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Úloha transkripčních faktorů MoaB2 a HelD v regulaci exprese mykobakteriálních genů

The role of transcription factors MoaB2 and HelD in mycobacterial gene expression regulation

Disertační práce

Vedoucí práce: doc. Mgr. Libor Krásný, Ph.D.

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Doctoral Thesis

Supervisor: doc. Mgr. Libor Krásný, Ph.D.

Prague, 2024

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Mgr. Barbora Brezovská

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ABSTRACT

RNA polymerase (RNAP) is the central enzyme of bacterial gene expression that controls the transcription of genetic information into RNA. Its activity is tightly regulated by transcription factors (TFs) that ensure precise gene expression in response to environmental conditions. The study of RNAP and its regulatory mechanisms is urgently needed, especially in the context of increasing antibacterial resistance. This thesis explores the transcriptional apparatus in *Mycobacterium smegmatis*, focusing on two selected TFs, MoaB2 and HelD. The study of MoaB2 describes the discovery of its interaction with the primary sigma (σ) factor, σ^A. Structural and biochemical analyses then reveal that MoaB2 forms a specific complex with σ^A but not alternative σ factors. Through this complex MoaB2 modulates the activity and stability of σ^A. The study of HelD first identifies the complexes of RNAP to which HelD binds. The structures of these complexes are solved, revealing that HelD binds to RNAP in a unique manner, penetrating both the primary and secondary channels of RNAP, clearing RNAP of nucleic acids. The role of HelD in the transcription cycle is then defined. HelD releases stalled nonfunctional RNAPs from DNA. This complex then associates with other transcription factors, σ^A and RbpA, and participates in transcription initiation. During this process, HelD is released in a sequential manner that is driven by ATP hydrolysis and promoter DNA binding. Importantly, HelD distorts the binding pocket of rifampicin, a clinically important antibiotic, in RNAP, mitigating its inhibitory effect on transcription. Collectively, these findings offer novel insights into the architecture of the mycobacterial transcription machinery and may guide future development of antimycobacterial compounds.

ABSTRAKT

RNA polymeráza (RNAP) je ústředním enzymem bakteriální genové exprese, který řídí přepis genetické informace do RNA. Její činnost je přísně regulována transkripčními faktory (TF), které zajišťují přesnou expresi genů v reakci na podmínky prostředí. Studium RNAP a jeho regulačních mechanismů je naléhavě nutné, zejména v souvislosti s rostoucí antibakteriální rezistencí. Tato práce zkoumá transkripční aparát u *Mycobacterium smegmatis* se zaměřením na dva vybrané TF, MoaB2 a HelD. Studie MoaB2 popisuje objev jeho interakce s primárním sigma (σ) faktorem, σ^A. Strukturní a biochemické analýzy pak odhalují, že MoaB2 tvoří specifický komplex s σ^A, ale nikoli s alternativními σ faktory. Prostřednictvím tohoto komplexu MoaB2 moduluje aktivitu a stabilitu σ^A. Studie HelD nejprve identifikuje komplexy RNAP, na které se HelD váže. Struktury těchto komplexů jsou vyřešeny a odhalují, že HelD se váže na RNAP jedinečným způsobem, proniká primárními i sekundárními kanály RNAP a zbavuje RNAP nukleových kyselin. Poté je definována úloha HelD v transkripčním cyklu. HelD uvolňuje z DNA zastavené nefunkční RNAP. Tento komplex se pak spojuje s dalšími transkripčními faktory, σ^A a RbpA, a podílí se na iniciaci transkripce. Během tohoto procesu se HelD uvolňuje postupně, což je řízeno hydrolýzou ATP a vazbou na promotorovou DNA. Důležité je, že HelD narušuje vazebnou kapsu rifampicinu, klinicky významného antibiotika, v RNAP čímž zmírňuje jeho inhibiční účinek na transkripci. Souhrnně tato zjištění nabízejí nový pohled na architekturu mykobakteriálního transkripčního mechanismu a mohou být vodítkem pro budoucí vývoj antimykobakteriálních sloučenin.

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LIST OF ABREVIATIONS

aa	amino acid
AS	active site
ATC	anhydrotetracycline
B. subtilis	*Bacillus subtilis*
bp	base pair
CDS	coding sequence
ChIP-seq	chromatin immunoprecipitation sequencing
CMP	cytidine monophosphate
cPMP	cyclic pyranopterin monophosphate
CRISPR	clustered regularly interspaced short palindromic repeats
cryo-EM	cryogenic electron microscopy
DNA	deoxyribonucleic acid
E	RNA polymerase core
E. coli	*Escherichia coli*
EC	elongation complex
EMSA	electro-mobility shift assay
EXP	exponential phase
Eσ	RNA polymerase holoenzyme
GC	guanine-cytosine
GMP	guanosine monophosphate
IgG	immunoglobulin G
iNTP	initiation nucleoside triphosphate
IP	immunoprecipitation
kDa	kilodalton
M. smegmatis	*Mycobacterium smegmatis*
MoCo	molybdenum cofactor
MPT	molybdopterin
mRNA	messenger RNA
MW	molecular weight
nm	nanometer
nt	nucleotide
NTM	non-tuberculous mycobacteria
NTP	nucleoside triphosphate

OD	optical density
PAA	polyacrylamide
RAE	rifamycin-associated element
RIF	rifampicin
RNA	ribonucleic acid
RNAP	DNA-dependent RNA polymerase
RPc	closed promoter complex
RPo	open promoter complex
RNAseq	RNA sequencing
σ	sigma
σ^A_N	N-terminal domain of σ^A
SAXS	small-angle X-ray scattering
STA	stationary phase
TEC	transcription elongation complex
TF	transcription factor
TIC	transcription initiation complex
wt	wild type

INTRODUCTION

Understanding gene expression and especially its first step, transcription, in bacteria is essential to unraveling the complex molecular mechanisms that govern bacterial survival, adaptation and pathogenicity. This Doctoral Thesis focuses on *Mycobacterium smegmatis*, a non-pathogenic fast-replicating mycobacterium closely related to *Mycobacterium tuberculosis*, a pathogen of global concern. Moreover, *M. smegmatis* is related also to other non-tuberculous mycobacteria (NTM) such as emerging pathogens *Mycobacterium abscessus* and *Mycobacterium avium* (Koh 2017; Sparks et al. 2023). With the rise of antibiotic-resistant strains of *M. tuberculosis* and NTM, and the public health threat they pose, research into mycobacterial transcription has never been more urgently needed (Waller et al. 2023). Insights gained from *M. smegmatis* provide invaluable contributions for developing new therapeutic strategies to treat tuberculosis and related severe bacterial diseases (Sparks et al. 2023).

The first step in transcription involves the assembly of the RNA polymerase (RNAP) holoenzyme (Eσ), a molecular complex consisting of the core RNAP (E) and a sigma (σ) factor. This Eσ is responsible for initiating gene transcription at specific locations on the DNA called promoters (Kouba et al. 2019).

σ^A is the primary σ factor in many bacteria, including mycobacteria, and plays a crucial role in the bacterial transcription machinery (Gomez et al. 1998). As part of the Eσ, σ^A is responsible for recognizing and binding to specific promoter sequences, allowing the initiation of transcription (Ruff, Thomas Record, and Artsimovitch 2015). Its primary function is to ensure the expression of essential housekeeping genes, which are necessary for fundamental cellular processes such as growth, metabolism, and replication. Without σ^A, bacteria would be unable to transcribe these critical genes, leading to a collapse of vital cellular functions (Paget, Wigneshweraraj, and Hinton 2015). In mycobacteria, σ^A is indispensable for maintaining cellular homeostasis, making it a key factor for survival under both normal and stress conditions (Gomez et al. 1998).

This Doctoral Thesis is based on research that investigates two key transcription factors, MoaB2 and HelD, in *M. smegmatis*. **Publications I** focuses on MoaB2, a newly discovered binding factor of σ^A, and its role in modulating mycobacterial transcription. **Publication II and III** examine HelD, a transcription factor that interacts with stalled RNAP, playing crucial roles in transcription regulation. All three studies advance our understanding of the transcriptional apparatus in mycobacteria. This knowledge may guide future development of antimycobacterial compounds.

LITERARY REVIEW

1. *Mycobacterium smegmatis*

Mycobacterium smegmatis is a non-pathogenic saprophytic (derives nutrients from decaying organic matter) bacterium belonging to the phylum Actinobacteria, family Mycobacteriaceae, and genus *Mycobacterium*. Recently, Gupta et al. (2018) proposed to divide the genus *Mycobacterium* into five different genera based on phylogenomic data (Gupta, Lo, and Son 2018). The current genus *Mycobacterium* would contain only members of the *Mycobacterium tuberculosis* complex, while most of the non-tuberculous species would be classified in the four genera: *Mycobacteroides* (e.g. *Mycobacteroides abscessus* complex), *Mycolicibacter* (e.g. *Mycolicibacter terrae* complex), *Mycolicibacterium* (e.g. *Mycolicibacterium fortuitum* and, among others, *Mycolicibacterium smegmatis*), and *Mycolicibacillus* (e.g. *Mycolicibacillus trivialis*) (Armstrong and Parrish 2021). However, in this Doctoral Thesis, I will not use the newly proposed nomenclature and taxonomy by (Gupta et al. 2018). The names of bacteria in this Thesis will follow the old nomenclature established by Lehmann and Neumann 1896 (Approved Lists 1980) and officially used by the List of Prokaryotic names with Standing in Nomenclature (LPSN).

M. smegmatis owes its name to the material from which it was originally isolated - from genital secretion, smegma (Röse, Kaufmann, and Daugelat 2004). It was first reported in 1884 by Lustgarten who had identified a bacterium in a patient with syphilitic chancres with staining characteristics similar to tubercule bacilli (the world had already recognized pathogenic *M. tuberculosis*, discovered in 1882 by Dr. Robert Koch (Cambau and Drancourt 2014)). Since its discovery, *M. smegmatis* has been ubiquitously found in natural environments, primarily soil and water (T et al. 2020).

M. smegmatis has gained prominence as a model organism for studying the biology of pathogenic mycobacteria, particularly *M. tuberculosis*, a rod-shaped obligate aerobic bacterium that is a causative agent of tuberculosis. *M. smegmatis* is non-pathogenic to humans, making it safe model organism for use in standard laboratory settings. *M. smegmatis* shares important genetic and physiological features with *M. tuberculosis*, such as the presence of a thick, waxy cell wall rich in mycolic acids, that makes it a useful model for studying mycobacterial genetics, metabolism, and for screening of antimycobacterial drugs (Greene, Calpin, and Guptill 2008).

1.1. Mycobacterial cell envelope

The protective mycobacterial cell envelope is composed of the cytoplasmic membrane, cell wall, surface lipids and a capsule. Cell wall core contains peptidoglycan, arabinogalactan and mycolic acid layers. Its defining feature is a waxy outer membrane, the "myco-membrane", rich in mycolic acids

(Chiaradia et al. 2017). A periplasmic space also exists in mycobacteria, between the cytoplasmic membrane and the peptidoglycan. In Gram-negative bacteria, periplasm is the space between the inner and outer membrane (Erickson 2021). A variety of proteins are necessary for construction and regulation of the mycobacterial envelope. These proteins include sensory signaling molecules like kinases, transporters, regulators involved in cell wall synthesis, enzymes for cell wall precursor production in the cytoplasm, and periplasmic proteins that assemble the cell wall (Figure 1) (Dulberger, Rubin, and Boutte 2019).

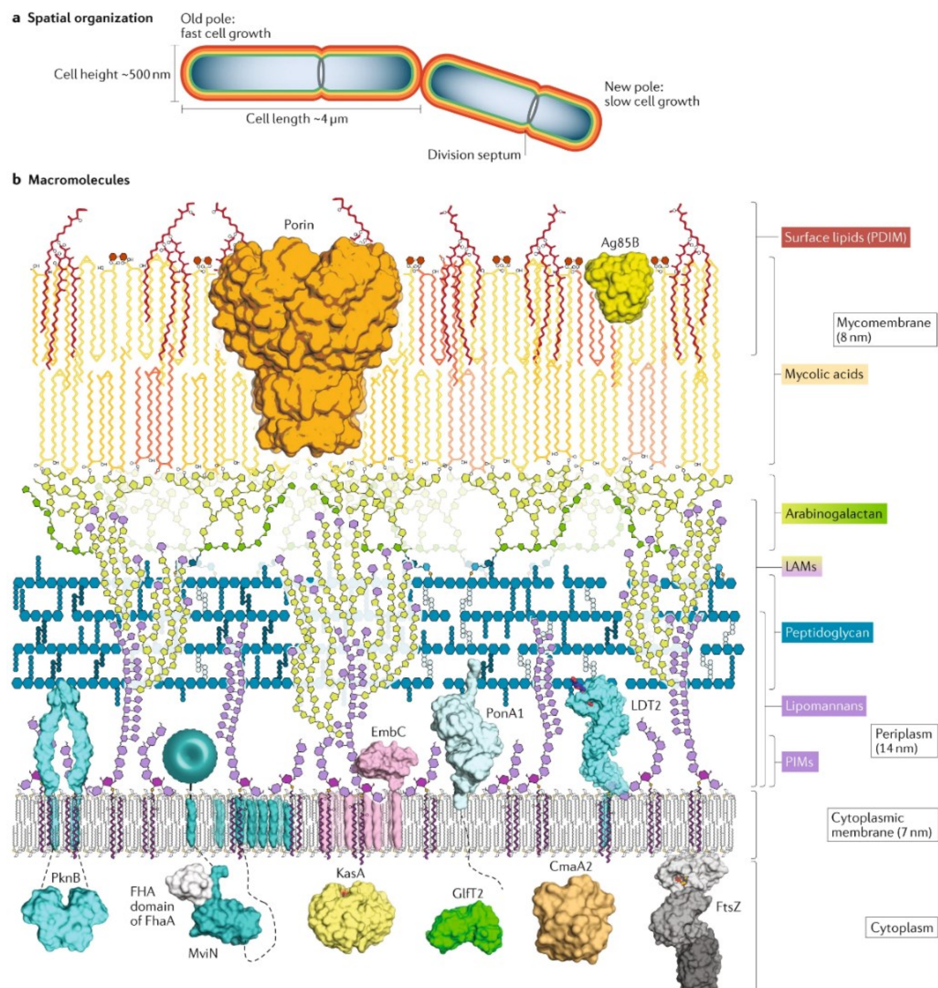

Figure 1. a) A scheme of different mycobacterial cell wall layers. Peptidoglycan in blue, arabinogalactan in green, mycolic acids in yellow and orange, phthiocerol dimycocerosate in red. Mycobacterial cells elongate at the poles, and cell division occurs slightly away from the middle of the cell. b) A detailed scheme of the cell envelope layers and proteins. Inositols are dark purple, and mannose is light purple. Lipomannans are shown by light purple mannose residues. Lipoarabinomannans are light green arabinose chains. Peptidoglycan is blue. Arabinogalactan is dark green (galactan) and light green (arabinan). Trehalose is represented by two dark orange hexagons and is linked to the mycolic acids. Adapted from (Dulberger et al. 2019).

1.2. Mycobacterial characteristics and growth

The genome of the *M. smegmatis* laboratory wild-type strain mc^{2}155 is 6,988,269 bp long and contains 6,790 genes, 6,625 coding sequences (CDS) and 54 RNAs (47 tRNAs, 6 rRNAs). Its guanine-cytosine (GC) content is 67.4 % (Mohan et al. 2015). This high content of GC pairs provides advantages such as increased DNA stability (due to the three hydrogen bonds between guanine and cytosine compared to two between adenine and thymine) and heat resistance (GC-rich DNA has a higher melting temperature) that promotes survival in harsh environments (Hu et al. 2022). However, high GC pair content also has its downside, including higher energy costs for DNA synthesis, limited codon usage (GC-rich codons are preferred and may limit the range of codons used for protein synthesis) (Li et al. 2015), and increased susceptibility to oxidative damage (among the four DNA bases, guanine has the lowest redox potential and is preferentially targeted for oxidation) (Matter et al. 2018; Poetsch, Boulton, and Luscombe 2018). In addition, it may complicate genetic manipulation techniques such as PCR, making laboratory research more difficult (Mamedov et al. 2008).

M. smegmatis is considered a fast-growing mycobacterium with a relatively rapid doubling time of 2 to 6 hours under optimal conditions (Stephan et al. 2005), unlike slow-growing *M. tuberculosis*, that has a doubling time of at approximately 24 hours and can take weeks to form colonies (James, Williams, and Marsh 2000). Still, compared to *E. coli*, which doubles every 20 minutes under optimal conditions (Gibson et al. 2018), and *Bacillus subtilis*, with a doubling time during exponential phase of growth of around 25 minutes (Böttinger et al. 2018), *M. smegmatis* is a slow grower while still fast for a mycobacterium.

1.3. Non-tuberculous mycobacteria

Non-tuberculous mycobacteria (NTM) are a diverse group of mycobacteria that do not belong to the *M. tuberculosis* or *M. leprae* complex (Griffith et al. 2007). They are ubiquitous in the environment, being found in soil, water and households (Falkinham 2015). NTM can cause a variety of diseases, with pulmonary infections being the most common, affecting mainly people with already existing lung diseases such as chronic obstructive pulmonary disease and cystic fibrosis. Infections caused by NTMs are a significant health threat mainly for immunocompromised patients due to their growing resistance. The main pathogens include *Mycobacterium avium* complex, *M. kansasii* and *M. abscessus* complex, which are slow- and fast-growing species, respectively. These organisms are notoriously difficult to treat because of their resistance to conventional antibiotics (Koh 2017).

