined for σ^{70} or σ^{28} , respectively. Start codons are shown in red capital letters. Mutated bases in -10 element are shown in bold red.

B. Images of 8 % polyacrylamide gels used to separate transcripts generated *in vitro* using *flgA1.1* fragment. Increasing concentrations of MarA (1 μ M – 2 μ M – 4 μ M – 5 μ M) represented as blue triangles. Increasing concentrations of FlhDC (0.05 μ M – 0.1 μ M – 0.2 μ M – 0.25 μ M) represented as yellow triangles. Yellow rectangles represent 0.2 μ M concentration of FlhDC. RNAP σ^{28} is indicated as green rectangles; 1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme and used to initiate transcription. RNAP σ^{70} is indicated as red rectangles and used at a concentration of 0.15 μ M. Bands formed by transcripts originating from *flgB* σ^{28} and *flgB* σ^{70} are indicated by green and red arrows, respectively. RNAI control is indicated by black dashes. Experiment was repeated three times with consistent results.

flgBP2 transcript were inversely correlated. Thus, *in vitro*, MarA alone directly activates the σ^{28} -dependent *flgBP1* promoter. Schematic models are shown in Figure 4.9.

To understand if we correctly identified the *flgBP1* and *flgBP2* promoters, we attempted to mutate their cognate -10 elements and generate *flgB2.2* and *flgB2.3* fragments, similar to the *flgA1.2* and *flgA1.3* fragments, described above (Figure 4.10A). In *flgB2.2* and *flgB2.3*, key residues of the σ^{28} or σ^{70} -10 elements are mutated, respectively. Briefly, positions -11 and -12, with respect to the σ^{28} -dependent TSS, were mutated to GC. The positions -12 and -13 of the σ^{70} -10 element, with respect to the σ^{70} -dependent TSS, were mutated to CC. Attempts to clone the *flgB2.3* fragment, lacking the σ^{70} promoter, in pSR plasmid were not successful. Experiments with *flgB2.3* fragment cloned in pRW50 are explained in later section, and its schematic sequence is shown on (Figure 4.10C).

In vitro transcription from *flgB2.2* was done with or without MarA, FlhDC and RNAP σ^{70} or RNAP σ^{28} (Figure 4.10). With RNAP σ^{70} alone, a transcript from *flgBP2* promoter is generated in a way similar to the one detected from *flgB2.1* fragment. The transcript from *flgBP2* was not generated in the absence of FlhDC (lane 1), and MarA addition did not have any effect (lanes 2-5). Addition of FlhDC activated the *flgBP2* promoter as expected (lanes 6-10). MarA did not have any effect on transcription, when it was added together with FlhDC (lanes 11-15). As expected, no transcription was observed when the *flgB2.2* fragment was analysed with RNAP σ^{28} , with or without MarA or FlhDC (lanes 16-30). When both RNAP σ^{70} and RNAP σ^{28} are added with FlhDC, only the transcript from *flgBP2* is generated (lanes 31-32). Addition of MarA did not have any effect (lanes 33-36). We conclude that the *flgBP1* promoter has been correctly identified.

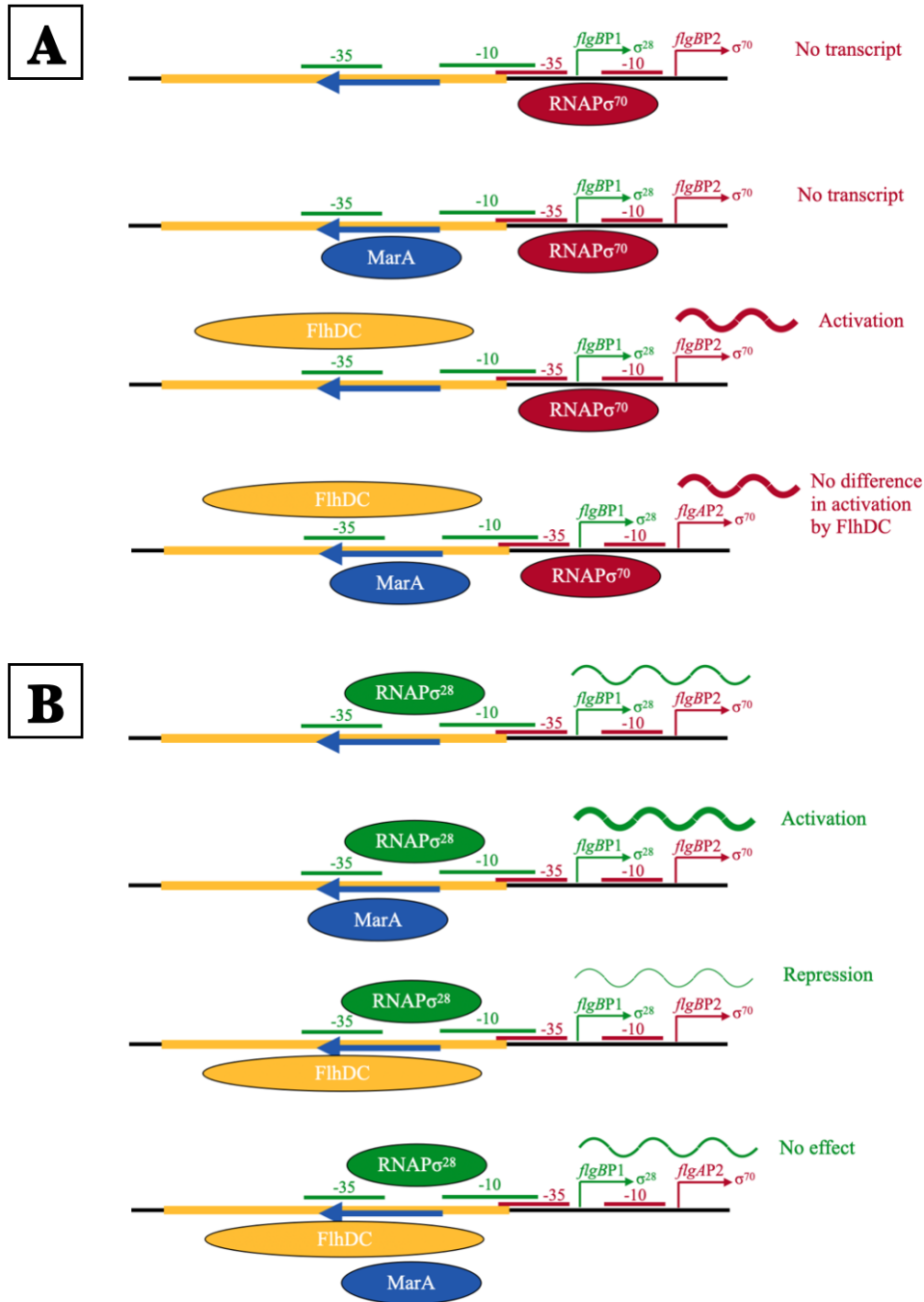


Figure 4.9: Schematic of *in vitro* transcripts from the *flgB2.1* regulatory region in the presence of FlhDC and/or MarA.

A. Model of transcripts generated in the presence of $\text{RNAP}\sigma^{70}$ and transcriptional activators. B. Model of transcripts generated in the presence of $\text{RNAP}\sigma^{28}$ and transcriptional activators. On the schematics FlhDC binding site is shown in yellow, marbox and its direction is shown in blue arrow, σ^{70} -binding and σ^{28} -binding sites are in red and green and underlined, respectively. Transcription start sites are shown in blended arrows, in red or green and underlined for σ^{70} or σ^{28} , respectively.

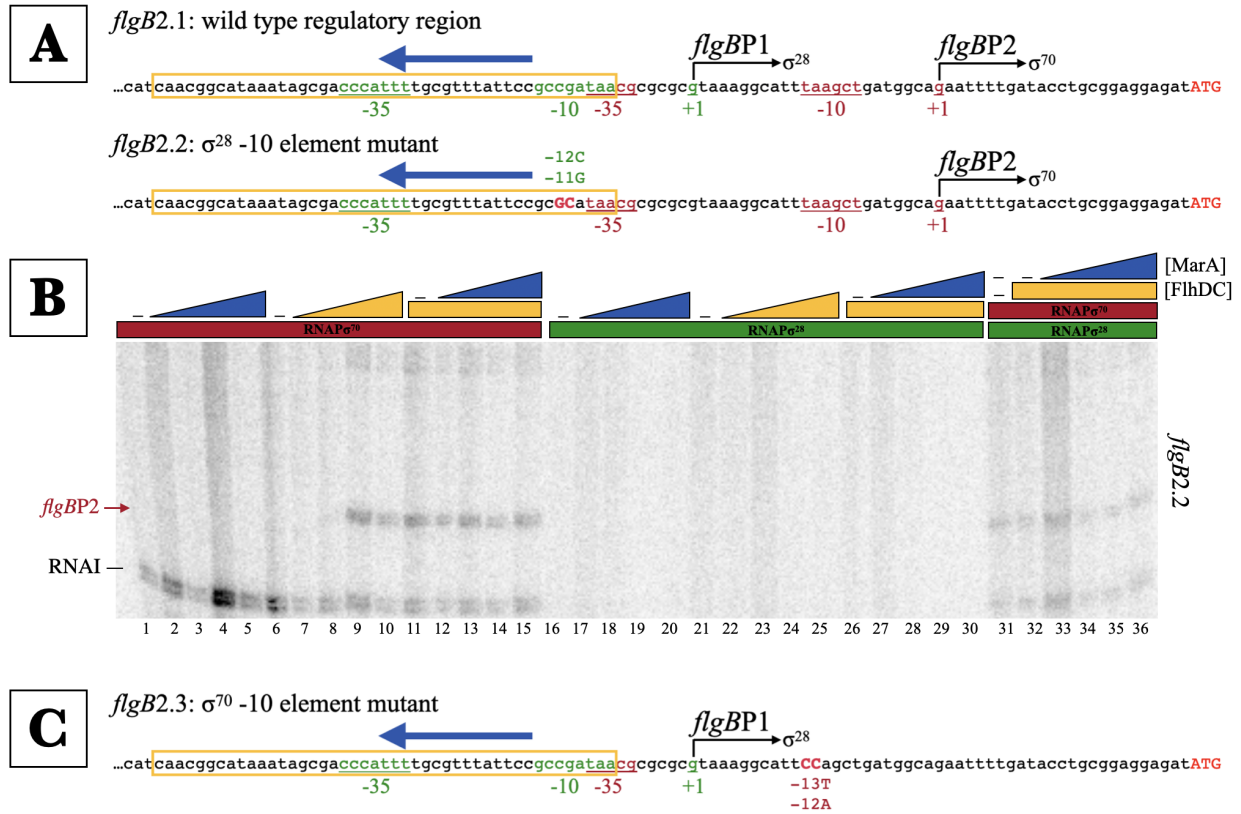


Figure 4.10: *In vitro* transcription from the *flgB2.2* regulatory region and its schematic. A, C. Schematic overview of the *flgB2.2* and *flgB2.3* fragment sequences with annotations, respectively. FlhDC binding site is shown as a yellow box, marbox and its direction is shown by blue arrow, σ^{70} -binding and σ^{28} -binding sites are in red and green and underlined, respectively. Transcription start sites are labeled as +1, in red or green and underlined for σ^{70} or σ^{28} , respectively. Start codons are shown in red capital letters. Mutated bases in -10 elements are shown in bold red. Positions of mutations are shown in green or red above or underneath the mutated sequences for the *flgB2.2* and *flgB2.3*, respectively. Sequence of the wild type *flgB2.1* fragment is shown above for a comparison.

B. Images of 8 % polyacrylamide gels used to separate transcripts generated *in vitro* using *flgA1.1* fragment. Increasing concentrations of MarA (1 μ M – 2 μ M – 4 μ M – 5 μ M) represented as blue triangles. Increasing concentrations of FlhDC (0.05 μ M – 0.1 μ M – 0.2 μ M – 0.25 μ M) represented as yellow triangles. Yellow rectangles represent 0.2 μ M concentration of FlhDC. RNAP σ^{28} is indicated as green rectangles; 1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme and used to initiate transcription. RNAP σ^{70} is indicated as red rectangles and used at a concentration of 0.15 μ M. Bands formed by transcripts originating from *flgA* σ^{70} are indicated by red arrow. RNAI control is indicated by black dashes. Experiment was repeated three times with consistent results.