Understanding the biology of these organisms and the mechanisms of their resistance to antibiotics, particularly through proteins such as HelD, offers insights into potential therapeutic targets. However, its gives resistance to *M. abscessus* that causes chronic lung infection that may lead to cystic fibrosis

(Esther Jr et al. 2010; Sudzinová et al. 2022). This thesis will further explore this difference to highlight the broader impact of studying non-tuberculous species such as *M. smegmatis* on bacterial gene expression research.

2. Bacterial growth

Bacterial growth is defined as an increase in the number of bacteria in a population over time. It can be represented by a growth curve, where the x-axis represents time and the y-axis represents the number of bacterial cells or cell density. Cell density is often measured as an optical density (OD) at a specific wavelength, usually 600 nm (Ughy et al. 2023). In nature, bacteria do not experience perfect environmental conditions for growth and bacterial populations change over time. However, optimal conditions can be created in the laboratory by growing bacteria in a nutrition-rich media so they can maintain continuous, rapid and balanced growth over a period of time (Nyström 2004).

2.1. Phases of bacterial growth

The characteristic bacterial growth curve pattern comprises four phases: lag phase, exponential phase, stationary phase, and death phase (Figure 2) (Ughy et al. 2023).

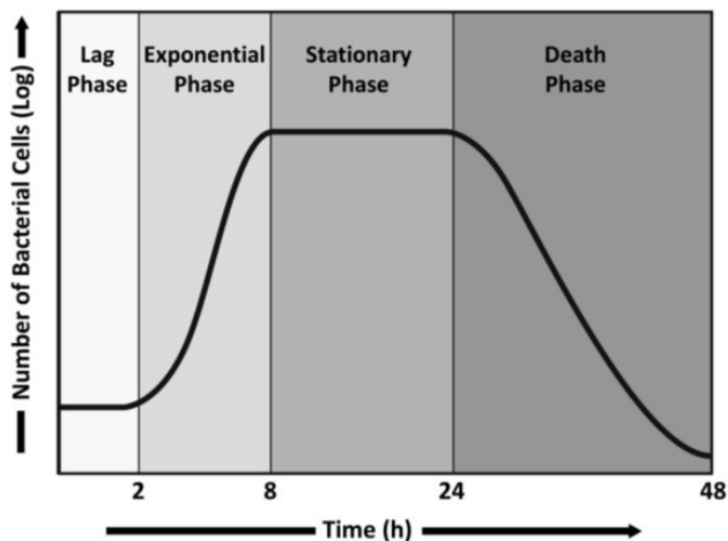

Figure 2. A scheme of bacterial growth curve in culture media. Individual phases indicated above the graph. Adapted from (Anon n.d.).

2.1.1. Lag phase

The lag phase is an understudied growth phase and its length varies depending on the bacterial species and the surrounding conditions (Ughy et al. 2023). In this initial phase of bacterial growth, bacteria need to adapt to the new environment in order to begin cellular metabolism. Minimal or no cell division occurs during this phase. Bacteria adapt to the surrounding conditions, synthesizing essential

enzymes, proteins, and other molecules needed for cell growth. Although bacterial cell numbers remain stable and low, metabolic activity is high (Rolfe et al. 2012). At the end of this phase, the numbers of cells start to increase, and the population enters the next growth phase (Bertranda 2019).

2.1.2. Exponential phase

The exponential phase (EXP), also known as the logarithmic phase, is characterized by rapid and constant bacterial growth that occurs at a maximal rate that depends on nutrition availability in the environment. Depending on the medium (*e. g.* different sources of carbon), bacteria can exponentially grow at different rates. During this phase, the bacteria divide exponentially and the growth rate varies depending on the culture medium, growing slower in nutrient-poor conditions and faster in nutrient-rich conditions. If cells are transferred from the EXP to fresh medium, they can bypass the lag phase and resume logarithmic growth. However, when nutrients are depleted and waste products accumulate, the growth rate eventually slows and cell division ceases, leading the bacterial population into the stationary phase. This phase marks the end of active growth in batch cultures because environmental conditions can no longer sustain exponential proliferation (Ughy et al. 2023).

2.1.3. Stationary phase

The stationary phase (STA) is characterized by a dynamic balance between dividing and dying cells, resulting in a constant number of cells and creating a plateau in the growth curve (Navarro Llorens, Tormo, and Martínez-García 2010). This phase is reached when nutrients become limited, waste products accumulate or other environmental conditions restrict cell growth. Although the total number of cells remains stable, the bacteria undergo physiological changes to adapt to stressful conditions, often increasing resistance to antibiotics and other stresses. Metabolic activity shifts to maintenance and survival, with some cells entering a dormant state (Navarro Llorens et al. 2010; Ughy et al. 2023).

2.1.4. Death phase

When the number of dying cells surpasses the number of newly formed cells, bacterial growth has reached its final phase, the death phase. When the environment cannot sustain the population, toxic catabolic products accumulate, leading to a decline in the number of viable cells and the population density decreases. Many cells lose metabolic activity, some may undergo autolysis, thereby degrading cellular components. Nowadays, a fifth phase is distinguished, referred to as the long-term STA where a small proportion of the population can survive and maintain viability for several weeks or even months. Some bacteria such as *M. tuberculosis* may enter a dormant non-replicative post stationary state that allows them to survive for a long time (Finkel 2006; Salina and Makarov 2022; Ughy et al. 2023).

3. Gene expression

The regulation of gene expression is a complex process crucial for bacterial survival and adaptation to changing environmental conditions (Sinha, Laursen, and Licht 2024). Gene expression occurs in two key steps: transcription and translation. In bacteria, these steps are tightly coupled both in time and space and are subject to strict regulation. This precise control is essential to avoid unnecessary use of resources and energy in a competitive and challenging environment (Figure 3) (Bervoets and Charlier 2019).

In all cells, from bacteria to humans, genetic information is encoded in DNA. The central dogma of molecular biology, postulated by Francis Crick in 1957, explains that DNA is transcribed into RNA in the process of transcription, which then serves as a template for protein synthesis through translation (Miller, Baluška, and Reber 2023). In the case of bacteria, the dogma is:

Replication: DNA is replicated, thus ensuring the precise transfer of genetic information during cell division.

Transcription: Bacteria lack a nucleus, so the DNA is transcribed into mRNA and immediately begins to be translated. Coupling of transcription and translation allows bacteria to respond rapidly to the environmental changes.

Translation: The mRNA sequence is then translated by the ribosome into the chain of amino acids (aa) that form the protein.

This streamlined and interconnected process embodies the efficient and regulated nature of bacterial gene expression.

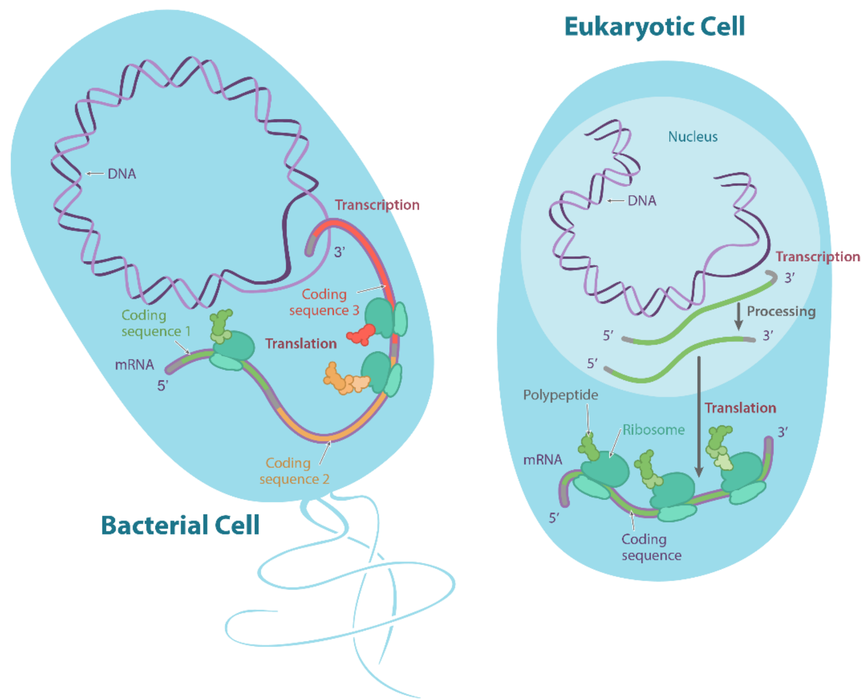

Figure 3. Schematic comparison of procaryotic and eucaryotic cell with depicted steps of gene expression: transcription and translation. Since bacterial cell is lacking the nucleus, transcription and translation is coupled and take place in cytoplasm. Eucaryotic cell has nucleus where transcription takes place, mRNA is processed, leaves the nucleus and in cytoplasm, translation can start as an individual and separate process (Untitled image. "Stages of translation" under "Initiation" *Khan Academy.* Accessed November 19, 2024. https://www.khanacademy.org/science/biology/gene-expression-central-dogma/translation-polypeptides/a/the-stages-of-translation.).

3.1. Transcription

Transcription is the process by which DNA is copied into a newly synthesized strand of mRNA using the key enzyme, RNA polymerase. In eukaryotic cells, RNA synthesis is catalyzed by at least three nuclear, DNA-dependent RNA polymerases (Pols)- Pol I, Pol II, and Pol III (Sekine, Tagami, and Yokoyama 2012). In plants, two additional RNA pols (IV and V) are present that transcribe non-coding RNAs (ncRNAs) (Barba-Aliaga, Alepuz, and Pérez-Ortín 2021). In bacteria, all RNA synthesis is typically carried out by a single RNA polymerase (RNAP).

Nevertheless, in some bacteria, such as *B. subtilis*, there are other RNAPs in addition to the multi-subunit RNAP. An example of such an RNAP is the single subunit YonO, which is encoded within the SPβ prophage of *B. subtilis.* YonO transcribes some of the prophage genes. YonO exhibits distant homology with multi-subunit RNAPs and functions as a highly processive DNA-dependent RNAP that represents a unique type of bacteriophage RNAP encoded in a bacterial genome (Forrest et al. 2017).

Bacterial claw-like RNAP is a multi-subunit enzyme. The core RNAP (E) is comprised of five subunits: β, β', ω, and two identical copies of α, and is capable of transcription elongation but not initiation. E

contains the active site (AS) that catalyzes the formation of the phosphodiester bond of nascent RNA and together with catalytically inactive primary σ factor (σ^A), that is responsible for distinguishing the promoter, it forms a Eσ (Hubin, Lilic, et al. 2017; Mejía-Almonte et al. 2020). In addition to a σ factor, RNAP binds many proteins or non-protein factors, so called transcription factors (TFs), that affect various steps of bacterial transcription (Browning and Busby 2016).

3.2. Phases of transcription

Bacterial transcription is a multistep process that can be divided into three basic phases: initiation, elongation, and termination (Figure 4). Each of these is characterized by distinct events and interactions (van Hijum, Medema, and Kuipers 2009).

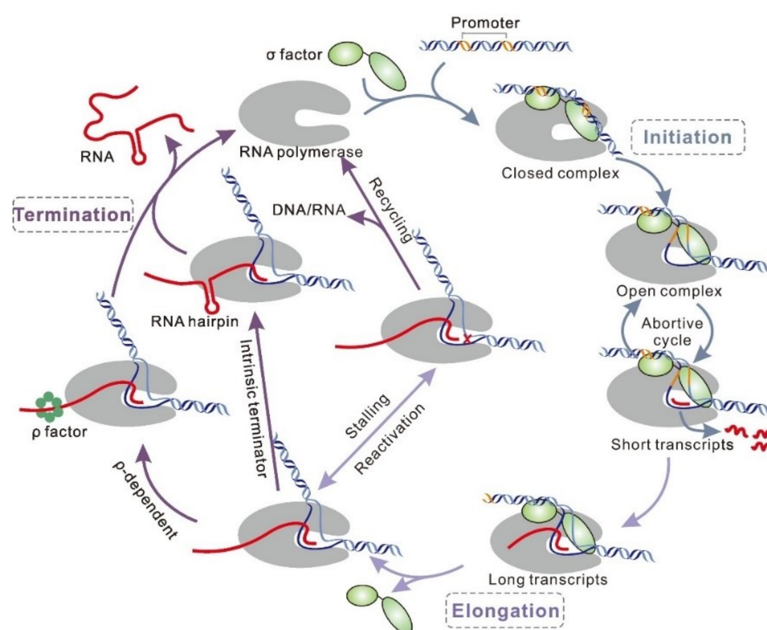

Figure 4. Scheme of bacterial transcription cycle. Promoter DNA is shown in light/dark blue, with the -35 and -10 elements of the promoter DNA shown in orange. RNAP is shown in grey, σ factor is shown in light green. Rho (ρ) factor is the green ring. RNA in red. Adapted from (Hu and Liu 2022).

3.2.1. Initiation

During initiation, Eσ recognizes the promoter region of DNA, binds to it and forms the closed promoter complex (RPc). After subsequent isomerization through one or more kinetic intermediates, Eσ forms an open promoter complex (RPo) containing a ~13 bp long melted DNA transcription bubble that allows RNAP and substrates (nucleoside triphosphates) access to the template strand (Saecker, Record, and Dehaseth 2011). Transcription can then initiate. RNAP initially does not leave the promoter. Rather, the DNA is reeled in by a process called scrunching. Some Eσ-promoter complexes undergo multiple rounds of abortive transcription initiation, with each round returning back to the RPo

(Winkelman and Gourse 2017). Transcription initiation ends with the synthesis of several nucleotides (nt) long nascent RNA strand, after which the σ factor dissociates from RNAP and leaves the promoter (promoter escape), forming the transcription elongation complex (TEC) (Basu et al. 2014; Heyduk and Heyduk 2018; Hubin, Lilic, et al. 2017).

3.2.2. Elongation

During elongation, TEC moves along the template strand of DNA and synthesizes RNA in the 5′ to 3′ direction by adding complementary nucleoside triphosphates (NTPs). As the elongating RNAP advances, the nascent RNA product remains transiently base-paired to the template DNA strand, forming 9-10 bp RNA:DNA hybrid. In the TEC, three nucleic acid-protein interactions play essential roles (Figure 5): i) the downstream DNA binding site, acts as a sliding clamp around the DNA duplex, holding it in place, ii) the rear zip lock secures the upstream edge of the RNA hybrid, ensuring that the RNA transcript is properly displaced from the DNA template and iii) the front zip lock, located at the RNAP active center, holds the downstream boundary of the hybrid and assists with RNA-DNA separation during RNAP backtracking (Mustaev, Roberts, and Gottesman 2017).

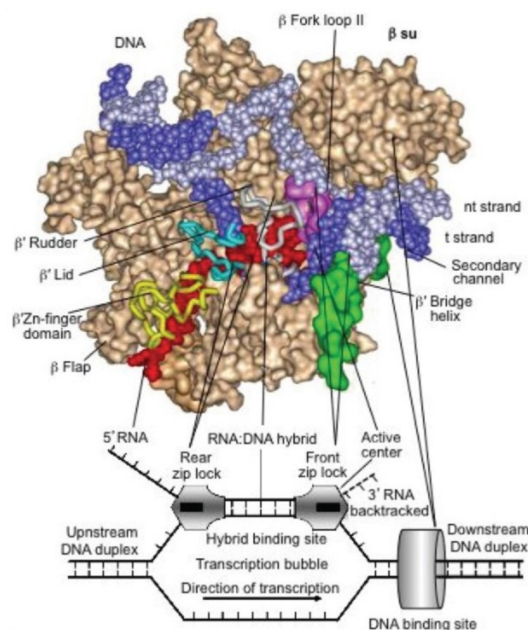

Figure 5. Scheme of the RNAP transcription elongation complex (TEC). RNAP and domains important for TEC formation are shown above the transcription bubble and the schematically represented binding sites. DNA is in blue, RNA is in red. The DNA:RNA hybrid is in the main channel between the β′ bridge helix, the β′ Rudder and β′ Lid loops at its upper end, the β fork loop II and active center at its lower end. It corresponds to the front and rear zip locks. Adapted from (Mustaev et al. 2017).

The transcription elongation rate is not constant. RNAP pausing is influenced by template sequence and DNA-binding transcription factors. This causes short-term pauses in elongation and affects the synchronization of translation-ready RNA production (Mustaev et al. 2017). In some cases, RNAP may

pause during elongation and can backtrack. Backtracked RNAP causes removal of the 3′ RNA growing end from the active center (Korzheva et al. 1998). Factors such as GreA and GreB (in *E. coli, e.g., B. subtilis* contains only GreA) enhance hydrolysis of the 3′ RNA nt, unblock the active center, and restart transcription (Mustaev et al. 2017).

3.2.3. Termination

During termination, the transcription process stops, RNAP dissociates from DNA and the newly synthesized mRNA is released. Termination can occur by a Rho-independent or Rho-dependent mechanism. In Rho-independent termination, or so-called intrinsic termination, the terminator is located at the end of the operon and is characterized by a GC-rich sequence followed by a stretch of Ts that, when transcribed into RNA, forms a stem-loop (or hairpin) structure followed by a 7- to 8-nt U-stretch in an RNA:DNA hybrid. The U stretch forces RNAP to stop and the formed palindrome causes RNA extraction from the RNA:DNA hybrid (Carafa, Brody, and Thermes 1990; Peters, Vangeloff, and Landick 2011).