4.6 MarA activates expression of the *flgBP2* promoter indirectly in the presence of FlhDC *in vivo* and have a direct effect on *flgBP1*

According to our *in vitro* transcription assays, MarA directly activates the σ^{28} -dependent *flgBP1* promoter. However, it is still unclear if MarA activates this promoter *in vivo*. To understand which *flgB* promoter is affected by MarA *in vivo*, the DNA fragments *flgB2.1*, *flgB2.2* and *flgB2.3* were cloned upstream of *lacZ* in reporter plasmid pRW50. These constructs were used to transform to *E. coli* JCB387 carrying pUC19*flhDC* and pJ203*marA*, or empty pJ203. The β -galactosidase activity was then measured in each strain (Figure 4.11).

The β -galactosidase activity generated by the wild type DNA fragment *flgB2.1* was as expected, and increased in the presence of MarA ($p=0.000002$). MarA also activated the *flgB2.2* fragment, that lacks the σ^{28} -dependent *flgBP1* promoter ($p=0.000005$). Interestingly, the activity of the *flgB2.1* and *flgB2.2* fragments was the same without MarA ($p=0.118223$). However, in the presence of MarA transcription driven by *flgB2.2*, lacking the σ^{28} -dependent *flgBP1* promoter, was significantly lower than transcription from the *flgB2.1*, where both promoters are present ($p=0.004976$). Activity was abolished with the *flgB2.3* fragment that lacks the σ^{70} -dependent *flgBP2* promoter.

We conclude that MarA can activate *flgBP1* *in vitro* and *in vivo*, but in living cells, in the conditions tested, this promoter makes only a minimal contribution to the overall activity of *flgB* regulatory region. The majority of the signal increase in detected transcription is indirect and due to *flgBP2*, when MarA is expressed.

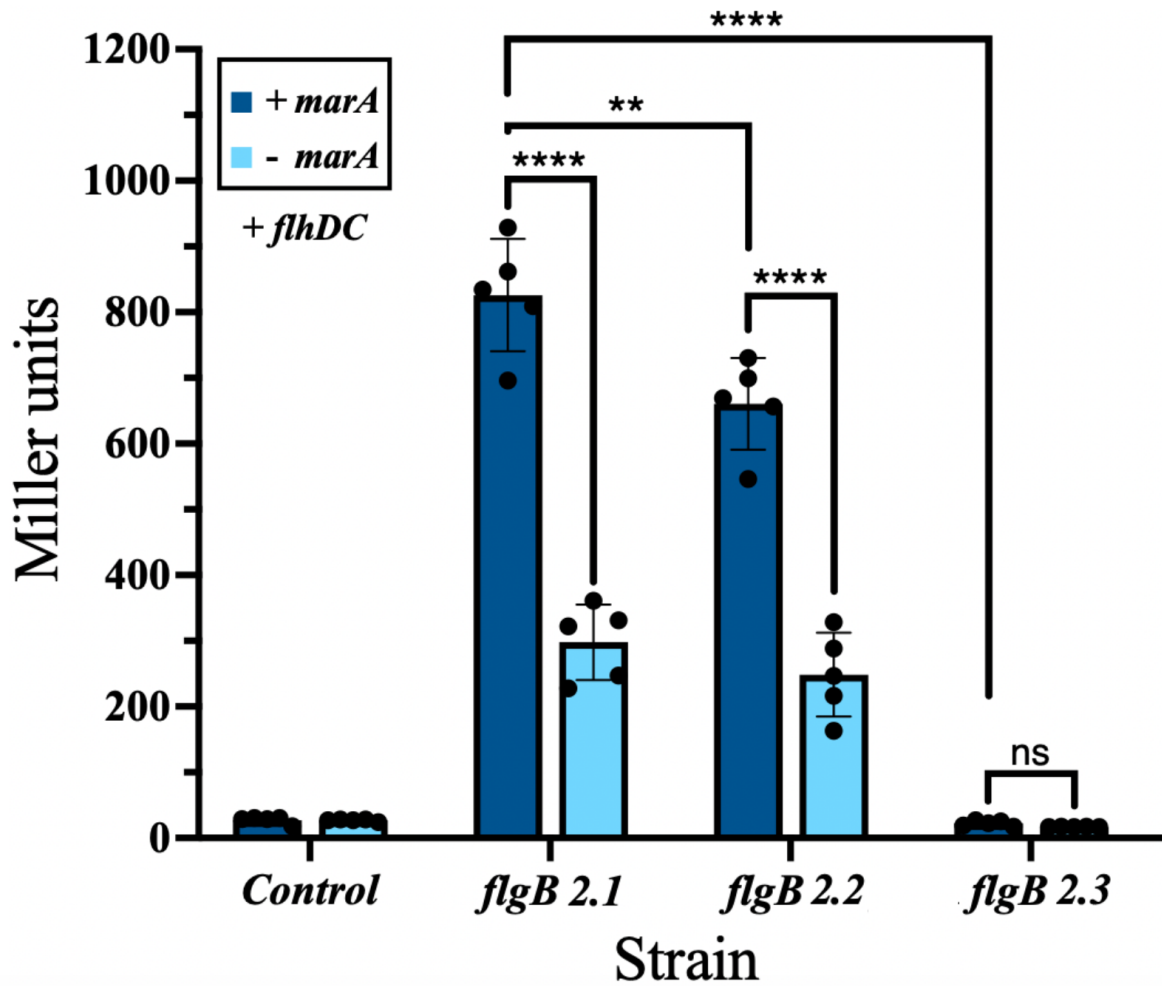


Figure 4.11: The β-galactosidase activity of the *flgB2.1*, *flgB2.2* and *flgB2.3* regulatory regions.

Expression of *lacZ* gene driven by the *flgB2.1*, *flgB2.2* and *flgB2.3* promoters derivatives assessed by β-galactosidase assay with MarA expressed from pJ203 plasmid. Assays were done using lysates of *E. coli* JCB387 grown to mid-log phase (OD₆₅₀=0.3-0.6). Standard deviation is shown for five biological replicates. A one-tailed two sample equal T-test was calculated in excel using the promoter activities, showing a statistically significant results (for *flgB2.1* and *flgB2.2* $p \leq 0.001$; for *flgB2.2* $p \leq 0.01$ against *flgB2.1*; for *flgB2.3* $p \leq 0.0001$ against *flgB2.1*).

4.7 Measurement of β -galactosidase activity from *flgA* and *flgB* promoters upon σ^{28} overexpression

In previous experiments, we saw that MarA has only a small stimulatory effect on the σ^{28} -dependent *flgBP1* promoter, particularly *in vivo*. To maximise any effect of MarA, we decided to overexpress σ^{28} , in *E. coli* T7 express cells. The DNA fragments *flgA1.1*, *flgA1.2*, *flgA1.3* and *flgB2.1*, *flgB2.2*, *flgB2.3*, upstream of *lacZ* in reporter plasmid pRW50, were used to transform to *E. coli* T7 express, which already carried plasmid pET21a encoding σ^{28} . Hence, high level of σ^{28} expression could be induced with IPTG. We used salicylic acid (SA) to induce MarA expression. Schematic of *E. coli* T7 cell and plasmids used is shown on (Figure 4.12A).

4.8 MarA and σ^{28} expression increase β -galactosidase activity from *flgA* promoter

First, we measured β -galactosidase activity from the *flgA1.1* fragment. When MarA was induced with SA, no significant increase in promoter activity was observed ($p=0.068671$). Induction of the σ^{28} with IPTG led to significantly higher activity ($p=0.031378$). Induction of both MarA and σ^{28} had a small additive effect ($p=0.033954$), (Figure 4.12B). We observed exactly the same pattern using the *flgA1.2* fragment lacking the σ^{28} -dependent promoter. Loss of the σ^{70} -dependent *flgAP2* promoter abolished all transcription. Hence, any effect of MarA on *flgA* expression is indirect, as we previously concluded.

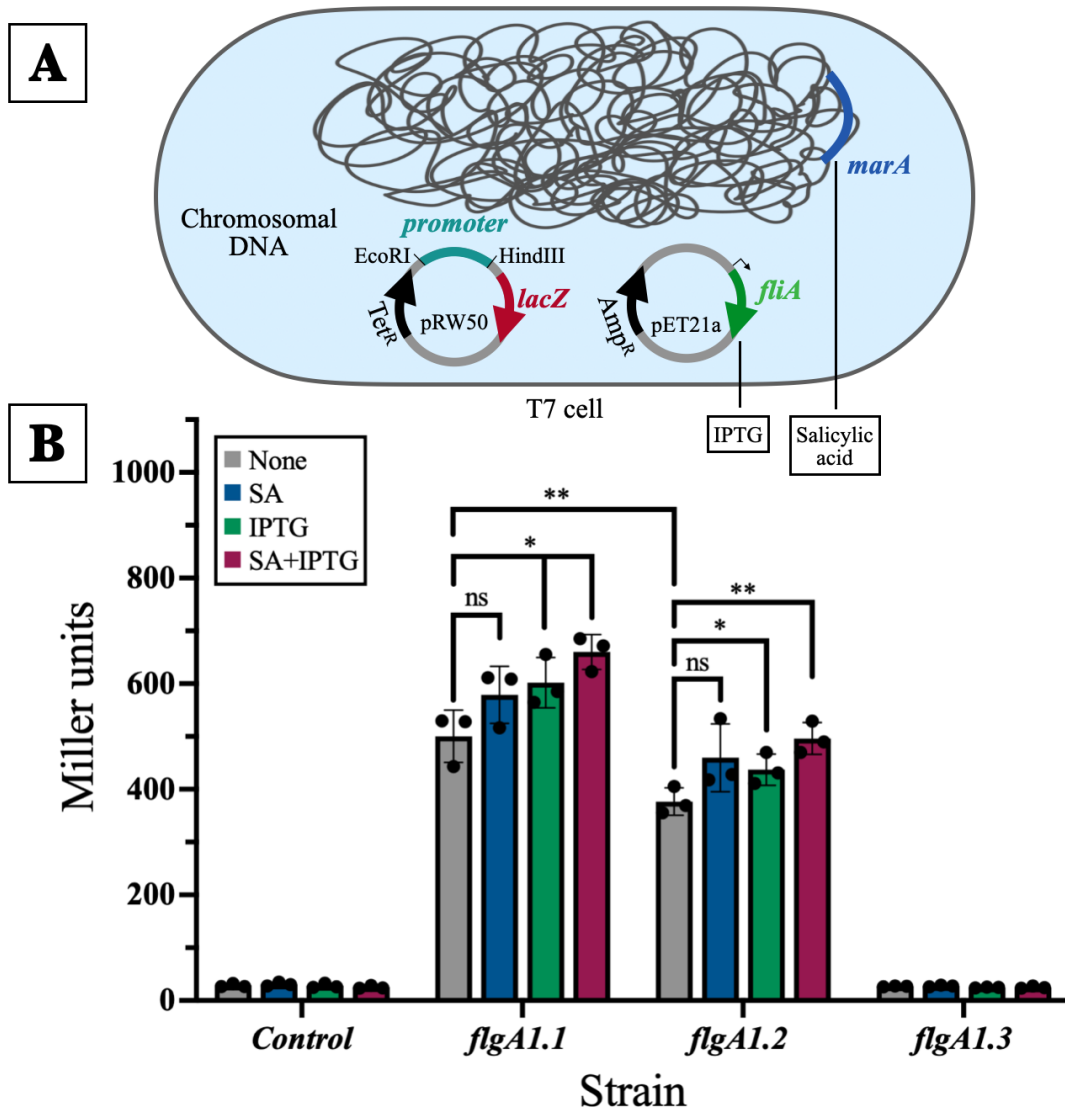


Figure 4.12: Individual and collective effect of MarA and FliA expression on *flgA1.1*, *flgA1.2* and *flgA1.3* regulatory regions in *E. coli* T7 background.