In Rho-dependent termination, a homo-hexameric ring protein called Rho (ρ) factor binds to the nascent RNA transcript and threads RNA 5′-3′ through the center of the ring in an ATP-dependent manner. The final step of Rho termination is Rho catching the TEC stopped at a pause site and its dissociation from DNA template (Moreira et al. 2024; Peters et al. 2011; Roberts 2019).

Together, these transcription steps ensure that the genetic information encoded in the DNA is accurately transcribed into RNA. Each stage is carefully regulated, allowing the bacteria to efficiently control gene expression. Also, a number of different TFs play a key role in these above-mentioned transcription stages, and as they are an important part of this thesis, a separate chapter is devoted to them.

3.3. RNA polymerase

DNA-dependent RNA polymerase (RNAP) is a critical enzyme involved in transcribing DNA into RNA, consisting of the core enzyme (E) with multiple subunits: two alpha (α), one beta (β), one beta-prime (β'), and a small omega (ω) subunit (Kouba et al. 2019). Two α subunits (*rpoA*, MW of α from *M. smegmatis* is 2x37.9 kDa) are involved in enzyme assembly and interacting with regulatory factors and promoter elements. The β subunit (*rpoB*, 128.5 kDa) forms part of the enzyme's catalytic core, playing a role in the AS where RNA synthesis occurs and the β' subunit (*rpoC*, 146.5 kDa) contains the catalytic site itself and with β forms a 'crab-claw' shape, which allows DNA to fit between the two subunits. ω subunit (*rpoZ*, 11.5 kDa) is the smallest subunit, it helps in the proper folding of β′ and stabilization of the whole RNAP complex (Figure 6) (Kurkela et al. 2021; Sutherland and Murakami 2018).

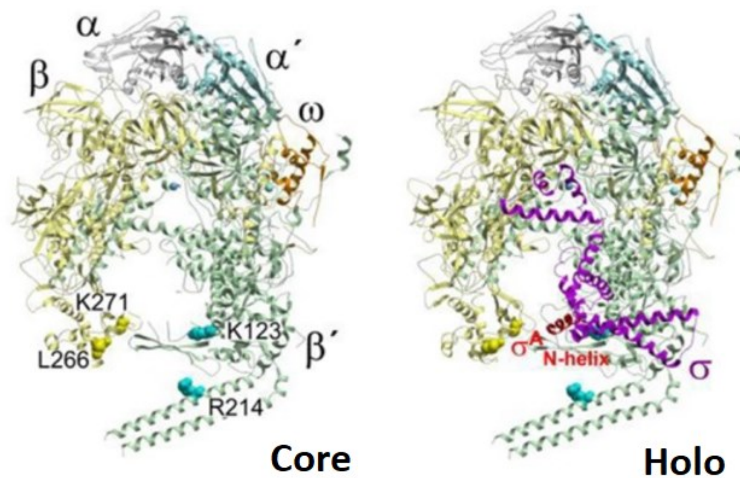

Figure 6. Cryo-EM structure determinations of *M. smegmatis* RNAP complex. Cryo-EM densities of secondary structures of two forms of RNAP from *M. smegmatis*: core (left) and Eσ (right, RNAP in complex with $σ^A$). Subunits are colored as follows: β, yellow; β', green; α, gray; α', cyan; ω, orange; $σ^A$, magenta. Adapted from (Kouba et al. 2019).

In prokaryotes, the β and β′ subunits are highly conserved. However, mycobacterial RNAPs contains the β′i1 insert (an insertion of approximately 90 aa residues at around position 140 of the β′ subunit) (Hubin et al. 2017) in the RNAP clamp module that interacts with the N-terminus of $σ^A$, which increases the stability of the RPo (Chen, Boyaci, and Campbell 2021).

RNAP contains three specialized channels that facilitate its function: (i) primary channel, where dsDNA enters, and it is flanked by the β and β' subunits. DNA is unwound in this region to expose the template strand for RNA synthesis. (ii) Secondary channel is used for NTPs (substrates) entry during transcription initiation and RNA elongation and is crucial for the incorporation of nt into the growing RNA chain. (iii) RNA exit channel, where, after synthesis, the nascent RNA is channeled out through this exit route, ensuring a smooth passage out from the enzyme (Kouba et al. 2019).

Other proteins, such as various transcription activators, may also associate with Eσ. In Mycobacteria there are specific TFs, RbpA (Hu et al. 2014; Perumal et al. 2018), CarD (Bae et al. 2015), and HelD (Kouba et al. 2020). A separate chapter is devoted to them later in the text.

3.3.1. Transcription inhibitors

RNAP is a major target of antibiotics because it is essential for transcription and crucial for the control of bacterial infections. An antibiotic such as rifampicin or fidaxomicin binds to RNAP and blocks RNA chain elongation. By interrupting RNAP activity, we can effectively limit bacterial infections, especially in pathogenic species (Darst 2004). Fidaxomicin is clinically used against *Clostridium difficile* infections and is also effective against *M. tuberculosis* RNAP *in vitro*. Fidaxomicin binds to RNAP and blocks the

RNAP clamp closure that occurs during RPo formation, thereby inhibiting transcription initiation (Boyaci et al. 2018; Lin et al. 2018).

3.3.1.1. Rifampicin

Rifampicin (RIF), also known as rifamycin, is an ansamycin antibiotic belonging to the rifamycin group. It is a semi-synthetic antibiotic, derived by several chemical reactions from rifamycin B, which is produced by the actinomycete *Streptomyces mediteranei* (*Nocardia mediteranei*). It was introduced into medical practice in 1968 as a first-line drug for the treatment of mycobacterial infections (Goldstein 2014; Grobbelaar et al. 2019; Vardanyan and Hruby 2006). Rifamycins as a group are potent inhibitors of DNA-dependent prokaryotic RNAP (Wehrli et al. 1968).

Rifampicin binds to the RNAP pocket (AS) in which the DNA-RNA hybrid normally binds in the absence of this antibiotic. The presence of rifampicin in this pocket during transcription initiation blocks the RNA exit channel and prevents the nascent RNA from elongating beyond 2-3 nt (Figure 7) (Campbell et al. 2001; Koval' et al. 2024). Rifampicin does not bind to eukaryotic RNAPs and does not affect the corresponding RNA synthesis in these organisms (Vardanyan and Hruby 2006).

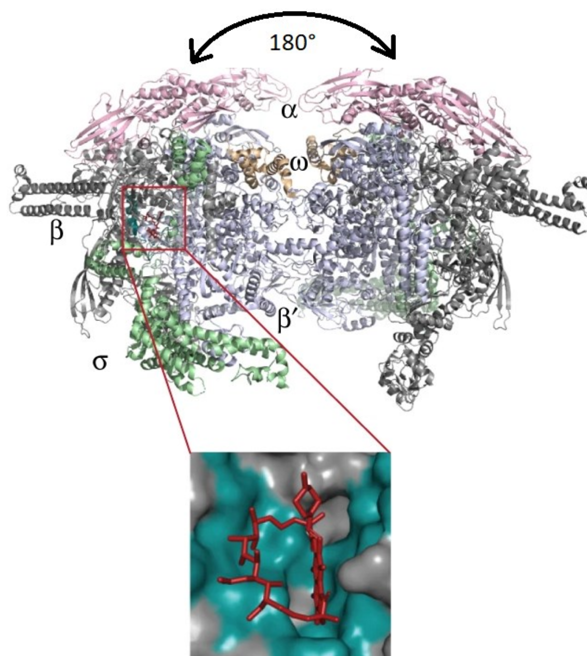

Figure 7. A structural view of the RIF binding pocket within the β subunit of *E. coli* RNAP (in blue), magnified in the lower panel (PDB 5UAC). Adapted from (Yang et al. 2023).

The disadvantage of rifamycin antibiotics is their high frequency of resistance, approximately 10^{-7} to 10^{-9}, which is why rifamycins are combined with other antibiotics (Waller et al. 2023). For the treatment of *M. tuberculosis* infections, rifampicin is combined with isoniazid and ethambutol (Organization n.d.; Surette et al. 2022). Resistance to rifampicin arises due to mutations in the *rpoB*

gene. Point mutations alter the structure of the RNAP rifampicin binding cavity and reduce or eliminate the affinity of rifampicin for RNAP (Molodtsov et al. 2017).

Genetic studies have identified an 81-bp region in *rpoB*, the rifampicin resistance-determining region (RRDR), that is responsible for 95% of clinical mutations, primarily substitutions at residues Asp516, His526, and Ser531 (Yam et al. 2004). They reduce the binding affinity of rifampicin to the RNA exit tunnel and account for ~85% of rifampicin-resistant *M. tuberculosis* cases (Ramaswamy and Musser 1998; Surette et al. 2022). Mycobacteria are capable of acquiring resistance due to non-adherence to treatment or irregular medication by patients (Molodtsov et al. 2017).

Gram-negative bacteria are intrinsically resistant to rifamycins due to low membrane permeability and active efflux. Many actinobacteria such as *Streptomyces*, *Nocardia* and several species of *Mycobacteria*, possess mechanisms of enzymatic inactivation of rifamycins, such as ADP-ribosyl transferases (Arrs), glycosyltransferases (Rgt), monooxygenases (Rox) and phosphorylases (Rph) (Dabbs 1987; Dabbs et al. 1995; Spanogiannopoulos et al. 2012; Surette et al. 2022). *M. abscessus* is highly resistant to rifampicin, partly because the Arr enzyme inactivates the drug (Hurst-Hess et al. 2022).

3.4. Procaryotic Promoter

The first recognition of promoters occurred when David Pribnow purified an RNAP-bound DNA fragment from *E. coli*. He isolated RNA sequences, annealed them to this region, and sequenced them (Pribnow 1975). He described to the understanding that promoters regulate gene expression in both bacteria and eukaryotes. Here, unless specified otherwise, I will be describing *E. coli* promoters dependent on the primary σ factor.

Promoters are DNA sequences with specific motifs to which RNAP and other TFs bind and initiate transcription. The promoters are located upstream of the start of the gene (in the 5' direction) and are highly specific for the genes they regulate (Helmann 2019). They contain several regions, such as two hexameric motifs centered at -10 and -35 positions relative to the transcription start site (TSS; +1) (Harley and Reynolds 1987). The -10 element, the Pribnow box, typically consists of the conserved TATAAT sequence and -35 element contains the conserved TTGACA sequence (Kanhere and Bansal 2005). Between the -10 element and the TSS is a sequence that is sometimes called as the discriminator. Originally, a discriminator was the G/C rich sequence between -10 and +1 in rRNA promoters of *E. coli*. However, over the years the term “discriminator” began to be used just for any sequence in this region (Forquet et al. 2021; Haugen et al. 2006).

In bacterial transcription, the σ factor is an essential component of Eσ and controls transcription initiation by recognizing -10 and -35 elements (Burton et al. 2016). Upstream of the promoter, an upstream (UP) A/T-rich element may be located about -40 to -60 bases relative to the TSS of certain genes (Browning and Busby 2016). These general bacterial promoter elements are similar to those found in mycobacteria, but with minor differences specific to mycobacteria only (Figure 8) (Newton-Foot and Gey Van Pittius 2013).

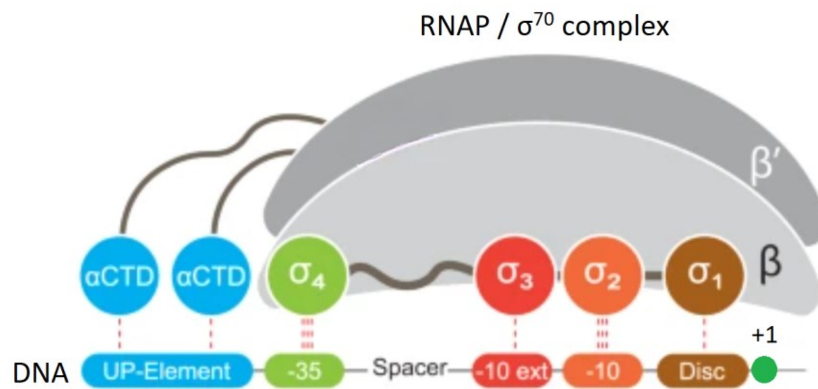

Figure 8. Scheme of the relative interaction strengths between mycobacterial RNAP, σ factor and promoter DNA. The lines between proteins and promoter DNA indicate the strength of the interaction, the more lines the stronger the binding. RNAP core subunits β and β' are in grey, σ factor domains $σ_4$, $σ_3$, $σ_2$, and $σ_1$ ($σ_N$) are depicted in different colors and individual promoter elements are also shown. αCTD, RNAP α-subunit C-terminal domain; - 10 ext, extended -10 element; Disc, discriminator. Adapted from (LaFleur, Hossain, and Salis 2022).

3.4.1. Mycobacterial promoter

Similar key features of *M. smegmatis* $σ^A$-dependent promoters and those of *M. tuberculosis*, *E. coli* or *B. subtilis* include a purine as the predominant transcription start site, a conserved -10 hexamer, consistent distances between hexamers (the sequence between the −10 and −35 element), and extended -10 motifs in strong promoters (Figure 9). In mycobacterial promoters, however, these components are more variable in sequence, and unlike *E. coli*, there is no clearly defined sequence for the -35 hexamer (LaFleur et al. 2022; Newton-Foot and Gey Van Pittius 2013).

The -10 element in mycobacteria is TATAAT (Ehrt and Schnappinger 2006). Mycobacteria further contain an extended -10 element, which provides extra binding support to stabilize the RNAP complex. The extended -10 class promoters contain a 5′-TGn-3′ motif (where "n" can be any base) placed in front of the conserved -10 element. Approximately 60% of the genes of Gram-positive bacteria are transcribed from extended -10 class promoters. Mutations in the TGn motif led to a reduction in the activity of mycobacterial extended -10 class promoters *in vivo* (Agarwal and Tyagi 2003). The extended -10 sequence helps bind RNAP to the promoter in the absence of a functional -35 element by increasing

the contact area between the RNAP and DNA and plays a role in RPo formation during initiation (Agarwal and Tyagi 2003; Bashyam and Tyagi 1998). The S16 promoter in *M. smegmatis* with a perfect extended -10 motif (TGTTATAAT) is one of the strongest mycobacterial promoters, suggesting that the TGn motif is critical for determining promoter strength in mycobacteria (Bashyam and Tyagi 1998; Newton-Foot and Gey Van Pittius 2013). The consensus -35 sequence with the greatest promoter activity in *M. smegmatis* is TTGCGA (Agarwal and Tyagi 2006).

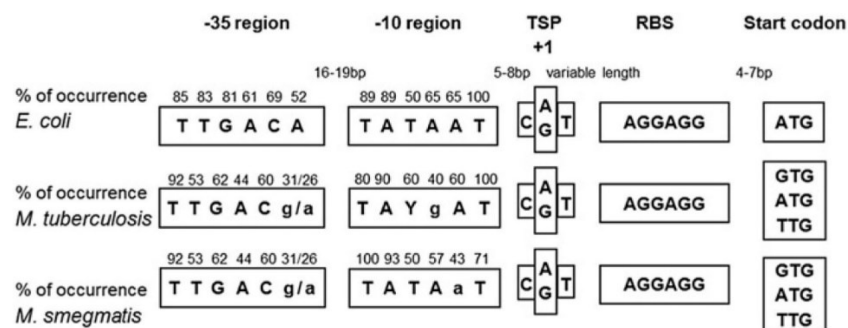

Figure 9. Schematic comparison of the *E. coli*, *M. tuberculosis* and *M. smegmatis* consensus promoter sequences. Y represents a pyrimidine base (T or C); lower case (g or a) shows consensus of less than 50%. Numbers above the nucleotide bases represent % of occurrence. TSP stands for transcription start point. RBS stands for ribosome binding site. Adapted from (Newton-Foot and Gey Van Pittius 2013).

The UP element, an A/T-rich sequence located upstream of the -35 element, is less common in mycobacteria due to their high GC content. The mycobacterial RNAP α-subunit C-terminal domain (αCTD) binds to AT-rich regions upstream of the -35 element, similar to its role in other bacteria, and is reinforced by interaction with UP element when present (Hubin, Lilic, et al. 2017; LaFleur et al. 2022).

3.5. σ factors

In the context of bacterial transcription, the σ factor plays a key role as an essential component of the Eσ that controls transcription initiation by recognizing the -10 and -35 promoter elements. σ was discovered in 1969 by Richard Burgess (Burgess 2021), and it was later demonstrated that the E is inactive without the σ factor (Burgess et al. 1969). Using a glycerol gradient, they isolated the active form of the enzyme, Eσ, providing an answer to the question of how RNAP identifies the transcription starting point (Burgess et al. 1969; Travers and Burgess 1969).

Bacterial cells contain multiple types of σ factors. Different σ factors can recognize distinct promoter sequences and allow them to regulate gene transcription in response to changing environment. Cells typically have one primary σ factor that regulates the majority of genes, so called housekeeping genes (Helmann 2019). Each σ factor controls a group of genes known as a regulon. Genes with related functions are usually regulated by a single σ factor and are often transcribed together in a single

transcript. These groups of genes are referred to as operons and are transcribed as a single polycistronic mRNA (Osbourn and Field 2009).

In most bacteria, the primary σ factor is termed σ^A (Kazmierczak, Wiedmann, and Boor 2005; Manganelli et al. 2004; Waagmeester, Thompson, and Reyrat 2005). In *E. coli*, the name for this factor is σ^{70} (Paget and Helmann 2003) and in *Streptomyces coelicolor* HrdB (Mazurakova et al. 2006; Šmídová et al. 2019).