A. Schematic of *E. coli* T7 cell and plasmids used in LacZ assay.

B. Expression of *lacZ* gene driven by the *flgA1.1*, *flgA1.2* and *flgA1.3* promoters derivatives assessed by β -galactosidase under MarA expression from the chromosome using 5 μ of salicylic acid or/and FliA expression from pET21a plasmid using 1 μ of IPTG. Assays were done using lysates of *E. coli* T7 grown to mid-log phase (OD₆₅₀=0.3-0.6). Standard deviation is shown for three biological replicates. A one-tailed two sample equal T-test was calculated in excel using the promoter activities, showing a statistically significant results (for *flgA1.1* IPTG, SA+IPTG $p \leq 0.05$ against *flgA1.1* None; for *flgA1.2* None $p \leq 0.01$ against *flgA1.1* None; for *flgA2.1* IPTG $p \leq 0.05$ against *flgA2.1* None and for *flgA2.1* SA+IPTG $p \leq 0.01$ against *flgA2.1* None).

4.9 MarA and σ^{28} expression significantly increase β -galactosidase activity from *flgB* regulatory region

β -galactosidase activity was detected from the full length *flgB*2.1 fragment with both *flgBP*1 and *flgBP*2 promoters present, without induction of MarA or σ^{28} (Figure 4.13). Induction of either MarA or σ^{28} , led to a significant increase in promoter activity, with maximum activity reached when both factors were induced. With the *flgB*2.2 fragment, lacking the σ^{28} -dependent *flgBP*1 promoter, we observed only small effects of MarA or σ^{28} induction that were mostly not significant. These data are consistent with MarA activating the σ^{28} -dependent *flgBP*1 promoter.

4.10 MarA does not up-regulate *flhDC* operon

Taking into account that most activity of the *flgA* and *flgB* regulatory regions comes from their cognate σ^{70} -dependent promoters, and that MarA can indirectly activate σ^{70} -dependent transcription of both genes, we decided to determine if MarA up-regulates promoter of the *flhDC* operon, that encodes the master activator of *flgA* and *flgB*. The *flhDC* promoter region was cloned upstream of *lacZ* in reporter plasmid pRW50. This construct was used to transform to *E. coli* JCB387 with pJ203 plasmid, expressing MarA. Empty pJ203 was also used as a control. Activity of β -galactosidase was then measured (Figure 4.14).

The β -galactosidase activity from the *flhDC* regulatory region did not increase in the presence of MarA ($p=0.193626$). Thus, the increase in activity of FlhDC-activated *flgA* and *flgB* σ^{70} -dependent promoters was not caused by MarA activation of the *flhDC* operon. Hence, MarA must indirectly regulate σ^{70} -dependent transcription via some other mechanism.

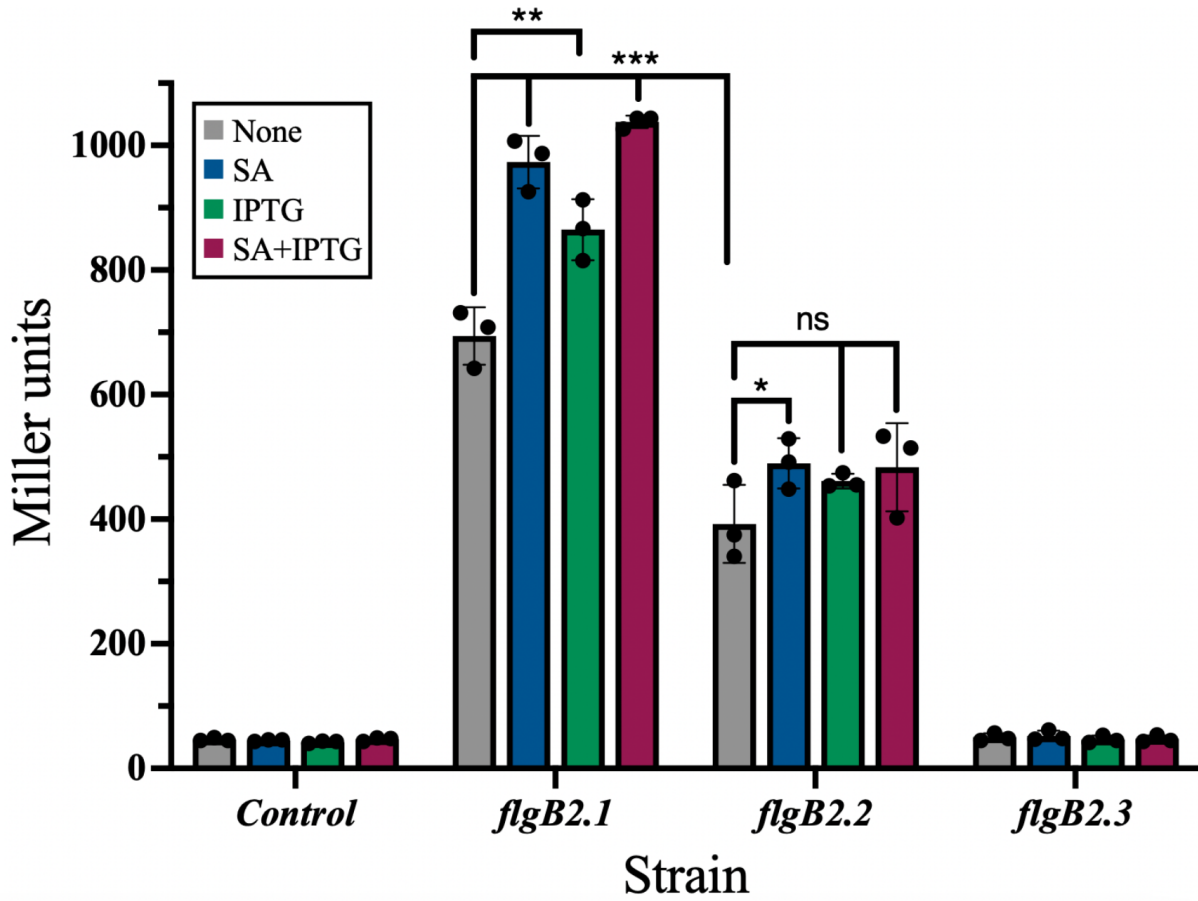


Figure 4.13: Individual and collective effect of MarA and FliA expression on *flgB2.1*, *flgB2.2* and *flgB2.3* regulatory regions in *E. coli* T7 background.

Expression of *lacZ* gene driven by the *flgB2.1*, *flgB2.2* and *flgB2.3* promoters derivatives assessed by β -galactosidase under MarA expression from the chromosome using 5 μ of salicylic acid or/and FliA expression from pET21a plasmid using 1 μ of IPTG. Assays were done using lysates of *E. coli* T7 grown to mid-log phase (OD₆₅₀=0.3-0.6). Standard deviation is shown for three biological replicates. A one-tailed two sample equal T-test was calculated in excel using the promoter activities, showing a statistically significant results (for *flgB2.1* SA, SA+IPTG and *flgB2.2* None $p \leq 0.001$ against *flgB2.1* None; for *flgB2.1* SA $p \leq 0.01$ against *flgB2.1* None; for *flgB2.2* SA $p \leq 0.05$ against *flgB2.2* None).

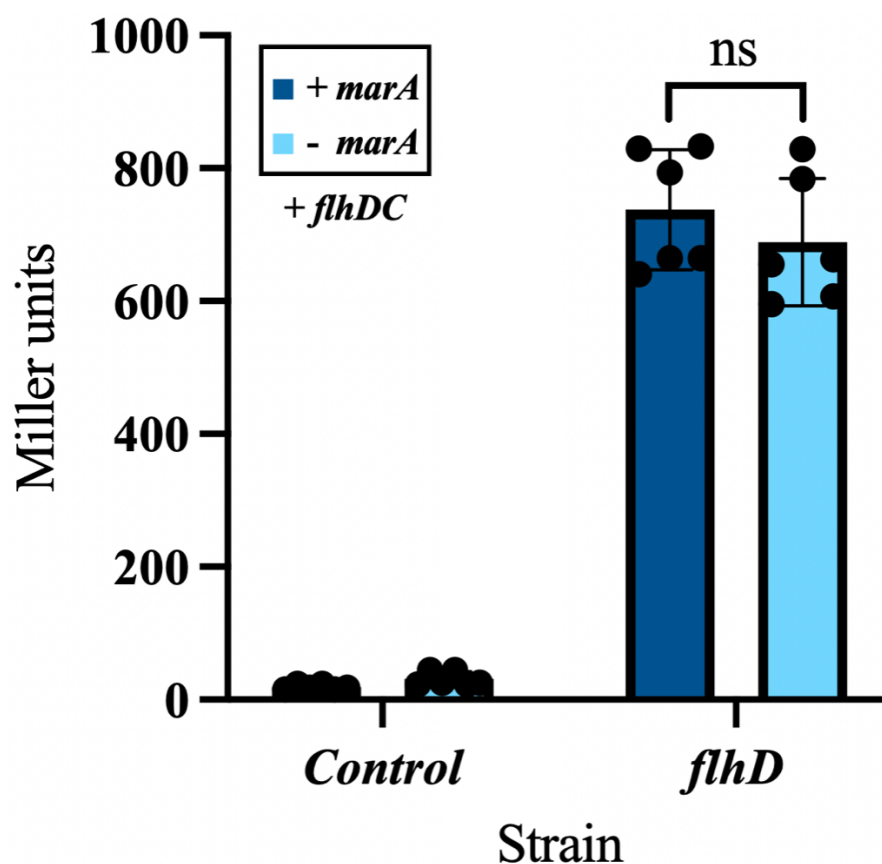


Figure 4.14: The β -galactosidase activity of the *flhD* regulatory region. Expression of *lacZ* gene driven by the *flhD* promoters derivatives assessed by β -galactosidase assay with MarA expressed from pJ203 plasmid. Assays were done using lysates of *E. coli* JCB387 grown to mid-log phase (OD₆₅₀=0.3-0.6). Standard deviation is shown for six biological replicates. A one-tailed two sample equal T-test was calculated in excel using the promoter activities.

4.11 Discussion

The regulation of flagellar biosynthesis is a complex process involving the coordinated action of transcriptional regulators and sigma factors directing RNAP to different sets of genes. In this chapter we studied regulatory mechanisms that control the expression of flagellar genes, focusing on interplay between the master regulator FlhDC, the transcriptional activator MarA, and the sigma factors σ^{70} and σ^{28} .

Results in previous Chapter 3 showed that MarA increases FlhDC-dependent activation at the *flgA* and *flgB* promoters, and modestly increase *flgB* expression in the absence of FlhDC. In this chapter, an *in vitro* approach was used to understand the *in vivo* activity. Using *in vitro* transcription experiments, FlhDC was shown to activate the σ^{70} -dependent *flgA* and *flgB* promoters, but MarA had no direct effect on those promoters. During these experiments, the presence of putative σ^{28} -dependent promoters, upstream of the σ^{70} -dependent *flgA* and *flgB* promoters, was noticed. Further *in vitro* transcription experiments confirmed that these new promoters are recognised by RNAP σ^{28} . We found that MarA alone had no effect on the σ^{28} -dependent *flgA* promoter. However, σ^{28} -dependent transcription of *flgB* was activated by MarA. We also observed activation of the σ^{28} -dependent *flgB* promoter by MarA *in vivo*. However, overall, this promoter do not contribute much to *flgB* expression, in conditions tested.

Since MarA has an indirect effect on the σ^{70} -dependent *flgA* and *flgB* promoters, we decided to check if MarA could up-regulate transcription of FlhDC itself. However, there is no evidence for a change in activity of the *flhD* promoter with MarA. Thus, MarA does not have any direct effect on either σ^{70} -dependent promoters or FlhDC expression. However, we can assume that this indirect effect caused by MarA might happen by either MarA activating the production of another regulator, that activates production of FlhDC, or MarA reorganising NAPs.

Overall, results from this chapter expand the regulatory network between flagellar genes, as the regulatory regions upstream *flgAMN* and *flgBCDEFGHIJ/KL* operons were considered to be σ^{70} -dependent only, and regulated in Class II manner by FlhDC. Taking into account previous studies revealing promoters of flagellar genes with double regulation [90], but active only with RNAP σ^{70} and FlhDC, we can suggest that their cognate σ^{28} -dependent promoters might be involved in fine-tuned regulation of flagellar biosynthesis in a specific conditions.

Chapter 5

MarA assists σ^{28} -dependent transcript
initiation at *flgB* promoter

5.1 Introduction

Transcriptional activation at promoters is a critical regulatory mechanism in bacteria, enabling them to respond rapidly to environmental changes. In bacteria, transcription initiation is facilitated by RNAP, that requires the assistance of σ -factors for promoter recognition and DNA unwinding. The sigma factors, such as σ^{70} and σ^{28} , play crucial roles in directing RNAP to specific promoters and forming the transcription bubble necessary for RNA synthesis. Both σ -factors belong to the σ^{70} family and contain conserved domains that facilitate their interaction with core RNAP and promoter DNA. Each σ -factor possess a domain 2, responsible for recognition and melting of the -10 promoter element to form the transcription bubble. Domain 4 recognises the -35 promoter element. However, those domains interact differently with the promoter DNA, and have overall differences in their structure [177, 65].

Interestingly, the cyclic AMP receptor protein (CRP) binds to σ^{28} -dependent promoters at an unconventional location, compared to the regulation of σ^{70} -dependent promoters [429]. This suggests that the mechanism by which σ^{28} recognises the -35 element might differ from the recognition process of σ^{70} [429]. So that, interactions with transcriptional activators such as MarA, that target domain σ_4^{28} might be also different, which explains why MarA does not activate *flgAP1* σ^{28} -dependent promoter in the previous chapter, even though the position of *marbox* is perfect for activation. However, the molecular mechanism of σ^{28} -dependent transcription initiation is poorly understood.

In rare cases, to assist transcription initiation, transcription activator proteins modify promoter structure itself, without any interactions with RNAP. These transcriptional activators, such as BmrR, CueR, SoxR and other MerR-like proteins, bind between the -35 and -10 elements of promoters with an unusually long spacer (19-20 bp). MerR-family members act as transcriptional repressors or activators, depending on cellular signals [199, 452, 349, 453]. In the presence of their ligands, these proteins bind on the opposite face of the DNA to

RNAP holoenzyme, making no contacts with the latter, but introducing a 90° central kink at the center of the spacer. This distortion makes the minor groove wider and the major groove narrower, which shortens the space between the -10 and -35 elements, restoring the spacer geometry closer to canonical 17-bp and optimal for RNAP recognition. This conformational change puts the first two positions of the -10 element at precise location needed to interact with tryptophans of σ^{70} domain 2 required for melting of the promoter DNA. There are also 2 additional kinks, upstream and downstream of the MerR binding site of the operator, well studied on BmrR TF, one of the MerR family members [349]. The upstream kink 1 created by BmrR binding, corresponds to a 37° bend at the center of the -35 element, and kink 2 corresponds to a 52° bend at the position -31 of the DNA region which is contacted by the wing loop and HTH motifs of BmrR. The downstream kink corresponds to a 48° bend at the downstream position -18, where the DNA region contacted by another wing loop and HTH motifs of BmrR. This kinks pre-distorts operator DNA and facilitates RNAP recruitment and access to the ssDNA template. This restores otherwise inactive MerR family TF-dependent promoters with long -10/-35 spacer, which helps melting of DNA to begin. However, members of MerR family do not typically create specific distortions in the -10 element itself.

In *V. cholerae*, the FruR transcriptional activator from the GalR-LacI family binds between the -35 and -10 elements of the promoter, which has a 20-bp spacer. This binding facilitates RNAP attachment to the promoter DNA by inducing distortions, similar to the mechanism employed by MerR TFs to regulate gene expression. These studies collectively underscore the significance and widespread nature of DNA distortion in transcription activation. The MarA binding site is located between the -10 and -35 elements of the *flgBP1* promoter, like MerR-family members. However, it remains unclear whether MarA activates transcription through a mechanism similar to MerR transcription factors or employs a distinct mechanism.

In this chapter, we found that MarA activates the *flgBP1* σ^{28} -dependent promoter by inducing a distortion at the conserved -11A base of the -10 element to facilitate melting and formation of the open complex by σ^{28} . This architecture of the *flgAP1* and *flgBP1* σ^{28} -dependent promoters, and the location of the *marbox* in the *flgAB* intergenic region appears unique to *E. coli* and closely related bacteria.

5.2 MarA activates *flgB* σ^{28} promoter *in vitro* from pLSR

In the previous chapter, we showed that *in vitro*, MarA represses both σ^{28} - and σ^{70} -dependent transcription from the *flgA* regulatory region, whilst increasing activity of the σ^{28} -dependent *flgB* promoter. Even though *flgA* and *flgB* are divergent transcription units, with a shared regulatory region, our experimental set up meant that we could detect transcription of only *flgA* or *flgB*, depending on orientation of the cloned DNA fragment.

To simultaneously monitor transcription of *flgA* and *flgB*, and better understand the role of MarA, the full wild type *flgB2.1* DNA fragment, possessing *flgAP1*/P2 and *flgBP1*/P2, was cloned in pLSR plasmid. This plasmid has loop terminators upstream and downstream of the inserted DNA, allowing detection of both forward and reverse transcripts simultaneously (a schematic of the *flgB2.1* DNA fragment is in Figure 5.1A). RNAP σ^{70} or RNAP σ^{28} , were added to reactions to initiate transcription. The [α - ^{32} P]-UTP labelled transcripts generated were analysed on 8% denaturing acrylamide gels. Gel with transcripts generated in the presence of RNAP σ^{70} and/or RNAP σ^{28} are shown in Figure 5.1B, and a schematic of this transcription is shown on Figure 5.2. In all reactions, the RNAI transcript, generated from the pLSR replication origin, serves as a control band for σ^{70} -dependent transcription. The 131bp transcript from *flgAP2* was generated in the absence of any transcriptional regulators by RNAP σ^{70} (Figure 5.1B, lane 1). Addition of MarA led to slight repression of this promoter (lanes 2-5). Increasing concentrations of FlhDC activated both the *flgAP2* and *flgBP2* pro-

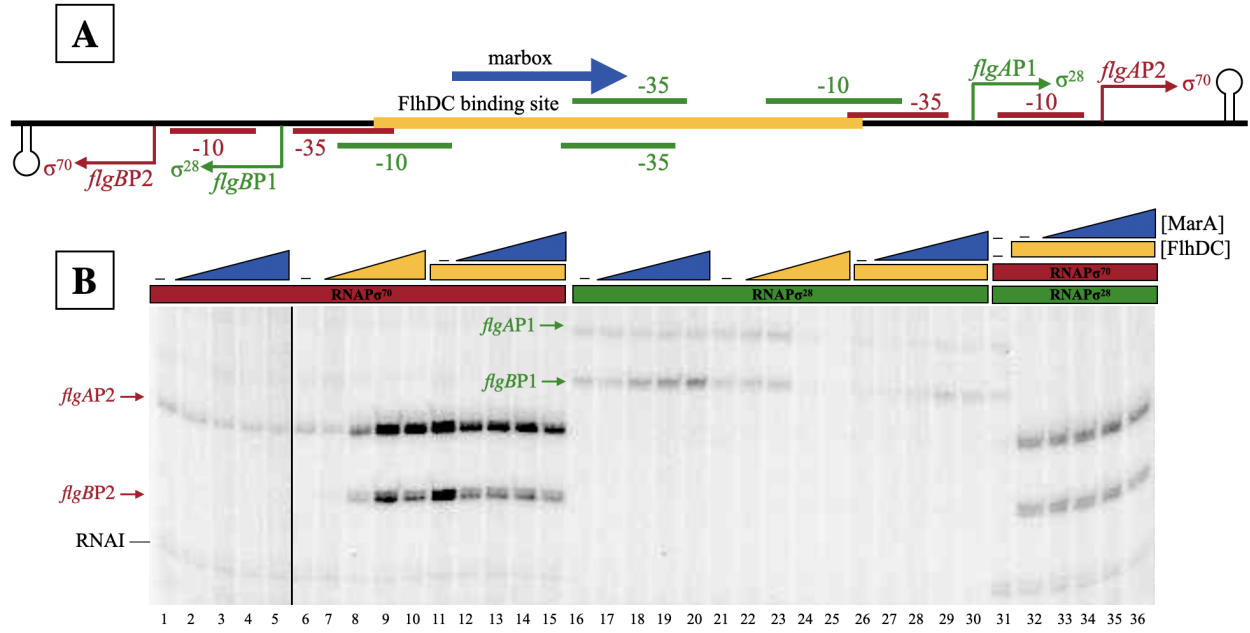


Figure 5.1: Transcription of the *flgAB* intergenic region with MarA.

A. Schematic of *flgAB* intergenic region.

B. *In vitro* transcription from the *flgB* derivative done with increasing concentrations of MarA (1 μ M – 2 μ M – 4 μ M – 5 μ M) represented by blue triangles.

Images of 8 % polyacrylamide gels used to separate transcripts generated *in vitro* using *flgAB* regulatory region derivative. Increasing concentrations of FlhDC (0.05 μ M – 0.1 μ M – 0.2 μ M – 0.25 μ M) represented by yellow triangles. Yellow rectangles represent 0.2 μ M concentration of FlhDC. RNAP σ^{70} is indicated by red rectangles and used at a concentration of 0.15 μ M. RNAP σ^{28} is indicated by green rectangles; 1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme and used to initiate transcription. Bands formed by transcripts originating from *flgAP1*/P2 and *flgBP1*/P2 are indicated by red or green arrows when RNAP σ^{70} or RNAP σ^{28} were used, respectively. The control RNAI band is shown by a black dash, and a black line between lanes 5 and 6 denotes where gels were merged. Experiment was repeated three times with consistent results.