The number of σ factors varies between bacterial species. Mycobacteria encode multiple σ factors, each with unique physiological roles, ranging from maintaining basic cellular functions (primary σ factor σ^A) to responding to stress conditions or interactions with the host (alternative σ factors) (Paget et al. 2015). For example, in *M. smegmatis* there are 28 σ factors (of which 27 are alternative σ), 13 σ factors in *M. tuberculosis* (12 alternative σ), and only 4 functional σ factors in *M. leprae* (Cole et al. 1998; Rodrigue et al. 2006; Tekaia et al. 1999). Interestingly, among all bacterial genera, *Mycobacterium* shows the greatest variation in the number of σ factors within its species (Sachdeva et al. 2010; Waagmeester et al. 2005). This diversity allows mycobacteria to fine-tune their transcriptional responses and adapt to different conditions (Davis et al. 2017).

3.5.1. σ^A

M. smegmatis σ^A is an essential σ factor responsible for the transcription of most housekeeping genes needed for basic cellular functions (Gomez et al. 1998). Primary σ factors usually lack corresponding anti-σ factors, though in *E. coli* the Rsd protein has been shown to interact with primary σ^{70} and influence the gene expression (Chauhan et al. 2016; Mitchell et al. 2007; Paget et al. 2015). σ^A mRNA levels remain relatively constant under various growth conditions, but the levels of σ^A protein have been observed to decrease during the STA (Gomez et al. 1998; Hnilicová et al. 2014; Hu and Coates 1999).

The σ^{70} family has been classified into four phylogenetically and structurally distinct groups. Group 1 consists of primary σ factors, and groups 2-4 consist of alternative σ factors with specialized functions (Paget et al. 2015). Group 1 to 4 differ by the presence and absence of four conserved regions (σ Regions σR1.1, σR1.2-2.4, σR3.0-3.2, σR4.1-4.2) (Figure 10). The σ_2, σ_3 and σ_4 domains interact with specific promoter DNA elements (-35 hexamer, extended -10 and -10 hexamer) and with RNAP. The σ_2 domain (σR1.2-2.4) is the most conserved and forms an extensive interface with RNAP α-helix comprising σR2.2 and the β' coiled-coil (Murakami, Masuda, and Darst 2002). Binding of σR2 to the β' subunit's coiled-coil, also known as the clamp helices (β'-CH), induces a cryptic conformational change in σR2, that increases its affinity for -10 ssDNA. Although σR2 can bind to -10 ssDNA, neither free σ nor σR2 alone can melt the promoter DNA duplex without the E (Vishwakarma et al. 2024). During DNA

melting, σ_2 interacts with the non-template ssDNA of the -10 element (σR2.3-2.4), capturing the DNA and stabilizing RPo. In this state, RNAP is ready to initiate RNA synthesis and forms a stable elongation complex after synthesis of a short 11-14 nt RNA. Regions of σ factor (σR1.1, σR3, and σR4) occupy the DNA and RNA binding channels of E and are progressively displaced by the DNA template (σR1.1 and σR3) and nascent RNA (σR3 and σR4) during transcription initiation (Figure 10) (Paget et al. 2015; Vishwakarma et al. 2024).

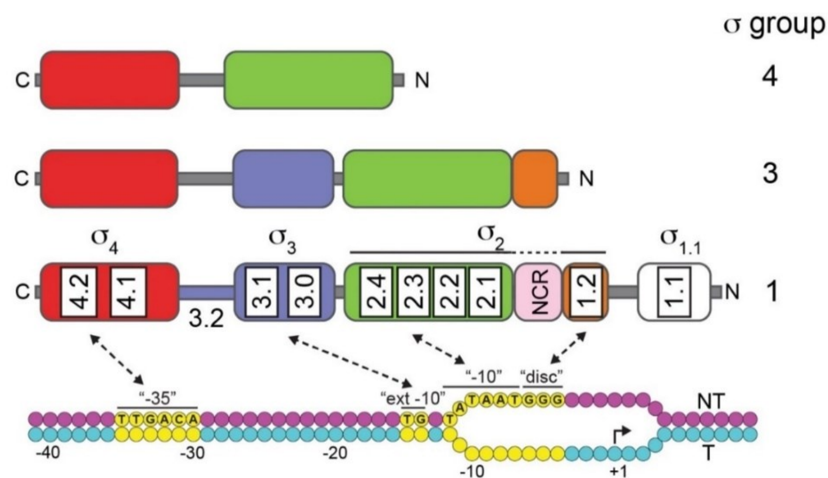

Figure 10. Scheme of σ factor domain organization from Groups 1, 3 and 4 of the σ^{70} family with corresponding consensus promoter DNA. Domains are colored: σ_4, red; σ_3, blue; σ_2, green/orange; $\sigma_{1.1}$ (σ_N), white. Non-template (NT) strand DNA is colored magenta and template (T) strand cyan, with key consensus promoter elements contacted by σ indicated in yellow: "-35", -35 element; "ext −10", extended -10 element; "-10", -10 element; "+1", start site. Adapted from (Paget et al. 2015).

The N-terminal domain of σ^A is a unique region called $\sigma^A_{1.1}$ in *E. coli*, *B. subtilis,* and similar bacteria, and is sequentially distinct from the N-terminal domain in mycobacteria that is called σ^A_N (Singha et al. 2023). $\sigma^A_{1.1}$ supports a compact conformation of free σ that conceals DNA-binding sites and prevents binding to the promoter in the absence of E (Zachrdla et al. 2017). However, during Eσ assembly, the negatively charged $\sigma^A_{1.1}$ mimics also negatively charged DNA, occupies the RNAP AS channel that will later be taken by duplex DNA in the RPo (Paget et al. 2015).

3.5.1.1. σ^A_N

The length of σ^A_N varies a lot. For example, N-terminal part of this domain in *M. smegmatis* σ^A_N has 163 aa and in *M. tuberculosis* σ^A_N has 225 aa (Hubin, Lilic, et al. 2017). In *M. smegmatis* and other mycobacteria, the N-terminal domains of σ^A exhibit divergent primary aa sequence from those of *E. coli etc.* BLAST searches identified sequence homologues for *M. smegmatis* σ^A_N only among Actinobacteria. Moreover, σ^A_N from *M. smegmatis* was separated in charge distribution. The N-terminal half of the sequence contains positively charged residues, while the C-terminal half contains more negatively charged residues (Rost, Yachdav, and Liu 2004). Importantly, it was reported to be

unfolded and intrinsically disordered (Hubin, Lilic, et al. 2017). Prediction of its secondary structure by Hubin et al. (2017) showed no secondary structure for residues 1–143, and one α-helix for residues ∼145-160. Phosphorylation of the disordered σ^A_N plays an important regulatory role in modulating σ^A activity in *M. tuberculosis* (Singha et al. 2023). The roles and function of this disordered domain in mycobacteria, though, are still poorly understood.

3.5.2. σ^B

σ^B is an alternative σ factor that transcribes genes required for bacterial survival in STA and in response to stress. Both *M. smegmatis* and *M. tuberculosis* have σ^B that shares 64% aa identity and −10 and −35 region-binding aa residues with σ^A (Hurst-Hess et al. 2019). σ^B regulates the transcription of genes involved during STA and stress response and may also promote RNAP oligomerization, potentially trapping it in an inactive state. The gene coding for σ^B is not essential. Nevertheless, in *M. tuberculosis*, deletion of *sigB* made the bacterium sensitive to low oxygen supply, oxidative stress (treatment with hydrogen peroxide), heat shock, and antibiotics (Fontán et al. 2009).

Further evidence for the role of σ^B in adaptation to STA and nutrient-poor conditions was that overexpression of *M. tuberculosis sigB* in *M. smegmatis* led to hyperglycosylation of cell-surface glycopeptidolipids, similar to what occurs during carbon starvation (Mukherjee et al. 2005). Additionally, the overexpression of *sigB* in *M. smegmatis* induced certain metabolic enzymes, including succinyl-coA synthetase, glycosyltransferases, β-ketoacyl coA synthetase, rhamnosyltransferase, and acetylcoA acetyltransferase (Mukherjee and Chatterji 2005).

Study of Hurt-Hess et al. (2019) showed that σ^B is also active during the EXP and binds to more than 200 promoter regions, several of which control transcription of housekeeping genes. Also, *sigB* mRNA is as abundant as *sigA* mRNA during the EXP of *M. smegmatis* growth, RNAseq shows. At least 61 promoter sites are recognized by both σ^A and σ^B, including genes for ribosome biogenesis and transcription (Hurst-Hess et al. 2019).

Previous studies have suggested that there are two more promoters upstream of *sigB*. One is recognized by RNAP with σ^E (Manganelli et al. 2001), σ^H (Manganelli et al. 2002), or σ^L, other one is recognized by RNAP with σ^F (Dainese et al. 2006). Transcription of *sigB* under standard physiological conditions and when exposed to surface stress depends on σ^E (Manganelli et al. 2001), induction of *sigB* during heat shock or oxidative stress depends on σ^H (Manganelli et al. 2002). No upregulation of *sigB* was observed when *sigF* was overexpressed (Williams et al. 2007). The conditions for σ^F- and σ^L-dependent transcription of *sigB* have not been described (Sachdeva et al. 2010).

Another important difference between σ^B and σ^A is, that σ^B lacks the N-terminal domain. This makes σ^B and σ^A structurally and functionally distinct from each other, preventing σ^B from fully functionally replacing σ^A (Singha et al. 2023).

3.5.3. Anti-σ factors

Anti-σ factors are proteins that regulate σ factors by binding them and blocking their interaction with RNAP. This inhibits their ability to initiate transcription. They predominantly bind to alternative σ factors because primary σ factors, like σ^A, are responsible for housekeeping transcription (Paget et al. 2015).

The regulation of σ factors and their associated regulators in *M. smegmatis* and *M. tuberculosis* has not been as thoroughly studied as in organisms like *E. coli* and *B. subtilis*. In a process known as "partner switching," anti-anti-σ factors can displace anti-σ factors from σ factors, allowing the σ factor to bind RNAP (Clark and Pazdernik 2012; Greenstein et al. 2007).

3.5.3.1. Rsd

Protein Rsd is considered an anti-σ factor, inhibiting σ^{70}-dependent transcription of housekeeping genes in *E. coli*. The interaction between Rsd and σ^{70} regulates the transcriptional response under stress conditions, and during the transition to STA (Hofmann, Wurm, and Wagner 2011).

When Rsd binds to σ^{70}, it prevents σ^{70} from interacting with the E and reduces its availability for transcription initiation, allowing alternative σ factors, such as σ^S (σ^{38}, RpoS) to compete more effectively for the E (Bouillet, Hamdallah, et al. 2024; Bouillet, Bauer, and Gottesman 2024). The alternative σ factors then redirect RNAP to transcribe genes required for stress responses or adaptation to new environmental conditions (Hofmann et al. 2011).

3.5.4. MoaB2

The first main focus of this Doctoral Thesis is the MoaB2 protein. Moa proteins are known to be involved in various biochemical processes and are primarily associated with molybdenum cofactor (MoCo) biosynthesis (Leimkühler 2020). In many prokaryotic and eukaryotic organisms, MoCo is a complex cofactor that includes a molybdenum ion coordinated by a pterin-based structure, often referred to as molybdopterin (MPT). This cofactor is necessary for the activity of a group of enzymes, molybdoenzymes, which participate in key metabolic reactions like nitrogen, sulfur, and carbon metabolism (Leimkühler 2020).

Moco has a tricyclic pyranopterin structure with a distinctive dithiolene group coordinating the molybdenum atom (Hasnat et al. 2021; Leimkühler 2020). Moco biosynthesis is a highly conserved

pathway, primarily studied in *E. coli* through biochemical, genetic, and structural approaches (Leimkühler, Wuebbens, and Rajagopalan 2011). In prokaryotes, Moco biosynthesis involves four key steps: (i) formation of cyclic pyranopterin monophosphate (cPMP), (ii) conversion of cPMP to MPT via sulfur insertion, (iii) insertion of molybdenum into MPT to form Mo-MPT, and (iv) formation of bis-Mo-MPT, with potential modifications of Moco by the attachment of nucleotides like cytidine monophosphate (CMP) or guanosine monophosphate (GMP) (Leimkühler 2020; Mendel and Leimkühler 2015; Raaijmakers and Romão 2006).

MogA and Moa proteins in *E. coli* are involved in the MoCo biosynthesis pathway. They are crucial for the function of molybdoenzymes, which play roles in electron transport and metabolism under anaerobic conditions (Nichols and Rajagopalan 2002). There is no scientific evidence of these proteins interacting directly with the σ^{70} in *E. coli*, neither other alternative σ factors nor in *Mycobacterium* species. Interestingly, *MoaB* in *E. coli* had been initially believed to be involved in MoCo biosynthesis but research by Kozmin and Schaaper (2013) revealed that disruptions in *moaB* did not affect MoCo-dependent activities, confirming its lack of direct involvement in the MoCo biosynthesis process (Kozmin and Schaaper 2013).

Interestingly, proteins involved in MoCo biosynthesis are known to have "moonlighting" functions. It is a term in biochemistry that refers to a single protein performing multiple, often unrelated, functions within the cell. This is applicable also to MoaB proteins that exhibit secondary roles beyond MoCo biosynthesis, for example, acting as chaperones or participating in stress responses, depending on the environmental conditions and the specific organism (Leimkühler 2017).

Correlating with the moonlighting functions of MoCo biosynthesis proteins, it was discovered in my lab by Jarmila Hnilicová before the beginning of my project, that MoaB2 from *M. smegmatis* interacts with σ^{A} (Figure 11). This suggested a potential role of MoaB2 in transcription, and it is the focus of the first part of my PhD Thesis.

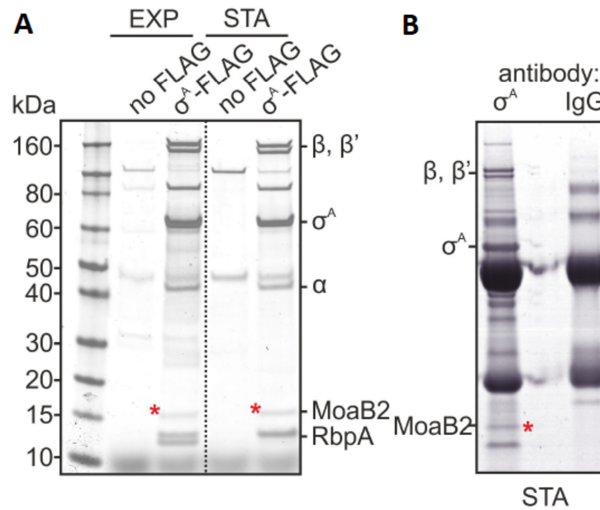

Figure 11. (**A**) SDS-PAGE of immunoprecipitation of FLAG-tagged σ^A (LK3207) using the anti-FLAG antibody. "No FLAG" strain is negative control (LK3016). MoaB2 is marked with red asterisks. (**B**) SDS-PAGE of immunoprecipitation of σ^A from the STA "no FLAG" strain cells (LK3016) using antibody against σ^{70} (anti-σ^{70}, clone 2G10). IgG is a mouse nonspecific IgG used as a negative control. MoaB2 is indicated with red asterisk. Adapted from (Brezovská et al. 2024).

3.6. Transcription factors

Bacterial TFs are proteins that regulate gene expression by interacting with RNAP and influencing transcription initiation, elongation, and termination. Their function is to activate or inhibit transcription of DNA by binding to RNAP or specific DNA sequences. They influence cellular responses to environmental changes by activating or repressing the transcription of specific genes (Browning and Busby 2016). In the *M. smegmatis* genome, about 500 TFs were predicted by the National Center of Biotechnology Information (Yang et al. 2012).

The second main focus of this Doctoral Thesis is dedicated to three key TFs from *M. smegmatis*: (i) RbpA, (ii) CarD, and (iii) HelD. Each factor plays a unique role in modulating RNAP activity. First, RbpA and CarD will be introduced, and Introduction will be closed with description of HelD, the primary focus of this part of the Doctoral Thesis.

3.6.1. RbpA

RbpA (13.4 kDa) is an essential TF that stabilizes the RPo (Figure 12). RbpA is not present in *E. coli*, suggesting distinct regulation of transcription across bacterial species (Stallings et al. 2009).

Figure 12. Structural scheme of the RNAP-σ^A holoenzyme (grey) from *M. tuberculosis* with RPo with CarD (blue) and RbpA (red). Cryo-EM structure containing the Mtb rrnAP3 promoter (-60 to +30) with a fully open DNA bubble. Adapted from (Jensen et al. 2019).

RbpA interacts with both RNAP and the σ^A by binding to the β' subunit near the N-terminal domain of σ^A. It enhances the affinity of RNAP for the promoter DNA and its binding position stabilizes the RPo complex. This interaction occurs near the RNAP DNA entry channel, helping maintain the complex's stability (Hu et al. 2012; Hubin, Lilic, et al. 2017). RbpA acts synergistically with CarD to enhance transcriptional fidelity and initiation efficiency.

RbpA was discovered in *Streptomyces coelicolor* as "RNA polymerase-binding protein A" and later was identified in mycobacteria. *M. tuberculosis* RbpA is 55% identical to *S. coelicolor* RbpA but lacks the C-terminal extension (Bortoluzzi et al. 2013; Paget et al. 2001). Later it was shown that RbpA bears basic level of rifampicin resistance and acts as a regulator of rRNA synthesis also in *M. smegmatis* (Dey, Verma, and Chatterji 2011; Newell et al. 2006).