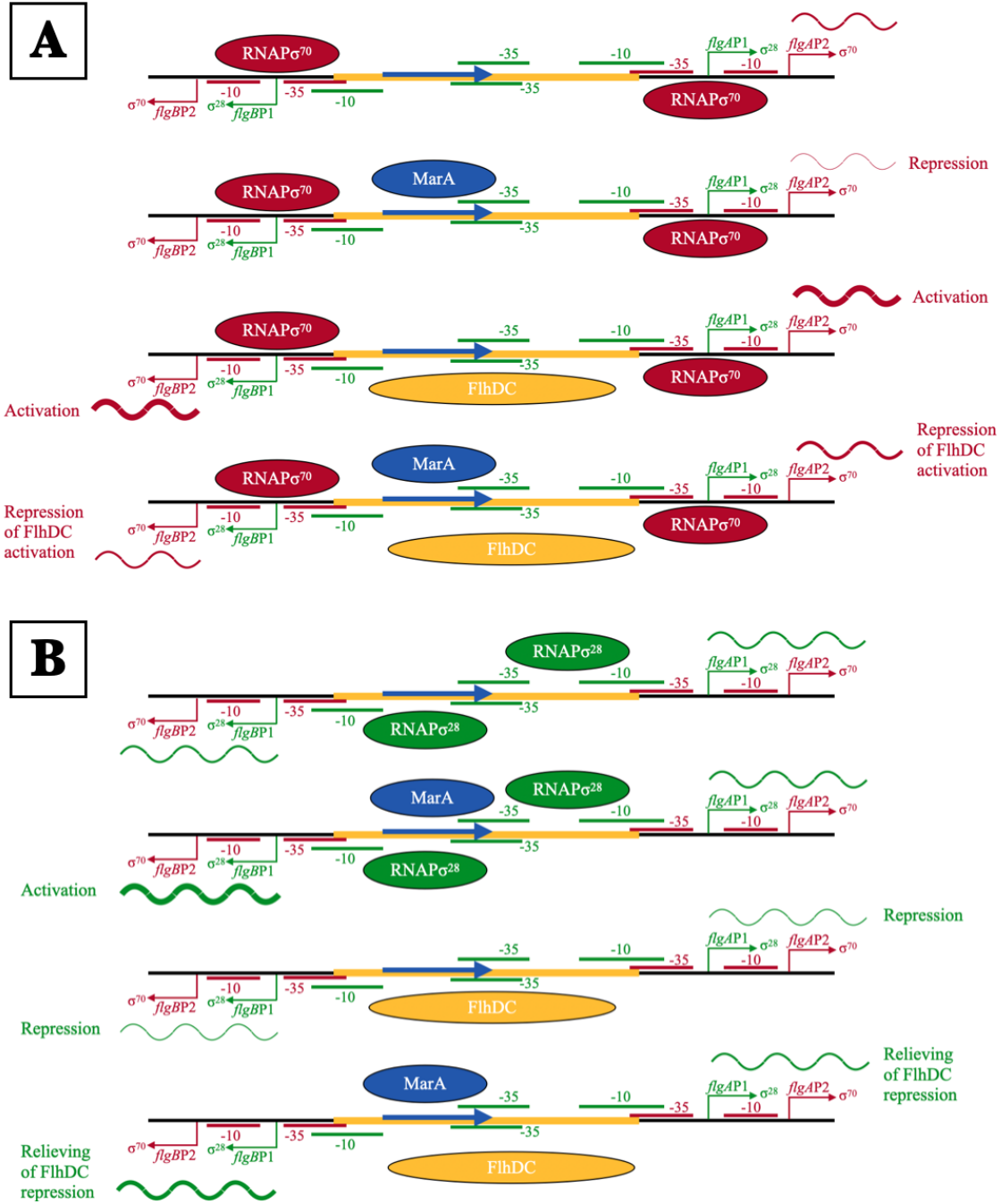


Figure 5.2: Schematic of *in vitro* transcripts from the *flgAB* regulatory region in the presence of FlhDC and/or MarA from pLSR plasmid.

A, B. Models of transcripts generated in the presence of RNAP σ^{70} and RNAP σ^{28} , respectively, with transcriptional activators.

On the schematics FlhDC binding site is shown in yellow, *marbox* and its direction is shown in blue arrow, σ^{70} -binding and σ^{28} -binding sites are in red and green and underlined, respectively. Transcription start sites are shown in blended arrows, in red or green and underlined for σ^{70} or σ^{28} , respectively.

motors, and a 116 bp transcript was generated from the latter (lanes 6-10). When both FlhDC and MarA were added, MarA slightly repressed both promoters, but affected transcription from *flgBP2* most (lanes 11-15).

With RNAP σ^{28} , 154 bp and 141 bp transcripts were generated from the *flgAP1* and *flgBP1* promoters, respectively (lane 16). Further addition of MarA activated the *flgBP1* promoter and did not have any effect on *flgAP1* (lanes 17-20). Addition of FlhDC led to repression of both σ^{28} -dependent promoters (lanes 21-25). Increasing concentrations of MarA, in the presence of FlhDC, relieved repression caused by FlhDC and partially restored transcription from *flgAP1* and *flgBP1* (lanes 26-30). We did not see this effect in previous experiments with pSR plasmid, as FlhDC did not repress transcription from the σ^{28} -dependent promoters clearly (Figures 4.3B, lanes 26-30 and 4.10A, lanes 26-30, respectively). Also, in previous assays, using the pSR DNA template, MarA activated *flgBP1* less efficiently (Figure 4.10A, lanes 16-20).

When both RNAP σ^{28} and RNAP σ^{70} were added, transcripts from *flgAP1* and *flgBP1* were generated as well as *flgAP2*, but the later was not as active as with RNAP σ^{70} only (Figure 5.1B, lane 31). When FlhDC was added, the σ^{28} -dependent *flgAP1* and *flgBP1* promoters were repressed (lane 32), whilst σ^{70} -dependent *flgAP2* and *flgBP2* promoters were activated. Addition of MarA did not affect transcription from those promoters (lanes 33-36).

5.3 SoxS activates *flgB* σ^{28} promoter in a way similar to MarA

According to the previous section, MarA activates the σ^{28} -dependent *flgBP1* promoter, even though it binds between the -10 and -35 elements for σ^{28} . MarA is a member of AraC/XylS family of transcriptional regulators that are characterised by two conserved HTH DNA-

binding motifs [348, 230, 349]. SoxS is 106 amino acids long, also belongs to AraC/XylS family, and shares 45% sequence identity with MarA protein [318]. SoxS is a component of the *soxRS* redox-sensing system responsive to superoxide regulon and binds to the *soxbox*, which is very similar to the *marbox* consensus. Hence, MarA and SoxS have overlapping regulatory effects [351]. Thus, we wanted to understand if SoxS have similar or different regulatory effect compared to MarA.

First, transcription with RNAP σ^{70} was done. With RNAP σ^{70} alone, only the *flgAP2* transcript is detected (Figure 5.3, lane 1). Increasing concentrations of SoxS led to repression even greater than observed with MarA at the same promoter (lanes 2-5). As observed previously, FlhDC activated both the *flgAP2* and *flgBP2* promoters (lanes 6-10). When both transcriptional activators were added, SoxS repressed this transcription (11-15). With RNAP σ^{28} alone, both *flgAP1* and *flgBP1* are active (lane 16). Addition of SoxS activated *flgBP1* and slightly repressed *flgAP1* (lanes 16-20). FlhDC addition led to *flgAP2* and *flgBP2* repression (lanes 21-25). When both SoxS and FlhDC were added, SoxS relieved repression caused by FlhDC only from *flgBP1* (lanes 26-30). With both RNAP σ^{70} and RNAP σ^{28} , weak transcripts from *flgAP1*, *flgAP2* and *flgBP2* were generated (lane 31). FlhDC activated σ^{70} -dependent *flgAP2* and *flgBP2* promoters and repressed σ^{28} -dependent *flgAP1* and *flgBP1* (lane 32). Addition of SoxS slightly repressed *flgAP2* and *flgBP2*, however, it did not restore activity of *flgAP1* and *flgBP1* (lanes 33-36).

Overall, the effect of SoxS is similar to MarA; both activate the σ^{28} -dependent *flgBP1* promoter and relieve the repression of this promoter caused by FlhDC binding. This might suggest that both MarA and SoxS activate the promoter in a similar manner. The data also suggest that conformation of σ_4^{28} is not optimal for direct interaction with MarA or SoxS at Class II promoters, since neither activate *flgAP1*. The position of *marbox* with respect to *flgBP2* suggests that MarA and SoxS do not make direct contacts with RNAP σ^{28} . Instead, we speculate that the regulators might change promoter conformation, between the core -10

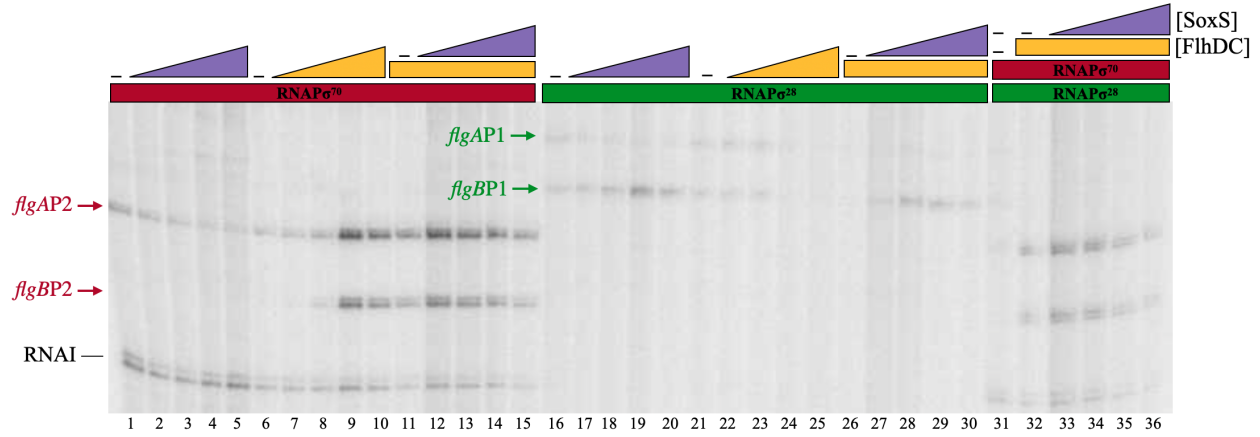


Figure 5.3: Transcription of the *flgAB* intergenic region with SoxS.

In vitro transcription from the *flgAB* regulatory region done with increasing concentrations of SoxS (1 μM – 2 μM – 4 μM - 5 μM) represented by purple triangles.

Images of 8 % polyacrylamide gels used to separate transcripts generated *in vitro* using *flgAB* regulatory region derivative. Increasing concentrations of FlhDC (0.05 μM – 0.1 μM – 0.2 μM - 0.25 μM) represented by yellow triangles. Yellow rectangles represent 0.2 μM concentration of FlhDC. RNAP σ^{70} is indicated by red rectangles and used at a concentration of 0.15 μM . RNAP σ^{28} is indicated by green rectangles; 1.75 μM of σ^{28} was mixed with 0.2 μM of RNAP core enzyme and used to initiate transcription. Bands formed by transcripts originating from *flgAP1/P2* and *flgBP1/P2* are indicated by red or green arrows when RNAP σ^{70} or RNAP σ^{28} were used, respectively. Control RNAI band is indicated by black dash. Experiment was done once.

and -35 elements for σ^{28} , like MerR, BmrR and CueR activators, as both SoxS and MarA bind between -10 and -35 element and presumably, change conformation of the DNA [349, 454, 452]. Indeed, MarA and SoxS is known to bend DNA 35° [212, 234].

5.4 EMSA analysis of MarA and RNAP σ^{28} binding to *flgAB* intergenic region

To understand if MarA assists RNAP σ^{28} in binding, we did an EMSA analysis (Figure 5.4). We used the 154 bp *flgB* regulatory region fragment, schematic of which is shown in Figure 5.1A. When MarA or RNAP σ^{28} are added, MarA:DNA or RNAP σ^{28} :DNA complexes are formed (lanes 2 and 3, respectively). As the MarA concentration increase in the presence of RNAP σ^{28} , we made two observations. First, the RNAP σ^{28} :DNA band shifts up on gel (lanes 4-8). This indicates formation of a ternary complex also containing MarA. Second, we did not observe a complex between the DNA alone and MarA at high MarA concentrations. This shows that MarA preferentially forms co-complexes with DNA and RNAP σ^{28} , rather than forming complexes with DNA alone. Hence, MarA and RNAP σ^{28} bind the *flgB* DNA fragment cooperatively.

5.5 Permanganate footprinting analysis of MarA and RNAP σ^{28} at *flgAB* intergenic region

Our EMSAs suggest that MarA and RNAP σ^{28} binding the *flgBP1* promoter DNA simultaneously and cooperatively activate transcription. To further investigate the mechanism of transcription activation, and to understand the impact of DNA bending, we did potassium permanganate footprinting (Figure 5.5A). This method detects perturbations in DNA organisation. In particular, DNA unwinding during transcription initiation is easily observed.

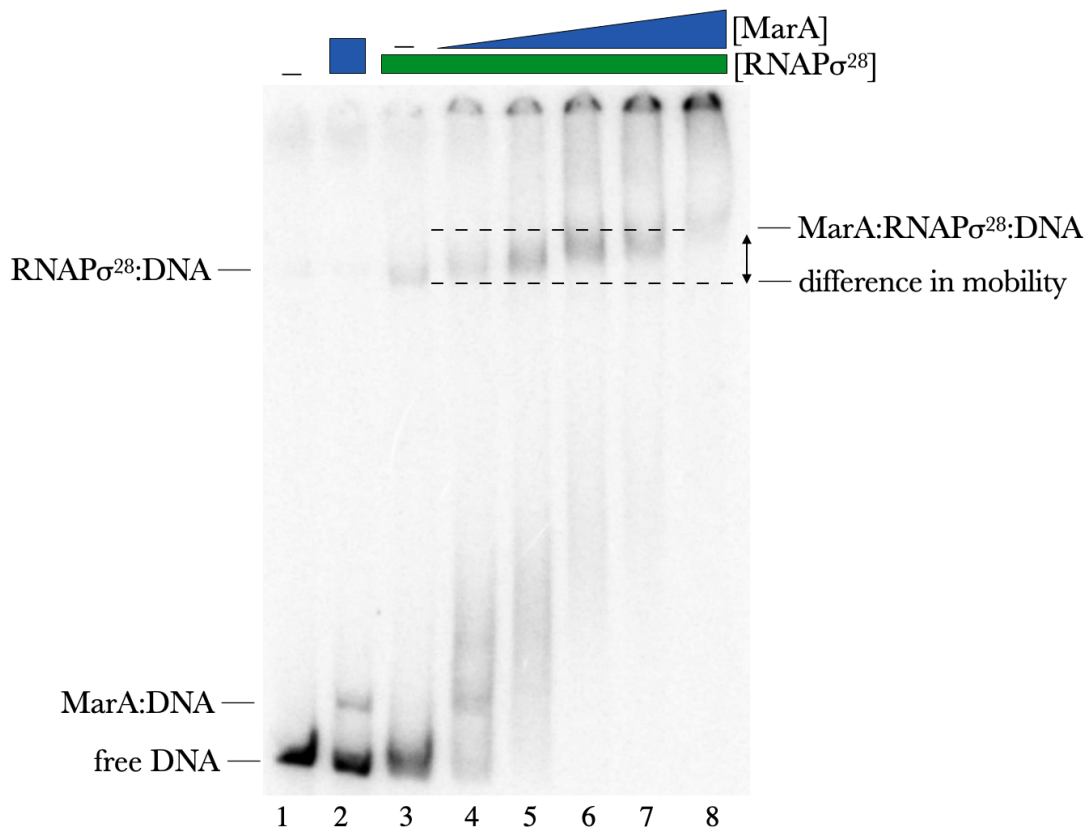


Figure 5.4: Binding of the MarA and RNAP σ^{28} to the *flgAB* intergenic region in EMSA. The EMSA assays were carried out with the *PflgB* derivative. Images show the separation of the MarA:DNA and RNAP σ^{28} :DNA complexes on the 6% polyacrylamide gels. Increasing concentrations of MarA (0.25 μ M – 0.5 μ M – 0.75 μ M – 1 μ M – 1.25 μ M) represented by blue triangles. The 1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme for each reaction; this concentration of RNAP σ^{28} is represented by green rectangle. MarA:DNA, RNAP σ^{28} :DNA complexes and free DNA are indicated. Experiment was repeated three times with consistent results.

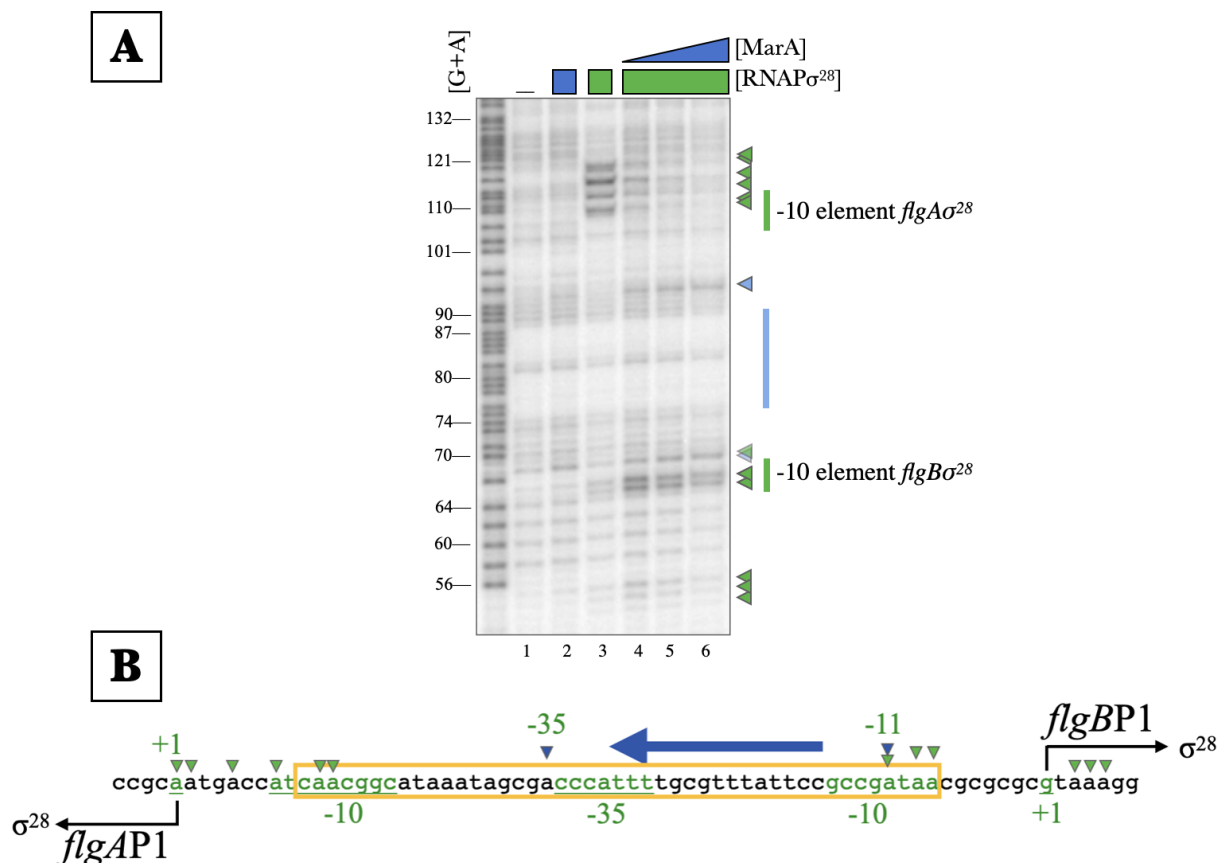


Figure 5.5: *In vitro* KMnO₄ footprint of MarA and RNAP σ^{28} .

A. *In vitro* KMnO₄ footprint from the *flgB* DNA fragment incubated with increasing concentrations of MarA (0.25 μ M – 0.5 μ M – 0.75 μ M) represented by blue triangle and RNAP σ^{28} (1.75 μ M of σ^{28} was mixed with 0.2 μ M of RNAP core enzyme) represented by green rectangle. In the second lane maximum 0.75 μ M MarA concentration was used, represented by blue square. Images of 8 % polyacrylamide gels used to separate DNA fragments of *flgB* DNA fragment derivative generated in KMnO₄ footprint. GA ladder is shown on the left of the gel image.

B. Sequence of the *flgAB* intergenic region with *flgAP1* and *flgBP1* promoters with highlighted bases of the open complexes revealed in KMnO₄ footprint.

Green triangles represent distortions in the open complexes revealed by the KMnO₄ footprint gel, green dashes represent -10 elements. Blue triangles represent distortions made by MarA, blue dash represent *marbox*.

Experiment was repeated more than three times with consistent results.

Permanganate selectively oxidizes thymine residues in ssDNA regions but not in dsDNA, revealing T residues of an open complex, if one is formed [428].

Surprisingly, when MarA was added alone (lane 2), sites of permanganate sensitivity appeared 5 nt upstream and downstream of the MarA binding site (MarA distortions are shown by blue triangles on Figure 5.5B). These distortions correspond to positions -11 and -35, with respect to the *flgBP1* TSS. This likely happen because MarA bends DNA 35° [212]. When RNAP σ^{28} is added alone, an open complex forms at *flgAP1*, indicated by distortions corresponding to positions +1, -1, -4, -7, -10 and -11, with respect to the *flgAP1* TSS (lane 3). The signal for the *flgBP1* transcription bubble is very weak and only partially formed. Specifically, distortions appeared at the +5, +4, +3, -8 and -9 positions with respect to the *flgBP1* TSS. However, conserved -11A remains double stranded [51].

Interestingly, the critical base for nucleation of DNA unwinding at position -11A of the *flgBP1* promoter is the same position distorted by MarA binding (lane 2). When both MarA and σ^{28} are present (lanes 4-6), a full *flgBP1* open complex forms at positions +5, +4, +3, -8, -9, as well as -11 according to the permanganate reactions (lanes 4-5). Simultaneously, DNA opening at *flgAP1* is lost. This suggests that MarA not only facilitates nucleation and formation of the *flgBP1* transcription bubble but also shifts transcriptional activity from *flgAP1* to genes transcribed from *flgBP1*. This may be because MarA induces a second distortion at position -26 relative to the *flgAP1* TSS, close to its -35 element. This might inhibit RNAP σ^{28} binding to *flgAP1*.

Overall, these data suggest that MarA binds between the -10 and -35 elements of *flgBP1*, introduces DNA distortions that promote unwinding and stabilisation of the *flgBP1* transcription bubble. This unwinding might provide an advantage to the otherwise weak *flgBP1* promoter over the stronger *flgAP1*.

5.6 Model of MarA interaction with an RNAP σ^{28} open complex

To understand how MarA and RNAP σ^{28} might co-bind *flgBP1*, we used models generated from structures of MarA (1BL0 [212]) and the RNAP σ^{28} open complex (6PMI [65]). The DNA of 6PMI was aligned with the *flgBP1* promoter to bring the structures into the correct position (Figures 5.6A). We further introduced a 35° DNA bend of MarA created upon binding. This places MarA DNA approximately 90° away from the bound RNAP σ^{28} . The base pairs deformed by MarA binding, according to permanganate footprinting data, are highlighted red in Figures 5.6B and 5.7. These locations correspond to the first base of the open complex and the base adjacent to the -35 element of the *flgBP1* promoter (highlighted in red on the Figure 5.6). According to our model, MarA makes no contacts with RNAP σ^{28} (Figure 5.7).

5.7 *flgAB* intergenic region is not widespread among other bacterial species

To investigate the conservation of *flgAB* regulatory region across different bacterial species, a BLAST analysis was performed using the *flgAB* intergenic sequence from *E. coli* JCB387. The aim was to identify homologous sequences in other bacterial genomes. For the BLAST analysis, we took the whole *flgAB* intergenic region from the *flgA* TSS to *flgB* TSS, including ATG of both genes, total length of the sequence is 160 nt long. The top BLAST hits, with 100% identity to the *flgAB* sequence from *E. coli* JCB387, revealed that 3307 strains have 100% identical sequence to the query. Most of them (3188) are *E. coli* strains; but this sequence is also found in 2 strains of *Shigella sonnei*; 2 strains of *Shigella boydii*, 3 strains of *Shigella dysenteriae*, 108 strains of *Shigella flexneri*, 3 strains of *Escherichia sp.* and 1 un-