RbpA consists of a central core domain (CD), an unstructured N-terminal tail (NTT), and a C-terminal σ-interaction domain (SID) linked to the CD by a basic linker (BL) (Bortoluzzi et al. 2013; Hubin et al. 2015; Hubin, Fay, et al. 2017; Prusa et al. 2018; Tabib-Salazar et al. 2013). The RbpA SID binds and forms a tight complex with $\sigma^A{}_2$, while not binding to other σ^A domains (Hubin et al. 2015). It induces a conformational change on the σ factor, which leads to the σ_2 and σ_4 domains being stretched over the surface of E (Vishwakarma et al. 2018, 2024; Vishwakarma and Brodolin 2020). RbpA also interacts with σ^B, forming a stable complex through its N- and C-terminal regions with the σ^B (Bortoluzzi et al. 2013; Wang et al. 2018). Without RbpA, the $E\sigma^B$ (unlike $E\sigma^A$) oligomerizes into an octamer in which the RNAP clamp is maintained in a fully opened conformation, resembling the structure observed when RNAP is inhibited by fidaxomicin (Morichaud et al. 2023). Binding of RbpA induces a σ^B remodeling that

subsequently promotes RNAP clamp closure and octamer dissociation (Boyaci et al. 2018; Morichaud et al. 2023; Vishwakarma et al. 2024).

Structural studies suggest that RbpA NTT binds to Eσ in a manner that contributes to fidaxomicin binding (Boyaci et al. 2018). When RbpA NTT was deleted, the positive effect of RbpA on the RNAP-fidaxomicin binding interface was lost. This reduced the sensitivity of *M. tuberculosis* RNAP to fidaxomicin *in vitro* and *in vivo* (Boyaci et al. 2018; Prusa et al. 2022).

3.6.2. CarD

CarD (17.9 kDa) interacts with the N-terminus of the RNAP β subunit near the upstream edge of the transcription bubble and contacts the β1 and β' regions of RNAP. The position of the N-terminus of CarD binding is close to the RNAP AS, stabilizes the RPo and prevents RNAP backtracking during transcription initiation (Stallings et al. 2009; Zhu and Stallings 2023). The RPo in mycobacteria (compared to e. g. *E. coli*) is relatively unstable, with a lifetime of 2 minutes or less, making it prone to be reversed (Davis et al. 2014). Molecular modeling of CarD and RPo suggested that CarD activates transcription by wedging a tryptophan residue into the top edge of the transcription bubble, thereby stabilizing RPo (Figure 13) (Srivastava et al. 2013).

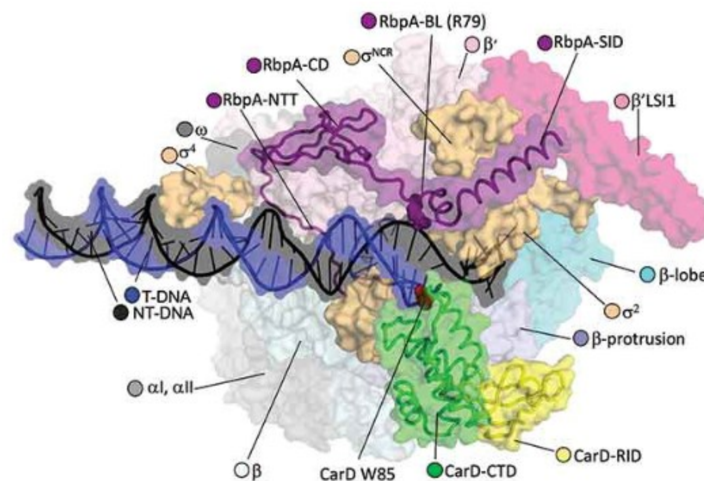

Figure 13. Structure of RNAP from *M. tuberculosis* with a *de novo* melted promoter and TFs, CarD and RbpA. The figure highlights RbpA serving as a tether immediately upstream of the transcription bubble, and CarD wedging into the upstream edge of the transcription bubble with W85 residue. Adapted from (Boyaci, Saecker, and Campbell 2020).

CarD was identified in a transposon screen aimed to discover genes involved in blue light-induced carotenogenesis in *Myxococcus xanthus*. CarD is conserved across eubacteria phyla and is essential in mycobacteria (Zhu and Stallings 2023). It functions as either an activator or repressor depending on the basal promoter kinetics and RPo stability (Davis et al. 2014; Jensen et al. 2019; Zhu et al. 2019; Zhu

and Stallings 2023). In coordination with RbpA, CarD slows RNAP escape from the promoter and acts during and after transcription bubble formation (Jensen et al. 2019; Zhu and Stallings 2023). ChIP-seq (chromatin immunoprecipitation) studies have shown that CarD co-localized with σ^A at promoters genome-wide, suggesting its role on σ^A-dependent regulons (Srivastava et al. 2013). Weakening the interaction between RNAP and CarD makes mycobacterial cells more sensitive to stress conditions including DNA damage, oxidative stress and antibiotics (Garner et al. 2017; Li et al. 2022). Also, CarD could functionally substitute for DksA, a regulator of (p)ppGpp in *E. coli*, that is absent in mycobacteria (Stallings et al. 2009).

The TFs RbpA, and CarD exhibit distinct yet complementary interactions with RNAP, allowing *M. smegmatis* to efficiently regulate transcription by binding in specific RNAP channels or domains, thereby enhancing transcriptional stability, promoter affinity, and elongation efficiency.

3.6.3. HelD

HelD, or recently proposed name HelR in Actinobacteria, is a helicase-like TF that was reported to act during transcription elongation rather than initiation. It was first identified in *B. subtilis* (Delumeau et al. 2011). HelD belongs to the UvrD family of helicases. Bioinformatic analysis showed it consists of three domains: an N-terminal domain, which has no homology with other known helicases, an ATPase domain and a C-terminal domain (Epshtein et al. 2014; Gwynn et al. 2013). HelD also belongs to the SF1A superfamily and can unwind DNA in the 3′-5′ direction in ATP dependent manner (Koval′ et al. 2019; Singleton, Dillingham, and Wigley 2007). 3D structure of UvrD is most closely related to HelD although their sequence identity is low (12%) (Koval′ et al. 2019). Another protein, RapA from *E. coli* shares, 21% sequence identity with HelD. It is involved in the release of stalled transcription complexes by reverse translocation and its domain structure resembles that of UvrD (N-terminal domain, ATPase domain) (Liu et al. 2015; Shaw et al. 2008).

In *B. subtilis*, SAXS experiments showed HelD can bind and hydrolyze ATP accompanied by protein conformational changes (Koval′ et al. 2019). The overexpression of HelD in *B. subtilis* stimulated spore formation and the absence of HelD on the other hand led to an extended lag phase during the outgrowth of STA cells when transferred into fresh medium (Meeske et al. 2016; Wiedermannová et al. 2014). This phenotype aligns with another role of HelD, suggested by Pei et al. (2020), and that is storing inactive RNAPs during the STA (Pei et al. 2020; Sudzinová et al. 2022).

HelD also actively displaces stalled RNA-DNA hybrid from RNAP, which facilitates the recycling of RNAP after transcriptional pausing or stalling and helping them be ready for subsequent rounds of transcription (Wiedermannová et al. 2014). Stalled RNAP complexes can be formed due to DNA obstacles or from RNAP inhibition by antibiotics similar to rifampicin (Hurst-Hess et al. 2022).

In *Streptomyces venezuelae*, HelD was associated with an intrinsic resistance mechanism to rifampicin. A 19 bp palindromic sequence called the rifamycin-associated element (RAE) encodes a rifamycin glycosyltransferase was found upstream of the HelR coding sequence, a rifamycin-associated helicase-like protein (homologue of HelD from *M. smegmatis*) (Spanogiannopoulos et al. 2014; Surette et al. 2022). RAE codes for rifamycin glycosyltransferase and appears in the 5' untranslated region (5' UTR), upstream of all known rifamycin-inactivating enzymes (Arr, Rgt, Rox, Rph) (Spanogiannopoulos et al. 2014; Surette et al. 2022).

In *M. abscessus*, transcriptomic analysis of strains exposed to sublethal rifampicin doses revealed a strong induction of putative HelD-like helicase from the SF1 superfamily of RNA/DNA helicases, HelR, while similar upregulation was observed in the homologous *helD* gene in *M. smegmatis* under rifampicin exposure (Hurst-Hess et al. 2019; Newing et al. 2020). Analysis showed that deleting *helR* causes hypersensitivity to both rifampicin and rifabutin, a spiro-piperidyl derivative of rifampicin (Hurst-Hess et al. 2022).

Prior to my Doctoral Thesis, the 3D structure of the complex between RNAP and HelD was unknown, as well as the mode how it released stalled RNAP from DNA. Likewise, it was unknown what complexes HelD can be in besides with RNAP, how HelD was released from RNAP, and whether it protected *M. smegmatis* directly against rifampicin.

AIMS

The primary objective of this Thesis was to expand our knowledge about the transcription machinery in mycobacteria, using the model organism *M. smegmatis*. My aims specifically were:

<u>The specific aims of MoaB2 project were:</u>

- Characterize the interaction between MoaB2 and σ^A.
- Determine whether MoaB2 is essential.
- Contribute to solving the 3D structure of MoaB2.
- Describe the role of MoaB2 in mycobacterial transcription.
- Explore the effect of MoaB2 on the stability of σ^A in the cell.

<u>The specific aims of HelD project were:</u>

- Identify binding partners of HelD.
- Determine the effect of HelD on the affinity of RNAP for iNTPs and the stability of RPo.
- Examine the role of HelD in transcription in the presence/absence of the antibiotic rifampicin.

LIST OF METHODS

Experimental work on both projects of this Doctoral Thesis was performed on the model organism *M. smegmatis* mc2 155. Plasmids were purified from *E. coli* DH5α, proteins were purified from *E. coli* DE3 (BL21 or Lemo21).

Work with bacteria

Bacterial cells cultivation (solid, liquid media)

Transformation (*E. coli* and *M. smegmatis*)

Characterization of bacterial phenotype (stress conditions)

Test of gene essentiality

Work with nucleic acids

Cloning, sequencing

DNA isolation, PCR

RNA isolation, reverse transcription, qPCR

Northern blot

Work with proteins

Protein purification (affinity chromatography)

Immunoprecipitation

Bacterial *in vitro* transcription assays (single- and multiple-round)

Western blot analysis (and dot blots)

LIST OF PUBLICATIONS

During my PhD studies, I participated on three scientific publications. In one of them I am a joint first co-author, in the other two I am a co-author. All three publications focus on the role of selected TFs in gene expression in *M. smegmatis*. First publication characterizes the interaction of the MoaB2 protein with primary σ factor, σ^A, and identifies its potential biological roles (Brezovská et al. 2024). The MoaB2 project was the main project of my PhD studies. The subsequent two publications investigate the role of the HelD protein, its interaction with RNAP, and its contribution to rifampicin resistance (Kouba et al. 2020; Kovaľ et al. 2024). Together, my Thesis deepens our understanding of the interplay between regulatory factors in gene expression in mycobacteria. The individual publications are appended at the end of this Thesis.

Publication I

MoaB2, a newly identified transcription factor, binds to σ^A in *Mycobacterium smegmatis.*

Brezovská B*, Narasimhan N*, Šiková M, Šanderová H, Kovaľ T, Borah N, Shoman M, Pospíšilová D, Vaňková Hausnerová V, Tužinčin D, Černý M, Komárek J, Janoušková M, Kambová M, Halada P, Křenková A, Hubálek M, Trundová M, Dohnálek J, Hnilicová J, Žídek L, Krásný L

*These authors contributed equally as joined first authors.

Journal of Bacteriology (2024): e00066-24. doi.org/10.1128/jb.00066-24.

PMID: 39499088

Accepted: 18 September 2024

Published: 05 November 2024

IF 2023/2024: 2.7

Contribution of the author: 40 %. I constructed the σ^A-FLAG construct integrated at its endogenous locus and $\sigma^A_{\Delta 60aaN}$-FLAG with N-terminal deletion of 60 aa to test if σ^A_N is involved in the binding of MoaB2 to σ^A in the cell. I performed immunoprecipitation of FLAG tagged σ^A to identify MoaB2 in the pull down and prepared samples for the LC-MS/MS analysis. I purified the MoaB2 protein for X-ray crystallography, and I was in close communication with my shared first author, Subhash Narasimhan (a crystallographer), discussing with him structural aspects of the study. I determined growth curves of the *moaB2* CRISPR-Cas9 depletion strain to see potential phenotypes. I tested the gene expression of this depletion strain for multiple RNA by RT-qPCR. I purified supercoiled DNA template P*rrnAPCL1* and proteins σ^A, MoaB2, RNAP for *in vitro* biochemical assays. Using multiple-round *in vitro* transcription assay I tested whether MoaB2 is able to affect transcription. By western blot, I

determined the relative levels of $σ^A$ and the β subunit of RNAP in *moaB2* depletion strain to see the stabilizing effect of MoaB2 on $σ^A$. I analyzed the data and prepared relevant graphs/figurers for the manuscript. I prepared the initial draft of the manuscript and participated on it writing throughout.

Publication II

Mycobacterial HelD is a nucleic acids-clearing factor for RNA polymerase.

Kouba T*, Koval′ T*, Sudzinová P*, Pospíšil J, Brezovská B, Hnilicová J, Šanderová H, Janoušková M, Šiková M, Halada P, Sýkora M, Barvík I, Nováček J, Trundová M, Dušková J, Skálová T, Chon U, Murakami KS, Dohnálek J, Krásný L

*These authors contributed equally as joined first authors.

Nature Communications 11.1 (2020): 6419. doi.org/10.1038/s41467-020-20158-4.

PMID: 33339823

Accepted: 17 November 2020

Published: 18 December 2020

IF 2023/2024: 14.7

Contribution of the author: 10 %. I cloned the gene encoding HelD-FLAG into an expression vector for subsequent biochemical assays. Furthermore, I performed immunoprecipitation with HelD-FLAG to investigate, whether FLAG-tagged HelD can pull down the RNAP core and $σ^A$. I contributed to manuscript's writing and preparation.

Publication III

Mycobacterial HelD connects RNA polymerase recycling with transcription initiation.

Kovaľ T*, Borah N*, Sudzinová P, Brezovská B, Šanderová H, Vaňková Hausnerová V, Křenková A, Hubálek M, Trundová M, Adámková K, Dušková J, Schwarz M, Wiedermannová J, Dohnálek J, Krásný L, Kouba T

* These authors contributed equally as joined first authors.

Nature Communications 15.1 (2024): 8740. doi.org/10.1038/s41467-024-52891-5.

PMID: 39384756

Accepted: 23 September 2024

Published: 09 October 2024

IF 2023/2024: 14.7

Contribution of the author: 15 %. I purified all the components (RNAP, σ^A, RbpA, CarD, HelD, plasmid containing the tested promoter) for multiple-round *in vitro* transcription assays and characterized the effect of HelD on transcription in the presence or absence of rifampicin. I also performed the *in vitro* experiments testing whether HelD affects the affinity of RNAP for iNTP. I performed subsequent quantitation and statistical analyses. I contributed to manuscript's writing and preparation.

This publication was one of the main projects of my colleague, a PhD student from my laboratory, Nabajyoti Borah.

I confirm that this Doctoral Thesis was written by Barbora Brezovská and that it is based on published data collected and generated during her Ph.D. study in between 2019-2024. I hereby declare that her contribution to the publications described above is based on the truth.

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Supervisor: Associate Prof. Mgr. Libor Krásný, Ph.D.

SUMMARY OF PUBLICATIONS

Publication I

MoaB2, a newly identified transcription factor, binds to σ^A in *Mycobacterium smegmatis.*

My first-author publication explored the role of MoaB2, a newly identified TF in *M. smegmatis*, and its interaction with the primary σ factor, σ^A. The aim of this study was to extend the understanding of transcription regulation by identifying and characterizing potential new proteins interacting with σ^A and to understand how this interaction influence transcription.

Through immunoprecipitation and mass spectrometry, we detected a number of proteins that co-purified with σ^A, and MoaB2 was identified among the most enriched ones. Their interaction was confirmed to be specific and independent of RNAP or alternative σ factors, including σ^B. In collaboration with researchers from Central European Institute of Technology (CEITEC) and Centre BIOCEV of the Czech Academy of Sciences, we applied analytical methods to explore whether MoaB2 binds directly to σ^A also *in vitro* or requires another unknown factor for their interaction. We found out they directly form a complex with no need of additional factor.

To characterize the binding of MoaB2 to σ^A, we studied the stoichiometry of their interaction. Results showed that σ^A is predominantly monomeric when alone in solution, while MoaB2 forms hexamers and minor trimers. Upon mixing σ^A and MoaB2, they formed a complex with a 1:1 stoichiometry. Binding affinity of this interaction was strong, with a submicromolar dissociation constant, that showed a strong and specific interaction. We also determined the crystal structure of MoaB2, which forms a hexamer, and our calculations revealed a prominently negative electrostatic potential on its surface. The structure revealed homology with MoaB proteins involved in molybdenum cofactor (MoCo) biosynthesis (Schwarz and Mendel 2006), although MoaB2 likely serves a different role in *M. smegmatis*.

Electrostatic surface analysis suggested that negatively charged MoaB2 might interact with positively charged regions on σ^A, located at its N-terminus. Specifically, this N-terminal domain of σ^A (σ^A_N) is the main difference that distinguish σ^A from alternative stress factor σ^B, that lacks this extended N-terminal domain. By immunoprecipitation using mutant σ^A strain lacking σ^A_N ($\sigma^A_{\Delta N}$) we showed that σ^A_N is involved in the interaction between σ^A and MoaB2. However, a more defined mutant of σ^A, which lacked the 60 N-terminal aa ($\sigma^A_{\Delta 60aaN}$), did not demonstrate the essentiality of these 60 N-terminal aa for the interaction with MoaB2. Subsequent NMR analysis of σ^A revealed σ^A_N to be intrinsically disordered but crucial for MoaB2 binding. Specific residues within the σ^A_N region between aa 111 and 143 showed chemical shifts upon MoaB2 binding, indicating their role in the interaction.

We subsequently demonstrated that MoaB2 is not essential for the growth of *M. smegmatis*. Our CRISPR-Cas9 knockdown experiments showed that while depleting σ^A caused significant growth inhibition, depletion of MoaB2 had no impact on the strain's growth, indicating that MoaB2 was not critical for survival under the tested conditions.

Although MoaB2 did not manifest any strong phenotype *in vivo*, we explored MoaB2's effect on σ^A-dependent transcription *in vitro*. We conducted multiple-round transcription assays using purified RNAP, σ^A or σ^B, RbpA, CarD, and MoaB2. Our results revealed that increasing concentrations of MoaB2 progressively inhibited σ^A-dependent transcription, suggesting that MoaB2-σ^A complex formation interferes with the formation of the Eσ^A (Figure 14). This inhibitory effect was alleviated by increasing σ^A levels, while σ^B-dependent transcription remained unaffected, highlighting MoaB2's selective interaction with σ^A.

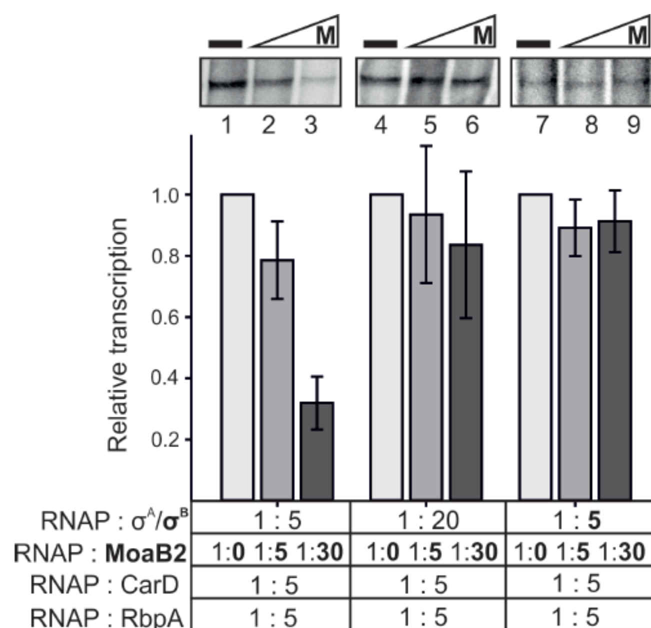

Figure 14. Column graph showing the effect of MoaB2 on σ^A-dependent transcription (lanes 1-3) but has no effect on σ^B-dependent transcription (lanes 7-9). *In vitro* transcriptions were done with RNAP reconstituted with σ^A (1:5 or 1:20 ratios) or σ^B (1:5). MoaB2 was present (1:5, 1:30) or absent (1:0). CarD and RbpA were present (indicated below the graph). Representative primary data are shown above the graphs (M, MoaB2). All graphs show averages from three independent experiments, and the error bars indicate ±SD. Adapted from (Brezovská et al. 2024).

The study further investigated whether MoaB2 influences the stability of σ^A *in vivo*. Comparing MoaB2-depleted and control strains, we found that σ^A protein levels decreased by approximately half within 4 hours after translation was blocked in the MoaB2-depleted strain, while remaining stable in the control. The β subunit of RNAP showed similar stability in both strains. While the MoaB2-σ^A

interaction seemed to be more pronounced during the STA, the low σ^A levels at this stage limited further analysis. This suggested that MoaB2 could play a role in stabilizing σ^A.

Collectively, our findings suggested that MoaB2 may function similarly to Rsd, potentially modulating gene expression by sequestering σ^A and affecting its stability in the cell. This mechanism would allow *M. smegmatis* to regulate σ^A-dependent transcription dynamically, particularly in response to environmental stress. We proposed that MoaB2 could serve as a target for developing antimycobacterial strategies by exploiting its role in transcriptional regulation.

Publication II

Mycobacterial HelD is a nucleic acids-clearing factor for RNA polymerase.

This publication investigated the mechanism by which HelD from *M. smegmatis* recycles RNAP. We showed that HelD interacts with RNAP, both the E and in complex with $σ^A$. When RNAP is stalled on DNA, HelD dissociates it, allowing other RNAPs to transcribe and to prevent transcription-replication collisions that can be deleterious for the cell. The results of this study provided further understanding of the regulation of gene expression and offered insights into the structural and functional role of HelD in transcriptional recycling and genome integrity.

HelD is a helicase-like TF that plays a crucial role in dissociation of stalled RNAPs. In collaboration with researchers from Centre BIOCEV and Institute of Organic Chemistry and Biochemistry of the of the Czech Academy of Sciences, we used cryo-electron microscopy (cryo-EM) and identified three major structural states (State I, II, III) of the HelD-E complex. This analysis revealed that HelD adopts a crescent-like shape that allows it to penetrate into both the primary and secondary channels of RNAP. HelD consists of primary channel (PCh) loop, clamp opening (CO) domain, ATPase domain and N-terminal domain (Figure 15). HelD's primary channel loop penetrates into RNAP AS and displaces stalled transcription elongation complexes and facilitates nucleic acid removal. The CO domain interacts with RNAP β' and holds the DNA clamp open. The N-terminal α-helical coiled-coil (NCC) domain inserts into the RNAP secondary channel.

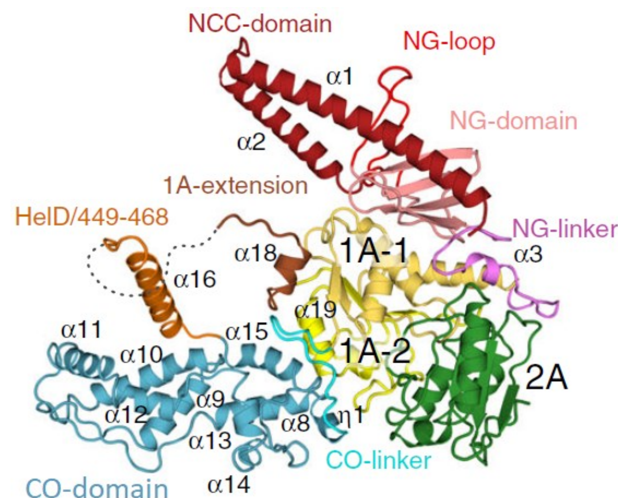

Figure 15. Structure of HelD from *M. smegmatis*. The N-terminal domain of HelD forming an antiparallel α-helical coiled-coil (NCC-domain in dark red), followed by four-β-strand globular domain (NG-domain in light red) and linker (NG-linker in pink), connecting 1A domain (yellow) with N-terminal domain. 1A-2A (green) is forming a heterodimer. CO stands for "clamp-opening" and CO-linker (light blue) is stabilizing interaction between CO-domain (blue) and 1A domain. HelD/449-468 in "HelD State I" later in "HelD State II" forms a primary channel loop (PCh-loop). Adapted from (Kouba et al. 2020).

Primary and secondary channels of RNAP are essential for nucleic acid binding and substrate delivery. By occupying these channels, HelD effectively locks RNAP in an inactive state, preventing non-specific DNA binding and clearing stalled elongation complexes. Our biochemical assays demonstrated that HelD has significant ATPase and GTPase but not CTPase activities that may help to release HelD from RNAP after recycling is complete.

Moreover, using electrophoretic mobility shift assay (EMSA) we tested whether the position of the HelD's CO-domain engaging in the primary channel of RNAP can disrupt non-specific interaction between the E and DNA. We used RNAP and a fragment of mycobacterial DNA in the presence or absence of HelD and showed that HelD significantly abolishes the non-specific binding of the E to DNA. In addition, HelD not only prevented DNA binding but also actively dismantled stalled elongation complexes (ECs). These stalled ECs, often caused by damaged DNA, present obstacles to the coupled transcription-translation machinery.

Another finding was that HelD, σ^A and RbpA can bind together to RNAP in one complex. It suggested that mycobacterial HelD perhaps participates in transcription initiation. By immunoprecipitation we confirmed the presence of these proteins in one complex that indicated HelD's assistance during the transition of RNAP from an inactive state to transcription initiation.

This study provided a detailed description of the structure and mechanism of HelD function, shedding light on how it dissociates stalled RNAPs. Our findings improved the understanding of transcriptional regulation in bacteria and highlighted the importance of TFs such as HelD in RNAP function.

Publication III

Mycobacterial HelD connects RNA polymerase recycling with transcription initiation.

This study was a follow-up of the previous paper. Here we focused on *M. smegmatis* HelD and investigated in detail its release from RNAP during transcription initiation, and defined how it protects RNAP against rifampicin. This publication employed cryo-EM, biochemical assays like ATP/GTP hydrolysis assay, HelD release assay, and *in vitro* transcription.

As we described in our previous publication, HelD is known for clearing stalled RNAP complexes by dissociating them from nucleic acids. However, the mechanism by which is HelD itself detached afterwards from RNAP to enable next rounds of transcription had been unclear. In this study we addressed this by examining the structural configuration and interactions of HelD with transcription apparatus in various transcriptional states.

Initially, we identified the *in vivo* interaction partners of HelD using pull-down assays with *M. smegmatis* HelD-FLAG strains. This confirmed HelD's association with RNAP subunits, σ^A, and the TF RbpA, while excluding the global regulator CarD. The structure and specific interactions within the RNAP-σ^A-RbpA-HelD complex were investigated in collaboration with researchers from Centre BIOCEV and Institute of Organic Chemistry and Biochemistry of the of the Czech Academy of Sciences. Our cryo-EM snapshots showed two 3D structural classes of the RNAP-σ^A-RbpA-HelD complex, where HelD was positioned within both RNAP channels, blocking DNA and RNA binding sites, that was a confirmation from our previous publication. In both classes, $\sigma^A{}_2$ in the complex was interacting with the binding site on the RNAP β′-clamp CC domain, which made an interaction platform for the HelD CO-domain. This contact region of the HelD CO-domain and RNAP β′-clamp CC domain became better defined and illustrated the importance of σ^A for the formation of a stable interaction of HelD with RNAP.

Next, we reconstituted the HelD-σ^A-RbpA-RNAP complex with a promoter DNA containing -35 and -10 promoter elements in order to test whether closed complex formation displaces HelD from HelD-Eσ complexes. We showed that binding of the DNA did not eject HelD from the RNAP complex, showing simultaneous binding of RNAP with HelD. This led to another three cryo-EM 3D structural classes, that presented structures of HelD-containing transcription initiation complexes. These 3D classes illustrated how the progressive removal of HelD domains from the RNAP primary channel led to the RNAP β′-clamp closure.

We also investigated the RPo formation, where we reconstituted the HelD-σ^A-RbpA-RNAP complex with a DNA promoter with an artificially opened transcription bubble. We identified three

cryo-EM 3D structural classes where in all of them, HelD N-terminal domain must have been released from the RNAP secondary channel for proper formation of the RPo.

In addition, as our pull-down experiments suggested that CarD was not present on RNAP together with HelD, we compared our cryo-EM data of HelD-σ^A-RbpA-RNAP-DNA with a similar structure containing CarD instead of HelD. This revealed that binding of HelD changed the location of the −10 promoter element away from the RNAP β-lobe, and these are the two main binding domains of CarD during transcription initiation. Thus, this relocation of promoter region and β-lobe caused by HelD weakened CarD binding and showed why CarD and HelD are not present on RNAP at the same time.

As the transcription complex underwent isomerization towards forming the RPo, HelD has to be released in a specific process enhanced by ATP or GTP binding, allowing full RNAP clamp closure necessary for transcription initiation showed in Publication II. One of our core questions was how HelD detaches from RNAP. We showed that ATP binding and hydrolysis, along with interactions with promoter DNA, were critical. The biochemical data showed that as RNAP approached DNA, ATP binding together with hydrolysis gradually displaced HelD from the primary channel, allowing RNAP to close its clamp fully and transition into an initiation-ready state.

We also showed that the presence of σ^A, RbpA, or their combination does not affect HelD release, even when the HelD-σ^A interaction was mutated. In contrast, DNA, specifically open complex (OC) DNA, enhanced HelD release. OC DNA alone stimulated release even without ATP, while the combination of OC DNA and ATP exhibited a synergistic effect and was the most efficient for HelD dissociation. These findings aligned with the structural analyses and highlighted the specific role of DNA in facilitating HelD release.

We further showed by *in vitro* transcription assays that RNAP in complex with HelD exhibited reduced inhibition by rifampicin. Binding of HelD to RNAP channels displaced rifampicin from its binding pocket, thereby providing protection from the antibiotic. This suggested a role for HelD in rifampicin resistance.

Taken together, this publication established a stepwise process of how HelD uncouples from RNAP, suggesting its dual role in recycling RNAP stalled on DNA and protecting RNAP against rifampicin. Our findings provided insight into the molecular dynamics of RNAP recycling process and show the impact of HelD on transcription *in vitro* in the presence or absence of rifampicin. This work further deepened our understanding of the structure of the mycobacterial transcription complex and what precise stages and steps must occur for RNAP to proceed to the next stages of transcription.

DISCUSSION

In this discussion of my Doctoral Thesis, I will delve into key findings regarding two critical TFs in *M. smegmatis*: MoaB2 and HelD. First, I will present MoaB2 as a newly identified TF that binds to the primary σ factor, σ^A, influencing transcriptional regulation through its potential role similar to that of Rsd (Brezovská et al. 2024). Next, I will explore the function of HelD as a nucleic acids-clearing factor, facilitating the release of stalled RNAP from nucleic acids (Kouba et al. 2020). Finally, I will discuss how HelD connects the recycling of RNAP to transcription initiation, the mechanism of HelD dissociation from RNAP and I will present its role in rifampicin resistance (Kovaľ et al. 2024).

MoaB2

The study of the interaction between MoaB2 and σ^A, and its effect on bacterial transcription, is detailed in the accompanying **Publication I**. The functional and structural aspects of MoaB2, a novel binding partner of the primary σ factor σ^A, and how this interaction affects the transcription and stability of σ^A in the mycobacterial cell are investigated.

The role of Moa proteins in bacteria

The MoaB family of proteins, mainly MoaA, MoaB, MoaC and MogA, has previously been associated with the biosynthesis of molybdenum cofactors (Moco), which have been studied mainly in *E. coli*. Bacterial molybdoenzymes, enzymes with incorporated Moco in their AS, are essential for the global cycling of sulfur, nitrogen, and carbon and can catalyze a wide variety of redox reactions in bacterial cell (Williams, Kana, and Mizrahi 2011; Zhong, Kobe, and Kappler 2020; Zupok et al. 2019). In *E. coli*, MoaB and MogA were initially thought to be involved in Moco biosynthesis (Leimkühler 2020; Nichols and Rajagopalan 2002), but further studies revealed that MoaB, unlike MogA, has no role in this process (Kozmin and Schaaper 2013). MoaB, despite its name, was shown to have no direct role in MoCo biosynthesis, as subsequent experiments revealed that its deletion or overexpression had no effect on the production of MoCo-dependent enzymes (Kozmin and Schaaper 2013). MoaB from *E. coli* shares 30% aa identity with MoaB2 from *M. smegmatis* (Brezovská et al. 2024).

On the other hand, MogA is essential for the final steps of MoCo biosynthesis, especially in the coordination of molybdenum insertion into cofactors (Leimkühler 2017; Srivastava et al. 2016).

Unlike in *E. coli*, MoaB2 in *M. smegmatis* does not participate in Moco biosynthesis but instead interacts with the transcriptional machinery (Brezovská et al. 2024). That suggests a novel regulatory role of Moco biosynthesis related protein not previously observed in other bacterial species. MoaB2 is encoded by the *MSMEG_5485* (*moaB2*) gene, the last gene transcribed from predicted *mprA-mprB-pepD-moaB2* operon (MSMEG_5488-MSMEG_5484). This locus is highly conserved in all

Mycobacterium species (Bretl et al. 2014; White et al. 2010; Zahrt and Deretic 2001). Genes *mprA* and *mprB* code for the two-component signal transduction system MprAB (Bretl et al. 2014) and *pepD*, the third gene in the operon, encodes an HtrA-like serine protease (White et al. 2010). Genome of *M. tuberculosis* also contains a locus *mprA-mprB-pepD-moaB2* (Rv0981-Rv0984) with high aa similarity to MprA, MprB, PepD, and MoaB2 from *M. smegmatis*, displaying a comparable genetic organization (White et al. 2010).

MoaB2 is annotated in Mycobrowser (*MSMEG_5485*) and Uniprot (A0R3I5) as a 178 aa long protein, with size of 17.9 kDa. But based on our mass spectrometry results, we revealed that MoaB2 that interacts with σ^A is 164 aa long, with size of 16.5 kDa. Translation of MoaB2 starts with methionine (ATG) 14 aa after the annotated predicted translation start (GTG) (Brezovská et al. 2024).