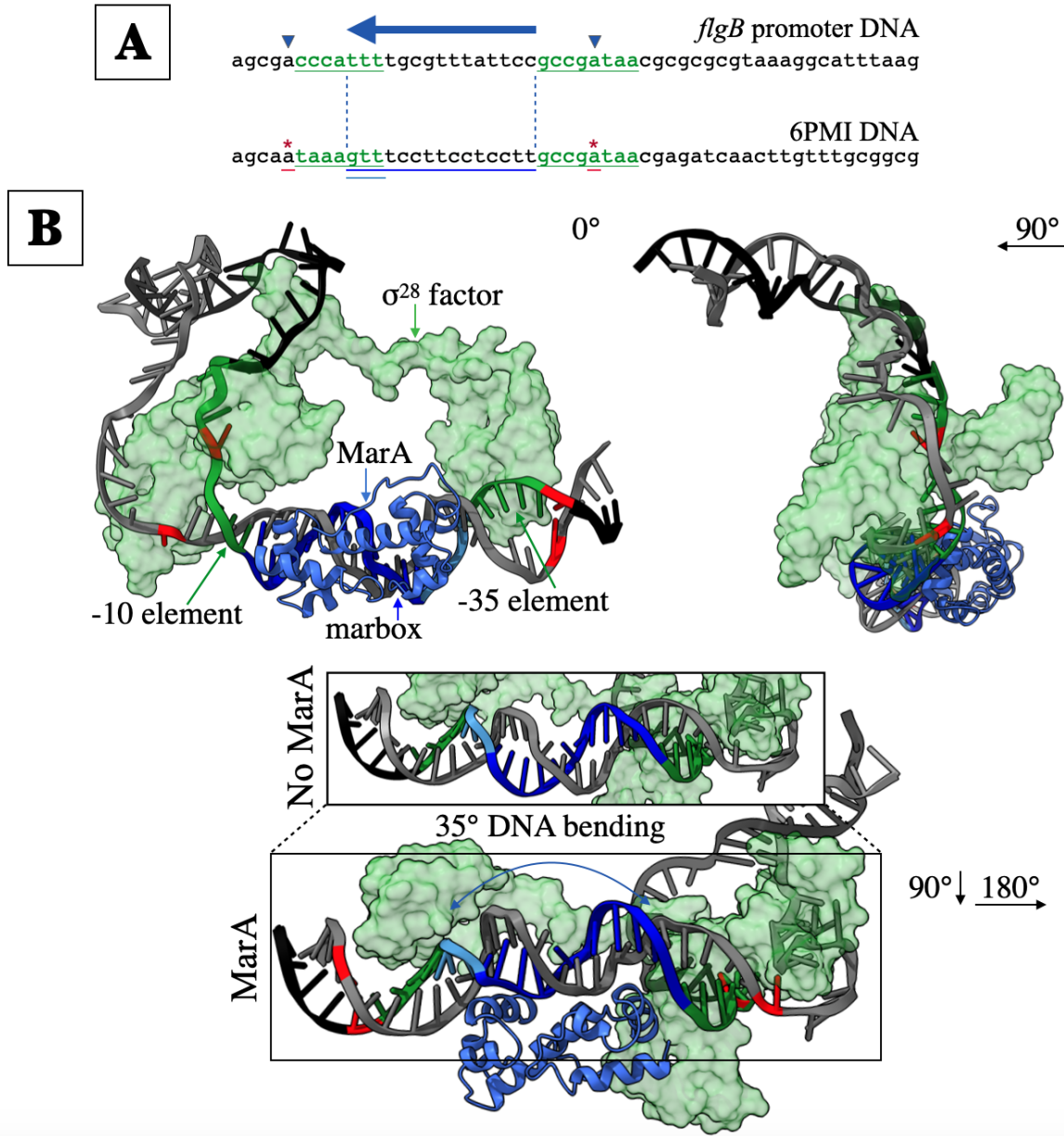


Figure 5.6: Model of MarA and σ^{28} interaction on *flgB* σ^{28} open complex.

A. Alignment of *flgB* promoter DNA and synthetic DNA used for obtaining cryo-EM open complex of RNAP σ^{28} .

B. Model of *flgB* σ^{28} open complex with σ^{28} and MarA. MarA is in blue, σ^{28} factor is in green. Template strand is in black, non-template strand is in grey. The -10 and -35 elements of σ^{28} are in green, *marbox* is in blue, bases where *marbox* and -35 element overlap are in light blue. Distortions made by MarA binding are colored in red.

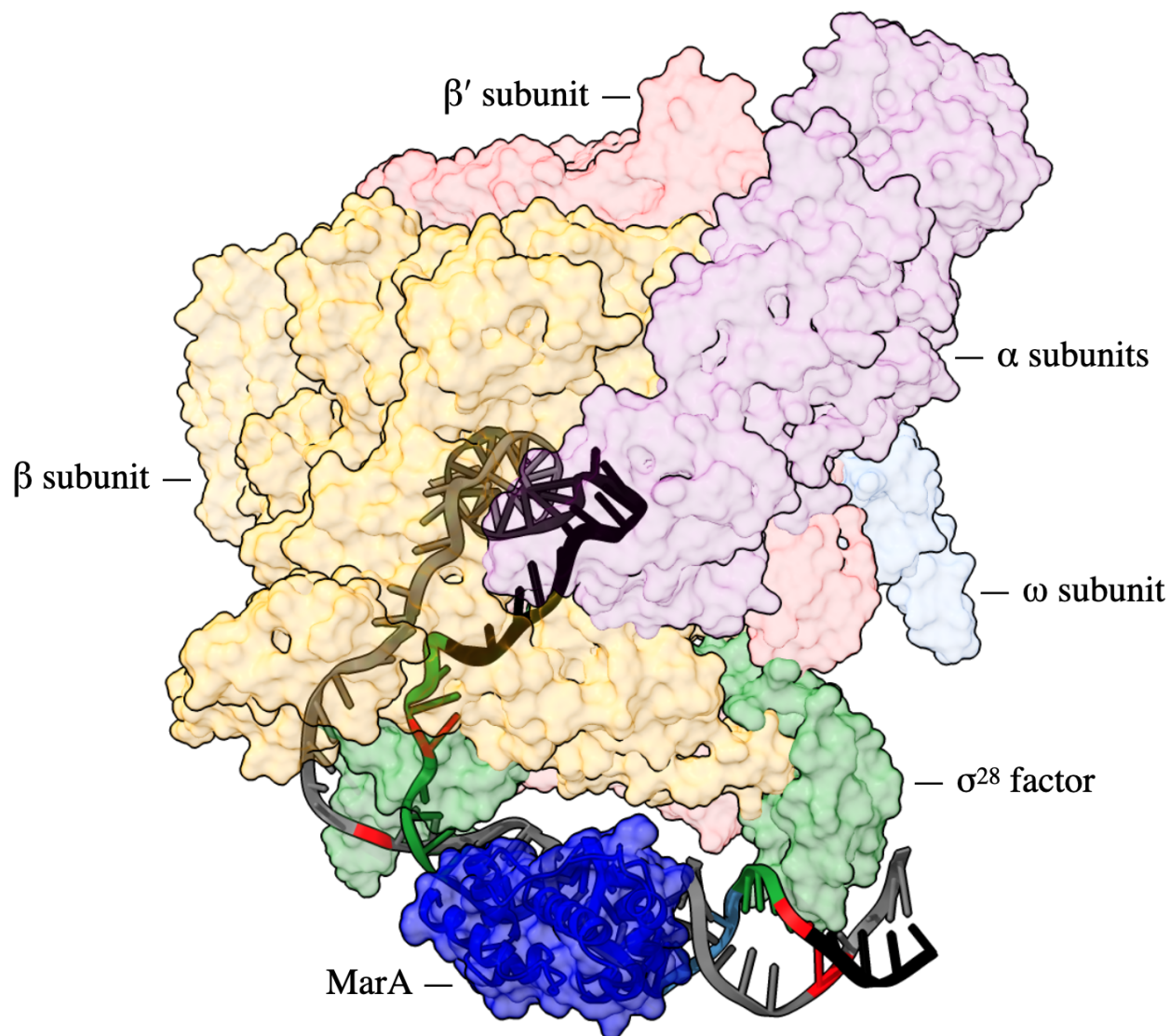


Figure 5.7: Model of MarA binding to the *flgBP1* promoter in the context of RNAP σ^{28} . MarA is in blue, σ^{28} factor is colored in green, both α subunits are colored in purple, β subunit is in orange, β' subunit is in red, ω subunit is in light blue. Template strand is in black, non-template strand is in grey. The -10 and -35 elements of σ^{28} are in green, *marbox* is in blue, bases where *marbox* and -35 element overlap are in light blue. Distortions made by MarA binding are colored in red.

cultured bacterium (Table 5.1). This suggests that the architecture of the *flgAB* regulatory region is not widespread among other bacterial species and might be unique for some *E. coli* strains.

5.8 Conservation of MarA and σ^{28} binding sites in other Enterobacteriaceae species

In the previous BLAST search we used the whole *flgAB* intergenic region to find sequence similarities. However, *flgAB* region is not conserved among the bacteria species, so the identity was quite low. To understand if MarA and σ^{28} binding sites are conserved in the context of the *flgAB* intergenic region in other Enterobacteriaceae species, we took a 47 nt sequence of the *E. coli* K-12 *flgB* regulatory region, which includes the *flgAP1*, *flgBP1* promoters and the *marbox* only, to make the BLAST search more targeted. Further, we performed BLAST analysis against other gram-negative motile bacterial species from Enterobacteriaceae family, such as *Escherichia fergusonii*, *Salmonella enterica*, *Klebsiella pneumoniae*, *Serratia marcescens*, *Enterobacter aerogenes*, *Enterobacter cloacae*, *Yersinia pestis*, *Yersinia enterocolitica*, and *Citrobacter freundii*.

The BLAST analysis revealed 806 strains with somewhat similar sequences to the 47 nt long regulatory region of *flgAB*, containing MarA and novel σ^{28} binding sites. We selected six most relevant strains from the BLAST search and performed a ClustalW alignment. We noticed that the aligned sequences have overall high similarity, particularly in regions where the *marbox*, *flgA* σ^{28} , and *flgB* σ^{28} promoter elements are expected to be located. Thus, we assumed that these strains likely contain putative MarA and σ^{28} binding sites, which we highlighted in Figure 5.8

However, ClustalW alignments show that the sequence is mostly conserved across only 75 strains of *E. fergusonii*. Matches were found with 93.33% identity, covering 95% of the

Table 5.1: Species with 100% Identity in the *flgAB* intergenic region

Scientific Name	Number of strains	Score (Max/Total)	E-value	Percentage Identity	Alignment Length (nt)
<i>Escherichia coli</i>	3188	296/296	1E-75	100.00	160
<i>Shigella flexneri</i>	108	296/296	1E-75	100.00	160
<i>Shigella dysenteriae</i>	3	296/296	1E-75	100.00	160
<i>Shigella boydii</i>	2	296/296	1E-75	100.00	160
<i>Shigella sonnei</i>	2	296/296	1E-75	100.00	160
<i>Escherichia sp.</i>	3	296/296	1E-75	100.00	160
Uncultured	1	296/296	1E-75	100.00	160

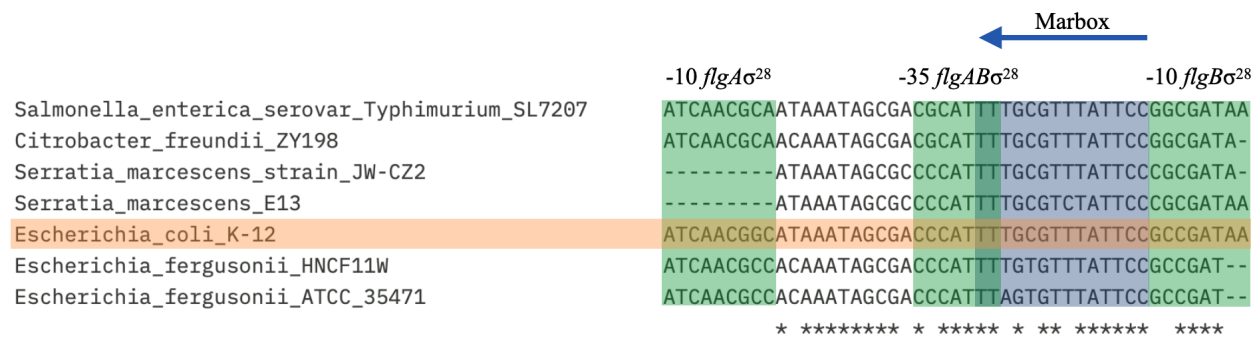


Figure 5.8: ClustalW alignment of flgAB intergenic regions in other Enterobacteriaceae species.

Query sequence is highlighted in orange, -10 and -35 elements are highlighted in green, *marboxes* are highlighted in blue. Bases common for all Enterobacteriaceae species tested are marked in asterisks.

sequence of interest, suggesting strong conservation of this regulatory region within this species. There are only 2-3 mismatches in the sequence, and 1-2 of them target the MarA binding site.