The identification of MoaB2 as a σ^A-interacting protein marks a shift in the understanding of the MoaB family in mycobacteria. In the **Publication I**, MoaB2 was found to specifically interact with σ^A but not with alternative σ factors σ^B, σ^E, σ^F, σ^G, and σ^H which was consistent with other reports in bacteria showing a strict division of labor between σ factors and their regulatory partners (Kazmierczak et al. 2005; Österberg, Peso-Santos, and Shingler 2011). This specificity is a key factor because it suggests that MoaB2 modulating σ^A-dependent transcription, while not interacting with RNAP, acts as an anti-σ factor-like protein, that compete for σ^A with RNAP. Thereby can influence gene expression during different phases of growth and stress responses, rather than playing a direct role in MoCo biosynthesis.

Moonlighting functions of Moa proteins

Moa proteins are an example of "moonlighting" proteins - proteins that perform several different functions in the cell. Silke Leimkühler (2017) pointed out that many proteins involved in MoCo biosynthesis have additional roles in other cellular processes, including Fe-S cluster assembly and tRNA thiolation (Leimkühler 2017; Leimkühler et al. 2011). In *M. smegmatis*, however, MoaB2 binds to σ^A and affects transcription. Moreover, at a catalytically important aa site of the protein, conserved in an enzyme family capable of molybdenum adenylation, a distinct aa is found in MoaB2 (Brezovska et al. 2024). It is possible that MoaB2 has diverged evolutionarily, its main and only role is binding to σ^A, it is no longer involved in MoCo synthesis, and it may therefor become even more functionally specialized or efficient in the future.

Structural and functional study of MoaB2 and σ^A_N

Structurally, MoaB2 shares homology with other Moa and MogA proteins of different bacterial species, although its interaction with σ^A is unique to *M. smegmatis*. The crystal structure of MoaB2, as reported in **Publication I**, revealed a highly conserved fold common to Moa proteins, but hides additional

features that enable its binding to σ^A (Brezovská et al. 2024). MoaB2 most likely binds with the unstructured, intrinsically disordered N-terminal region of σ^A ($\sigma^A{}_N$), that is absent in σ^B (Singha et al. 2023). To reveal the structure of MoaB2, we used X-ray crystallography and NMR to investigate that $\sigma^A{}_N$ plays a role in MoaB2 binding and transcription modulation.

The 3D crystal structure of MoaB2 revealed that it forms a hexamer, composed from dimers or trimers into the asymmetric unit (Figure 16). Such a hexameric structure is prevalent among MoaB protein structures (Havarushka et al. 2014; Sanishvili et al. 2004). Nevertheless, our MoaB2 structure is unique, as in this study we report the first peer-reviewed published structure of MoaB2 from the genus *Mycobacterium* (PDB: 8BYR). Other MoaB structures from mycobacteria have been published in the Protein Data Bank (PDB) database but have not undergone peer review. Also, the Adaptive Poisson-Boltzmann Solver analysis showed that the surface of MoaB2 has a strongly negative electrostatic potential (Brezovská et al. 2024).

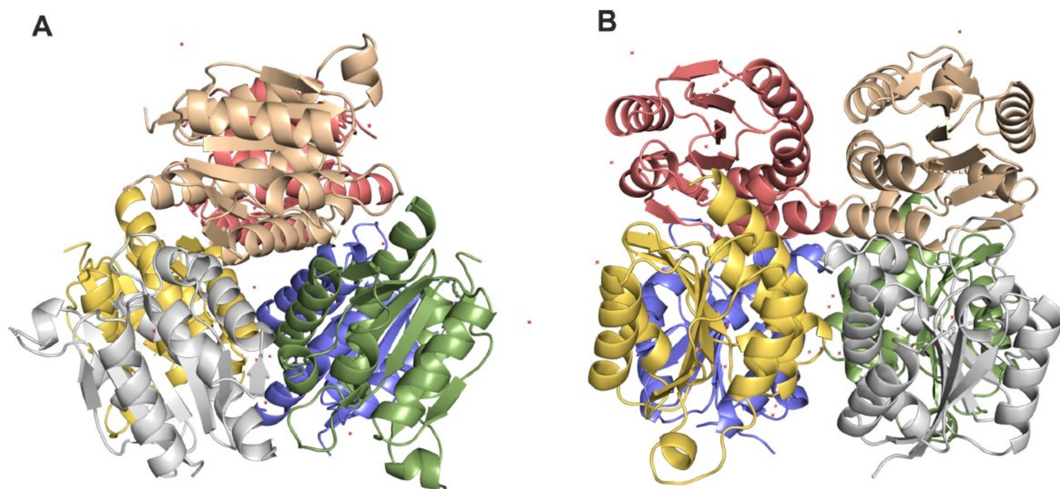

Figure 16. 3D structure of MoaB2 from *M. smegmatis*. (**A**) Top view of the MoaB2 hexamer composed of 3 trimers, subunits are shown in different colors. (**B**) Side view of the MoaB2 hexamer colored as in (A). PDB: 8BYR. Adapted from (Brezovská et al. 2024).

Our pull-down immunoprecipitations showed that MoaB2 interacts with σ^A but does not interact with σ^B, σ^E, σ^F, σ^G, and σ^H. RNA-seq analysis of Singha et al. (2023) showed, that σ^A directly or indirectly regulates approximately 57% of the *M. tuberculosis* transcriptome. Despite sharing approximately 64% similarity with σ^A, σ^B fails to compensate for its loss when overexpressed, highlighting the limited functional redundancy between these two factors (Singha et al. 2023). These two σ factors, σ^A and σ^B, share a domain organization of 4 highly conserved regions 1, 2, 3, and 4. However, they differ from each other in that the long, extended domain of $\sigma^A{}_N$ as it is not present in σ^B (Singh et al. 2022) (Figure 17). Deleting the whole region $\sigma^A{}_N$ resulted in a loss of interaction between MoaB2 and σ^A.

The sequence analysis of σ^A showed that the first 60 aa of σ^A_N are highly positively charged (pI=11.3) due to arginine and lysine aa residues. Deleting this region ($\sigma^A_{\Delta60aaN}$), however, did not disrupt σ^A binding to the negatively charged MoaB2, suggesting another part of σ^A_N mediates the interaction. NMR analysis pointed to the 112 AATPAVATAKAA 123 aa stretch, within a strongly acidic region (Pro61–Pro130), as a likely binding site (Brezovská et al. 2024). Additionally, NMR results further confirmed that σ^A_N adopts a disordered conformation, which is in correlation with recently published σ^A_N from *M. tuberculosis* (Singha et al. 2023). Further experiments are needed to pinpoint the exact binding region, though the disordered nature of σ^A_N makes this challenging.

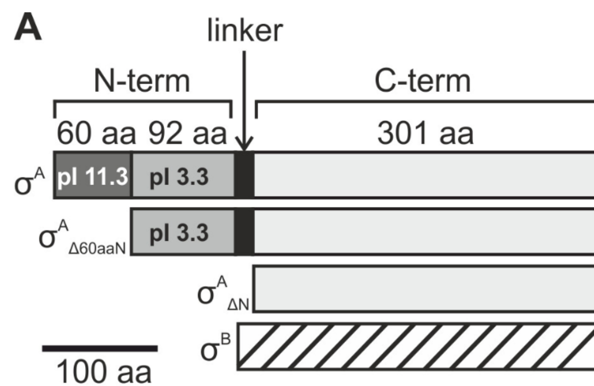

Figure 17. A scheme of linear representation of σ^A, $\sigma^A_{\Delta60aaN}$, $\sigma^A_{\Delta N}$, and σ^B constructs. The scale bar represents 100 amino acids (aa). The length of σ^A is 466 aa, $\sigma^A_{\Delta60aaN}$ is 401 aa, $\sigma^A_{\Delta N}$ is 301 aa, and σ^B is 319 aa. Adapted from (Brezovská et al. 2024).

We found that MoaB2 depletion did not inhibit bacterial growth *in vivo*, indicating that it is not essential for *M. smegmatis*, unlike the housekeeping σ^A. We showed the modulatory effect of MoaB2 on transcription *in vitro*, specifically by inhibiting σ^A-dependent rather than σ^B-dependent transcription. MoaB2 could fine-tune transcription initiation, possibly by influencing the stability and availability of σ^A for the Eσ (Brezovská et al. 2024). Similar to this effect of MoaB2, Rsd in *E. coli* specifically binds to the σ^{70} factor and modulates its activity by interfering with the E and promoter-binding functions (Patikoglou et al. 2007). Also, Rsd does not associate with alternative σ factors, analogous with MoaB2, but rather compete with σ^{70} and alternative σ factors for the available E (Jishage and Ishihama 1998).

At times when the cell does not need σ^A, such as during STA (Hnilicová et al. 2014), MoaB2 binds σ^A and reduces its availability for Eσ (Brezovská et al. 2024). The observation that depletion of MoaB2 led to a decrease in σ^A protein levels after blocking translation by streptomycin, analyzed by western blotting, suggested that MoaB2 could act as a stabilizer of σ^A and help maintain its function, especially under stressful conditions, such as during STA and nutrient limitation (Smeulders et al. 1999). Given

this, MoaB2 could have broader roles than just transcription regulation. The ability of MoaB2 to stabilize σ^A points to a chaperone-like function, as chaperones often interact with proteins to prevent their degradation or misfolding (Lund 2001; Saibil 2013).

These findings suggest that MoaB2 forms a specific complex with σ^A that regulates transcription in a phase-dependent manner and increases the stability of σ^A in the cell (Figure 18).

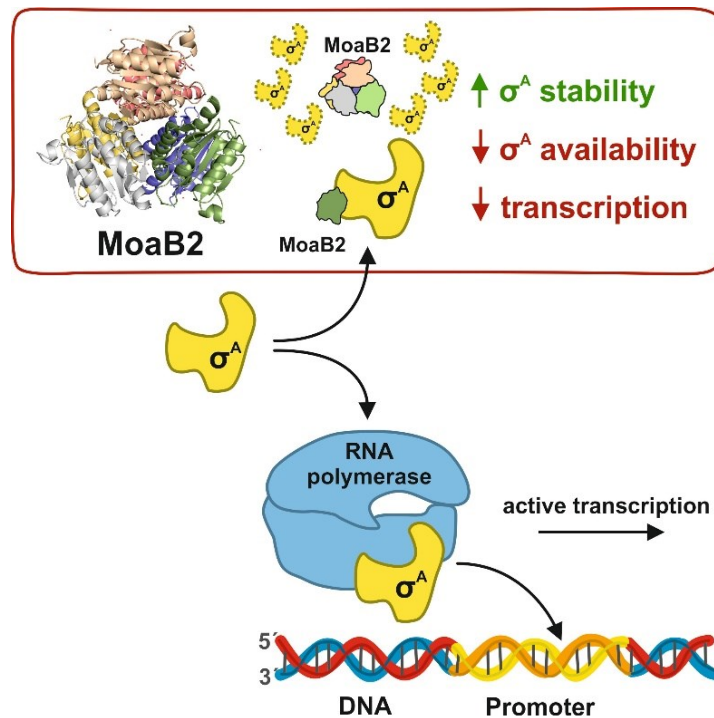

Figure 18. Model of interaction between MoaB2, σ^A, and RNAP in the mycobacterial cell. The model shows that MoaB2 binds to σ^A in a 1:1 ratio, reducing the available σ^A pool and modulates transcription by competing with the E for σ^A. Once bound to MoaB2, σ^A cannot associate with RNAP. Additionally, MoaB2 may stabilize σ^A through this interaction. Adapted from (Brezovská et al. 2024).

Future experiments are needed to determine the role of MoaB2 in further detail. Structural biology techniques such as cryo-EM or prediction tools like AlphaFold3 modeling could provide detailed insight into the MoaB2-σ^A interaction. In addition, performing mutational analysis of specific residues involved in the MoaB2-σ^A interaction could reveal critical contact points necessary for their functional interaction. *In vivo* experiments, including co-immunoprecipitation assays, that previously posed challenges as we struggled to perform a reciprocal pull-down due to the affinity tag being obscured within the quaternary structure of MoaB2. Also, other CRISPR-based knockdown studies, could help to elucidate the role of MoaB2 in σ^A stabilization under different growth conditions, such as during EXP and STA.

In addition, the use of genome-wide approaches such as RNA-seq in MoaB2 depletion/deletion strains could provide insight into the broader regulatory effects of MoaB2 on global transcription. ChIP sequencing of σ^A could also be used to determine the precise MoaB2 binding sites on the bacterial chromosome, shedding light on whether MoaB2 directly regulates σ^A-dependent promoters. Together, these approaches would help determine whether MoaB2 has additional roles beyond TF regulation and whether it acts as a molecular chaperone or plays a role in broader regulatory pathways (Brezovská et al. 2024).

HelD – an overview of the whole story

The study of bacterial TFs has evolved considerably over the past decade, providing new insights into the regulation of RNAP activity. That includes the discovery and characterization of HelD, a helicase-like protein, found in a number of bacteria such as *B. subtilis* and *M. smegmatis*.

The first report of HelD associating with RNAP dates back to a 2011 study where, using affinity purification and mass spectrometry of RNAP complexes of *B. subtilis* cells from different growth phases, the authors identified HelD as one of the RNAP binding partners (Delumeau et al. 2011). Later, HelD was shown by my laboratory to play a key role in RNAP recycling and demonstrated the ability of HelD to help RNAP recover from stalled transcription complexes (Wiedermannová et al. 2014).

Subsequent research on HelD focused on resolving its structure and defining its interactions with RNAP. Initial attempts to crystallize HelD were unsuccessful, and research turned to SAXS. By SAXS, my laboratory demonstrated in that *B. subtilis* HelD changes conformation in dependence on binding and hydrolysis of ATP (Kovaľ et al. 2019). Later, by cryo-EM, we obtained structural data on HelD from *M. smegmatis* that was in complex with RNAP (Kouba et al. 2020). Studies from other teams then in parallel determined the same complex but from *B. subtilis*. We and the other teams became aware of each other and published our results simultaneously in the same issue of Nature Communications (Kouba et al. 2020; Newing et al. 2020; Pei et al. 2020). These studies revealed structural and possibly functional differences between HelD from *M. smegmatis* and *B. subtilis.* Two subsequent studies showed that HelD in Actionobacteria protects RNAP against rifampicin (Hurst-Hess et al. 2022, Surette et al. 2022). This was confirmed by our recent study where we also showed how HelD is released from RNAP (Kovaľ et al. 2024).

Classes of HelD and their interaction with RNAP

HelD from both *B. subtilis* and *M. smegmatis* penetrates both the primary channel (where DNA binds) and secondary channel (where NTPs enter the active site), that causes conformational changes in RNAP

(Kouba et al. 2020; Sudzinová et al. 2022). Structural and phylogenetic studies on HelD from these species revealed there are three distinct classes of the HelD (Figure 19).

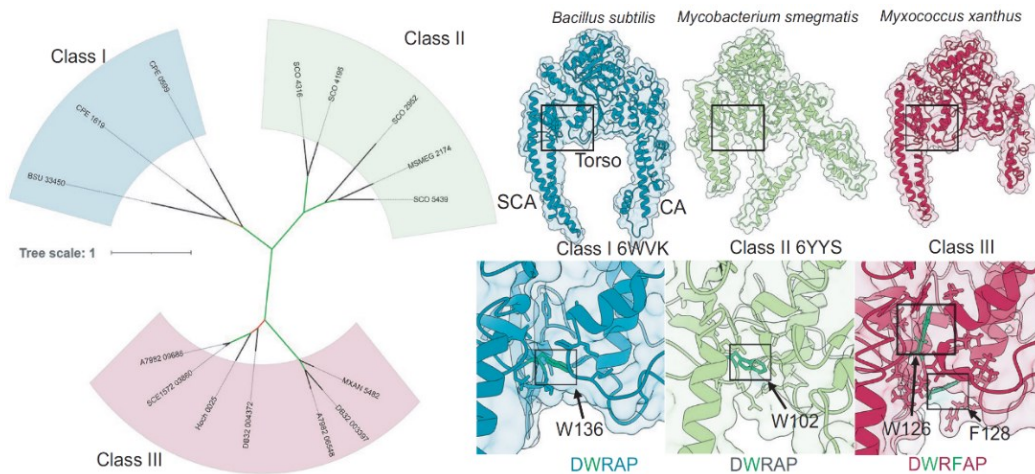

Figure 19. Three classes of HelD. Left panel shows a phylogenetic tree constructed using multiple HelD sequences. Right panel shows structures of whole HelD (top) and Trp-cage regions (bottom) of Class I (*B. subtilis*), Class II (*M. smegmatis*), and Class III (*M. xanthus*). Conserved Trp (all classes) and additional amino acid (aa) (Class III) are shown as green sticks. Adapted from (Larsen et al. 2021).

Class I HelD was described for *B. subtilis* (Newing et al. 2020; Pei et al. 2020), present in the low G+C Gram-positives and some Gram-negative *Bacteroidia*, where they probably originated through horizontal gene transfer from anaerobic gut *Clostridiales*. Class I HelD lacks the PCh-loop (see Summary of Publication II for a description of the HelD domain structure) (Sudzinová et al. 2022; Surette et al. 2022).

Structurally distinct Class II HelD was identified in high G+C Gram-positive *M. smegmatis* (Kouba et al. 2020). Some genera of Gram-positive bacteria such as *Streptomyces* or *Nonomuraea* contain several Class II HelD paralogs (Larsen et al. 2021). Class II is characterized by the presence of the PCh-loop, which stretches out into the RNAP primary channel to the RNAP AS, where a 2 nt long duplex of the nascent DNA–RNA hybrid would be located and where rifampicin binds as well (Campbell et al. 2001; Kouba et al. 2020).

Class III HelD was bioinformatically predicted to be found in the Gram-negative *Deltaproteobacteria*, has an extended motif within the N-terminal domain, DWR<u>X</u>[A/S]P (extended by one aa, <u>X</u>), but its functional characteristics remain unknown. Its N-terminal domain, expected to bind the RNAP secondary channel, is shorter compared to Class I HelDs and longer than in Class II HelDs (Larsen et al. 2021).

The length of the N-terminal domains of HelD implies how HelD functions in rifampicin dissociation. Class II HelD does this using a PCh-loop (Kovaľ et al. 2024), which is not present in class I HelD. In *B.*

subtilis, the role of class I HelD in rifampicin resistance is not well characterized. The dissociation of rifampicin from RNAP AS may be mediated by aa residues in the N-terminal domain that directly interact with aa residues of the RNAP rifampicin binding pocket (Pei et al. 2020; Sudzinová et al. 2022).

The conserved DWR (Asp-Trp-Arg) aa motif in class III HelD's N-terminal domain is crucial for its function. The Trp residue, nestled in a hydrophobic Trp-cage, stabilizes the interaction between the N-terminal domain and the helicase 1A domain, enabling effective insertion into the RNAP secondary channel and modulation of its activity (Koval' et al. 2024; Larsen et al. 2021).

The overall structure of the N-terminal domain of HelD is structurally analogous to TFs such as GreA or the ppGpp cofactor DksA, which interact with the RNAP secondary channel (Figure 20) (Abdelkareem et al. 2019; Molodtsov et al. 2018; Ross et al. 2016; Sudzinová et al. 2022).

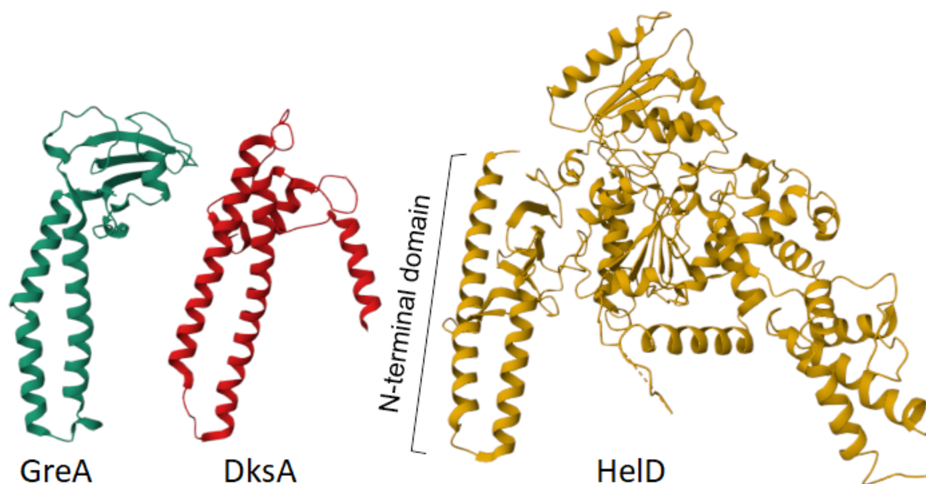

Figure 20. Structural comparison of GreA (green, PDB: 1GRJ, *E. coli*), DksA (red, PDB: 7KHE, *E. coli*) and N-terminal domain of HelD (yellow, PDB: 6YXU, *M. smegmatis*). Full HelD structure is shown, N-terminal domain is indicated. Figure was created using Mol* */molstar/* web app.

Gre factors (GreA, GreB) are conserved across bacterial species and have a similar function to DksA, Rnk and TraR (Blankschien et al. 2009; Rutherford et al. 2007; Vinella et al. 2012). Gre factors, cleaving RNA, and DksA functionally overlap in *E. coli* (Vinella et al. 2012). *M. smegmatis* have only one Gre factor, it cleaves the RNA several nt from the 3′ end and prevents abortive initiation (China, Mishra, and Nagaraja 2011). In GC-rich mycobacterial genomes, GreA plays an important role in overcoming transcriptional blockage caused by stable RNA-DNA hybrids (Belotserkovskii et al. 2010). Unlike Gre factors, DksA is absent in mycobacteria and CarD is believed to functionally substitute it (Stallings et al. 2009). DksA is a regulator of (p)ppGpp in *E. coli* and inserts its extended coiled-coil motif into the RNAP secondary channel up to the AS and blocks NTP access (Lennon et al. 2012; Parshin et al. 2015; Paul et al. 2004; Paul, Berkmen, and Gourse 2005; Perederina et al. 2004).

GreB and DksA in *E. coli* also affect RNAP affinity for initiating nucleotides (iNTPs). They bind to the RNAP secondary channel, coincides with RPo formation and influence the RPo stability (Rutherford et al. 2006). However, since these factors are not found in *M. smegmatis* and HelD also binds to the secondary channel (Kouba et al. 2020), I tested if HelD changes the affinity of RNAP for iNTPs. This would reflect the stability of RPo, since less stable RPo require higher concentrations of iNTP for maximum transcription compared to more stable RPo (Krasny et al. 2008; Sojka et al. 2011; Zenkin and Yuzenkova 2015). My results showed that HelD does not affect this affinity and thus its binding does not coincide with RPo formation (Koval' et al. 2024).

HelD and other TFs

The *M. smegmatis* HelD forms a crescent-shaped structure that penetrates into both primary and secondary RNAP channels, distinguishing it from other TFs. Other TFs in *M. smegmatis*, *M. tuberculosis*, *E. coli* and *B. subtilis* bind to only one channel, either the primary (Mfd) (Selby 2017) or secondary (GreA, GreB, DksA) channel but not both (Fernández-Coll et al. 2020; Parshin et al. 2015). HelD blocks both channels, locking RNAP in an inactive state, **Publication II** showed. It prevents nonspecific binding of RNAP to DNA and dissociates stalled transcription elongation complexes. Once released from these complexes, RNAP can either remain inactive or restart transcription upon HelD uncoupling (Kouba et al. 2020). The exact mechanism in which HelD is released from RNAP was unknown at the time of Publication II (Kouba et al. 2020), so was the subject of study in Publication III (Koval' et al. 2024).

We showed that HelD, σ^A and RbpA were simultaneously bound to the E. I experimentally confirmed it through immunoprecipitation experiments, where FLAG-tagged HelD, σ^A and RNAP were identified together in one complex (Kouba et al. 2020). This concurrent binding of HelD and σ^A has not been shown in *B. subtilis* (Wiedermannová et al. 2014).

Publication III extended these findings to how HelD is involved in transcription initiation. HelD remained associated with RNAP-σ^A complexes during the initial stages of transcription and protected RNAP from rifampicin. Cryo-EM snapshots revealed formation of the RNAP-σ^A-RbpA-HelD complex and showed how HelD is progressively released during transcription initiation when RNAP associates with promoter DNA. In addition, we have shown that when HelD is bound to RNAP, it ruled out the possibility of CarD binding (Koval' et al. 2024).

Roles of HelD in the transcription cycle

The results described above allowed to create an illustration of our current knowledge of the roles of HelD in bacterial gene expression. Figure 21 thus demonstrates a simplified model of HelD roles in the transcription cycle.

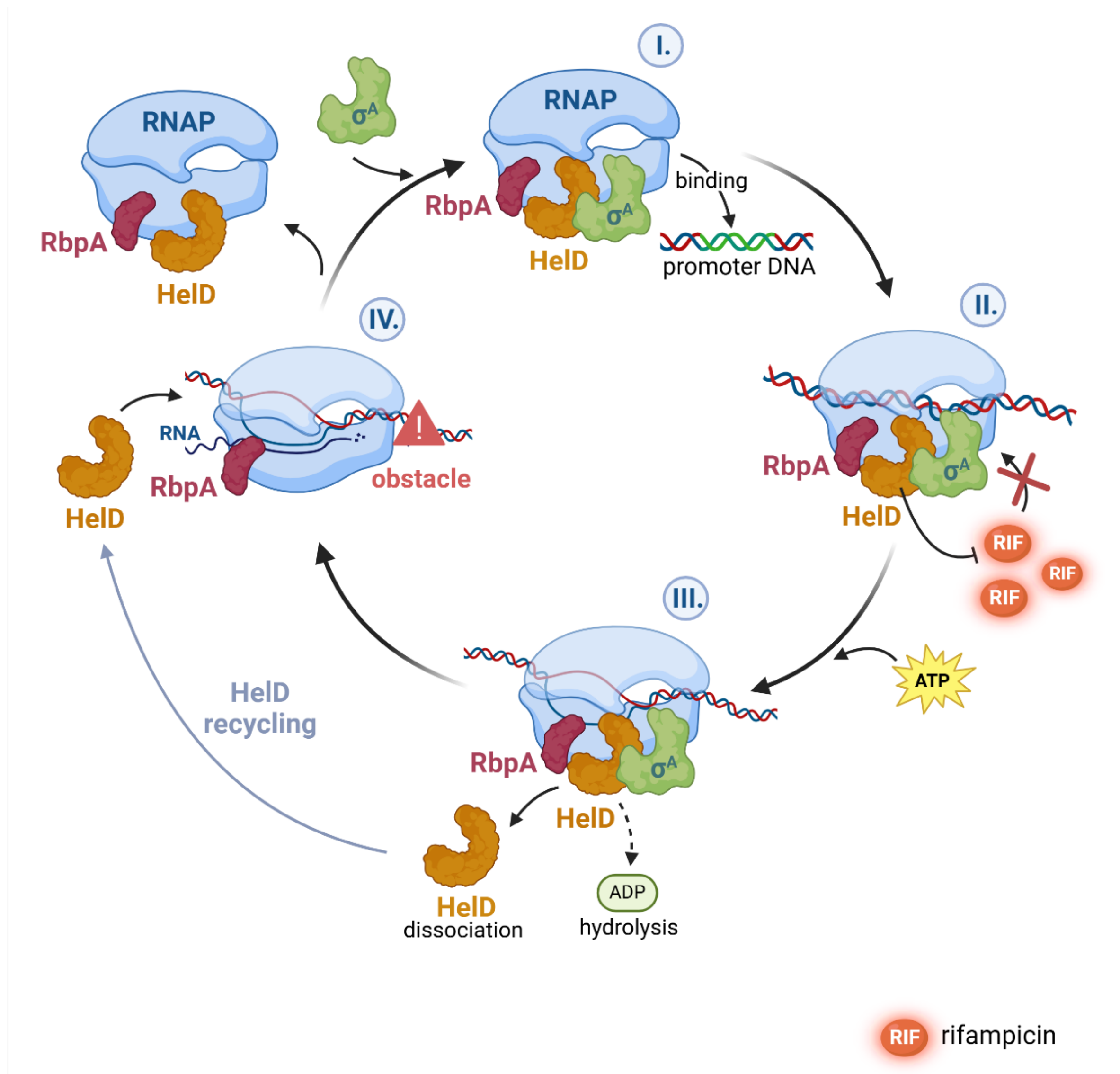

Figure 21. A simplified model of HelD function in transcription. Created in https://BioRender.com based on (Kouba et al. 2020; Kovaľ et al. 2024; Sudzinová et al. 2022).

The individual phases in this cycle (Figure 21) are as follows:

I. Binding to the promoter DNA. Disassembled elongation complex, consisting of RNAP, σ^A, RbpA and HelD is able to initially interact with the promoter DNA.

II. Role of HelD in rifampicin inhibition. HelD forms quaternary RNAP-σ^A-RbpA-HelD complex bound to promoter DNA in a RPc-like manner. At this stage, HelD by its PCh-loop inserted into the RNAP primary channel up to the RIF binding site (Figure 22A) inhibits rifampicin and protects RNAP.

III. RPo formation during initiation. During isomerization to RPo formation, HelD must be released from RNAP. HelD completely dissociates in order for transcription to begin. HelD exits both the primary and secondary RNAP channels, the DNA is loaded, and transcription bubble is formed. HelD release is

stimulated by ATP hydrolysis, and even more so if the RNAP complex interacts with the open complex DNA (Kovaľ et al. 2024). Transcription continues towards elongation and HelD is recycled. HelD dissociation prevents possibly harmful transcription-replication crash (Sudzinová et al. 2022; Wiedermannová et al. 2014).

IV. Stalled elongation complex. The elongating RNAP may stop on DNA due to an obstacle such as DNA damage (Kohler et al. 2017; Pani and Nudler 2017). Class I and II HelDs can both bind to such stalled elongation complexes and dissociate them. HelD conformationally changes RNAP, RNAP primary channel expands, and DNA dissociates from the complex. This is an important capability of HelD for recycling RNAP and ensuring the availability of the enzyme for further rounds of transcription.

The effect of HelD against rifampicin

As outlined in Figure 21, by occupying both RNAP channels, HelD physically displaces rifampicin from its binding pocket (Figure 22A) and protects RNAP from rifampicin. This protective function is particularly critical in the context of pathogenic mycobacteria, where rifampicin resistance poses a problem in the treatment of tuberculosis (Howell et al. 2022). Though it is important to mention *M. tuberculosis* appears to lack HelD homologs (Sudzinová et al. 2022). The ability of HelD to protect RNAP during transcription initiation introduces a new level of regulation.

Protective effect of HelD against rifampicin was illustrated by *in vitro* multiple-round transcription assays (Figure 22B). I showed that HelD enhanced transcription in the presence of rifampicin, allowing transcription to continue more efficiently despite the presence of the antibiotic. In the absence of HelD, rifampicin more strongly inhibited transcription.

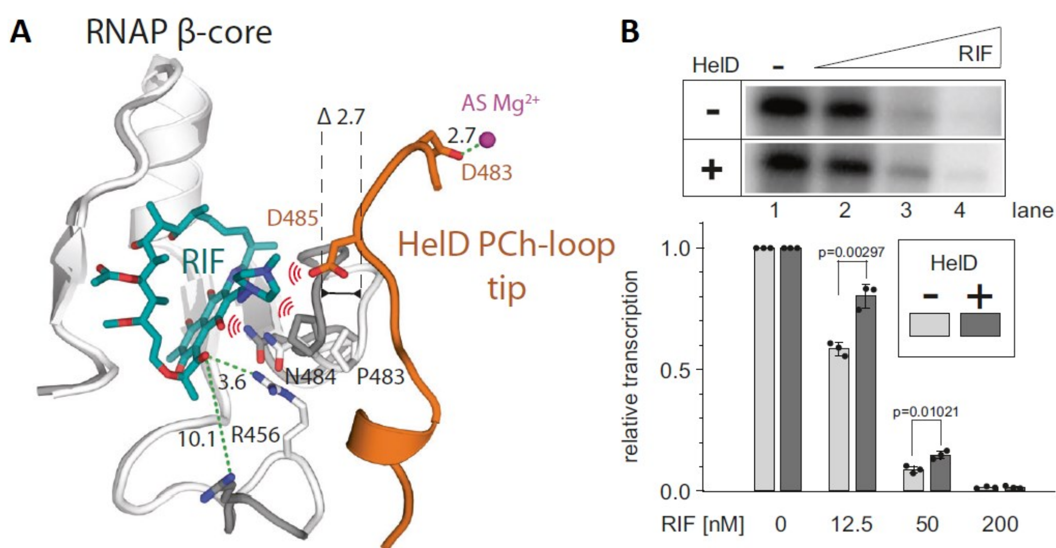

Figure 22. (**A**) Conformational changes in the RNAP rifampicin binding site (grey) caused by binding of the HelD PCh-loop (orange). Red waves indicate atomic collisions between RIF (blue), the RNAP distorted RIF binding site and HelD. (**B**) A graph showing the protective effect of HelD against rifampicin

in *in vitro* transcription assay. Representative primary data are shown above the graphs. Transcription reactions were done in the absence/presence of HelD with increasing amounts of rifampicin (RIF). The 1:1 RNAP:HelD ratio was used for protein reconstitution. Transcription at zero RIF was set as 1 for both groups (+/-HelD). The error bars show averages from three independent experiments, the dots show individual experimental data, the error bars are ±SD. Adapted from (Kovaľ et al. 2024).

This result was consistent with the inhibitory effect of HelD on *in vitro* transcription showed by Hurst-Hess et al. (2022) in *M. abscessus* together with the protective role of HelD against rifampicin (Hurst-Hess et al. 2022). Surette et al. (2022) showed that class II HelD from *S. venezuelae* protects cells from rifampicin upon transcription (Surette et al. 2022).

CONCLUSIONS

This Doctoral Thesis reveals novel aspects of transcription regulation in *M. smegmatis* through the characterization of two transcription factors, MoaB2 and HelD.

MoaB2, identified as a novel binding partner of σ^A, specifically interacts with its N-terminal domain, modulates its activity and stability, and plays a unique regulatory role distinct from traditional MoCo biosynthesis-associated proteins. This discovery expands our understanding of σ factor regulation and highlights the potential chaperone-like functions of MoaB2 under stress conditions in mycobacteria.

HelD, on the other hand, emerges as a multifunctional factor involved in RNAP recycling and transcription initiation. Structural analyses revealed a unique ability of HelD to bind both primary and secondary RNAP channels, mediate the release of stalled RNAP-DNA complexes, and protect RNAP from inhibition by antibiotic rifampicin.

Collectively, these findings, based on extensive biochemical, functional, and structural studies, provide new insights into the transcriptional machinery of mycobacteria and highlight the evolutionary and functional diversity of transcription factors and their roles in bacterial survival and adaptation. This provides a framework for development of antimycobacterial compounds targeting the transcription apparatus.

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