The *E. coli* region also shows 91% identity to 304 *Salmonella enterica* serovar *Typhimurium* strains, with a 100% query cover. Nevertheless, 2 and 1 mismatches target the *flgA* and *flgB* extended -10 element sequence, respectively, which might make both of those promoters weaker or inactive. *Marbox* sequence remains identical to the query. In 167 strains of *Citrobacter freundii*, this region shows 89% identity to the query sequence with 100% query cover. There are 6 mismatches to the query, 4 of which are located in similar positions to those in *Salmonella enterica* serovar *Typhimurium* strains, targeting the *flgA* and *flgB* extended -10 elements, however, *marbox* stays identical to the one in *E. coli* K-12 sequence. The 260 strains of *Serratia marcescens* are 89% identical to the query sequence, with 3-4 mismatches and 79%-80% query cover. Two of the mismatches are located in the first 2 positions of the *flgBP1* extended -10 element, which probably makes this promoter weak. Moreover, there is no *flgAP1* -10 element in this strain. Another mismatch targets the *marbox*, but it is not in the conserved positions of the *marbox* motif. The rest of the selected bacteria do not show sequence identity to the region in BLAST.

Overall, according to BLAST searches, the architecture of the *flgAB* intergenic region, with the *marbox* overlapping the *flgBP1* σ^{28} -dependent promoter, is widespread among *Escherichia*, but not in other species, even if they are from the same family and have motile lifestyles. *E. fergusonii* sequence is the closest to the query compare to other bacteria chosen for BLAST. In *Salmonella enterica* serovar *Typhimurium*, *Citrobacter freundii*, and *Serratia marcescens*, mismatches are found in the most important positions for σ^{28} promoter recognition. However, in all of those strains we found *marboxes* which are similar or very close to the *marbox* consensus sequences found in *flgAB* intergenic region in *E. coli*. This *marbox* position must be important for gene regulation in those species, but not in the context of

σ^{28} -dependent promoters.

5.9 Discussion

The results in this chapter reveal potential regulatory mechanism for activation of σ^{28} -dependent *flgBP1* promoter by MarA. The data also show how differently this activator influences transcription from the nearby σ^{70} - and σ^{28} -dependent promoters. Both MarA and its homolog SoxS bind to the *marbox*, that overlaps the upstream -35 element of *flgAP1* σ^{28} -dependent promoter. On the opposite strand of the DNA, *marbox* locates between -10 and -35 element of the *flgBP1* σ^{28} -dependent promoter. Despite the canonical positioning of the *marbox* for activation of the *flgAP1*, both MarA and SoxS activate the σ^{28} -dependent *flgBP1* promoter. This unexpected activation pattern might be due to differences between σ^{70} and σ^{28} domains 4 and 2, which interact with the -35 and -10 elements, respectively.

The EMSA analysis suggests that MarA may assist in the binding of RNAP σ^{28} , which leads to enhancing of transcription initiation from *flgBP1*. When MarA binds to the DNA, it bends *flgBP1* promoter DNA 35° [212]. In the permanganate footprint with MarA alone, two distortions on the DNA appeared 5 nt upstream and downstream of the *marbox*, probably due to the DNA bending by MarA. One of those distortions coincides with the first base of the open complex at *flgBP1*, which facilitates nucleation of DNA unwinding.

Transcriptional activation by targeting the promoter structure never has been reported for the AraC/XylS family of transcriptional activators. However, this type of activation is quite common for MerR family of transcriptional activators in *E. coli*. MerR TFs bind the abnormally long spacer (19-20 bp) and introduce distortions, that shortens the space between -10 and -35 elements, restoring the spacer geometry closer to canonical 17-bp and optimal for RNAP recognition. Similar to MerR TFs, MarA binds between -10 and -35 elements and distorts promoter DNA without contacting RNAP σ^{28} , however, there are several crucial

differences in how MarA activates the *flgBP1* promoter.

When MarA binds between the -10 and -35 elements of the *flgBP1* promoter, it introduces 2 kinks in the DNA, 5nt upstream and downstream the *marbox*. The latter kink induces specific distortions in the -10 element itself. The upstream kink is positioned at the -35, adjusted to the -35 element. According to our model, MarA binds two major groove sections approximately 90° offset from RNAP binding. Similar to MerR, MarA probably does not interact with RNAP. Instead, MarA helps to unwind duplex DNA via pre-opening of the conserved -11A base of the *flgBP1*, make it more accessible for σ factor. Thus, the distortion, created by MarA, helps σ_2^{28} domain to access -11A base more easily to initiate nucleation of the dsDNA.

The *flgBP1* -10 element is identical to the canonical motif of σ^{28} (GCCGATAA), and the *flgAP1* -10 element is a more distant match (GCCGTTGA). Even so, σ^{28} is able to form a good open complex with *flgAP1* -10 element, but not with *flgBP1*. This is probably because the -35 element (AAAATGG) of the *flgBP1* promoter is a very poor match to the consensus (TAAAGTT), and binding of the -35 element is needed to direct the RNAP to form an open complex with -10 sequence. When MarA binds *flgBP1 marbox*, binding of RNAP σ_4^{28} is stabilised (Figure 5.4), and the downstream kink distorts the first base of the *flgBP1* -10 element where the unwinding of the otherwise partially closed open complex nucleates [51]. The upstream MarA kink disturbs the *flgAP1* -35 element in a way that positioning, and subsequently, binding of RNAP σ_4^{28} to the *flgAP1* promoter becomes harder. Thus, *flgBP1* promoter gets an advantage and is being activated when MarA binds to it.

We also run a BLAST analysis, which indicates that the *flgAB* regulatory region is somewhat conserved among *Escherichia* species but not widespread among other Enterobacteriaceae with the motile lifestyle. The presence of the *marbox* in several strains suggests its importance for gene regulation, though not necessarily in the context of σ^{28} -dependent promoters. This specificity could suggest a unique regulatory mechanism in *E. coli* and some

Shigella strains that may not be present in other closely related species. Thus, the regulatory architecture of the *flgAB* intergenic region, involving MarA, particularly its activation of the σ^{28} -dependent *flgBP1* promoter, is a distinctive feature of the *flgAB* intergenic region in *E. coli*. Our data shows the complexity of transcriptional regulation involving different sigma factors and transcriptional activators, and the need for fine-tuned gene regulation to achieve optimal gene expression.

Chapter 6

Final conclusion

Gene expression is a critical process for function and adaptation to environmental changes in bacteria. Regulation of gene expression allows bacteria to efficiently respond to various stimuli, ensuring survival and optimal growth in diverse conditions [138]. In *E. coli*, fine-tuned regulation of flagellar gene expression is particularly important, as it enables the bacterium to balance the energy requirements of motility with the need to adapt to environmental conditions. This control not only optimises energy use, but also plays a key role in pathogenicity, biofilm formation, and invasion in the host [397, 455, 379].

The traditional model of flagellar biosynthesis suggests ordered assembly of the flagellum, started by σ^{70} - and FlhDC-dependent activation of genes required in early stages of the process. Early genes encode for the base plate of the flagellum, located in the interior of the cell, and for σ^{28} , which switches on expression of late flagellar genes [145, 456]. Late genes are required for completion of the flagellum on the cell exterior. In more recent studies, σ^{28} -dependent promoters were found upstream σ^{70} -dependent flagellar genes, challenging long standing narrative [388, 389, 90]. The reason of double regulation of some flagellar genes by σ^{70} and σ^{28} is poorly understood.

In this work, the second layer of flagellar genes regulation was studied. I found new *flgA* σ^{28} , *flgB* σ^{28} , *flgJ* σ^{28} and *flgM* σ^{70} promoters, further expanding the existing regulatory cascade of flagellar genes. Current research shows that MarA, a well known activator of intrinsic resistance to antibiotics in *E. coli*, also play a role in regulation of some of those novel promoters. More specifically, MarA assist RNAP σ^{28} at the promoter found upstream *flgBCDEFGHIJ/KL* operon, and represses σ^{28} -dependent promoter of *flgAMN* *in vitro*. It is also important to highlight that *flgAMN* operon encodes FlgA and FlgN chaperons for flagellar assembly and FlgM anti- σ^{28} factor, that represses σ^{28} activity [386]. The *flgBCDEFGHIJ/KL* operon encodes structural subunits for the flagellar upper basal body and hook [388]. Thus, *in vitro* MarA assists RNAP σ^{28} to redirect transcription flow away from *flgAMN* operon, where anti- σ^{28} factor is encoded and promote transcription of *flgBCDEFGHIJ/KL*

operon. In this case, redirection of transcriptional flow might be advantageous for other σ^{28} -dependent promoters, as less anti- σ^{28} factor may be produced. Nevertheless, MarA activate both *flgAMN* and *flgBCDEFGHIJ/KL* σ^{70} -dependent promoters *in vivo* (presumably NAPs knock out, or activation of FlhDC's ligand or activator). It is also important to notice that in both *in vivo* and *in vitro* experiments non-motile *E. coli* JCB387 strain was used, which could affect *in vivo* results.

MarA is a well-known activator of σ^{70} -dependent promoters, and there is no prior evidence of MarA assisting polymerase other than RNAP σ^{70} . However, in current research, it has been shown that MarA assists RNAP σ^{28} at the *flgB* promoter, by creating a distortion in the conserved -11A base of the cognate open complex, where unwinding nucleates. This unusual mechanism of transcription activation has not been previously observed, but it is possible that MarA could activate other genes in the same manner. Similarly, other transcriptional regulators might activate other promoters through the same mechanism.

If MarA reduces transcription of anti σ^{28} -factor, MarA could indirectly activate other σ^{28} -dependent promoters, which are active in late flagellum synthesis. This might be important for a cell that had previously expressed the motility apparatus, but became non motile if flagella were damages or ejected. In the case of antibiotics exposure, a cell might require a flagellum to be rebuilt from a relic of the previous flagellum to escape antibiotics. This might be the reason why the σ^{28} -dependent promoters, which are usually active in the late stages of flagellar biosynthesis, might be utilised instead of activating flagellar synthesis from *de novo* [457, 458, 459]. Bacteria can disassemble their flagellum, leaving behind the flagellar outer membrane complex (FOMC), which composition varies between species, but usually include the L- and P- rings. So in this case, redirection of the transcription flow to the *flgBCDEFGHIJ/KL* operon away from *flgAMN*, might be beneficial for the cell. In this scenario, σ^{28} -dependent flagellar promoters will be activated, and flagellar biosynthesis will start from later genes, as the flagellar base is already present. However, further studies on

motile MarA mutants must be done to prove this suggestion.

Our *in silico* analysis shown that architecture of the *flgBCDEFGHIJ/KL* regulatory region might not be widespread among other bacterial species. The bioinformatics search revealed that the σ^{28} promoter in this region is unique to *E. coli* strains and not found in other motile members of Enterobacteriaceae. However, the *marbox* positions is quite fixed. Thus, MarA regulation of *flgBCDEFGHIJ/KL* operon might be different for other bacteria under antibiotic stress.

Nevertheless, further studies are needed to confirm MarA role in flagellar biosynthesis. For instance, ChIP-seq should be performed with both RNAP σ^{28} and MarA to determine the genome-wide localisation of their binding sites and to understand if there is any overlap between them. ChIP-seq can be conducted under conditions different from the standard. For example, with and without salicylic acid on motility agar, to evaluate whether the induction of MarA and its homologs affects genes involved in flagellar biosynthesis, including novel σ^{28} -dependent *flgA* and *flgB* promoters. In this experiment, *E. coli* MG1655 can be used as a motile strain.

ChIP-qPCR can also be utilised to evaluate MarA occupancy at potential binding sites upstream of flagellar genes. If additional MarA binding sites are identified in the ChIP-seq, they should be validated using EMSA and *in vitro* transcription experiments to determine the regulatory effects MarA has on these promoters. Additionally, in both EMSA and *in vitro* transcription experiments, quantification of the signal in different lanes would help in interpreting the results.

Overall, this work examined the regulatory role of MarA in flagellar biosynthesis. We showed that MarA regulate some promoters of flagellar genes both *in vivo* and *in vitro*. However, more studies must be done to understand if MarA have a bigger role in flagellar biosynthesis, and whether its activation of genes involved in this process represents an additional intrinsic mechanism of antibiotic resistance in *E. coli*.

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