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Mgr. Tamara Knežová Balgová

Bacillus subtilis rozpoznává antibiotikum rifampicin prostřednictvím konvergentních promotorů, které řídí expresi genů zajišťujících ochranné mechanismy

Bacillus subtilis senses the antibiotic rifampicin through convergent promoters that control expression of genes providing protective mechanisms

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Doctoral thesis

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

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Abstract

Bacteria inhabit various environmental niches and compete for survival. One of the survival strategies of some bacteria is to produce antibiotics. The selection pressure of antibiotics on other bacterial species has led to the evolution of antibiotic resistance mechanisms. Many of these mechanisms are still unknown.

Adaptation to inhospitable conditions can be achieved most rapidly at the transcriptional level. The central enzyme of bacterial transcription is RNA polymerase (RNAP), whose activity is precisely regulated by various factors. Its essentiality for bacterial cells makes it a perfect target for antimicrobial compounds. One of these antimicrobial compounds is rifampicin (RIF), which prevents the synthesis of RNA beyond two to three nucleotides and forms stalled RNAP complexes on DNA, thereby inhibiting transcription. Bacteria possess various transcription factors (TFs), that help release or restart stalled RNAP complexes. One of these TFs is HelD.

HelD interacts with RNAP and binds to it in both the primary and secondary channels. This leads to the release of RNAP from the DNA and helps restart transcription. There are at least two different classes of HelD, which are structurally different: Class I (*e.g.* Firmicutes) and Class II (*e.g.* Actinobacteria). Previous studies have shown that Class II HelD mediates RIF resistance. However, no such effect has been observed for Class I HelD.

This thesis focuses on the role of Class I HelD in RIF resistance and its expression control in response to this antibiotic. We show that Class I HelD in *Bacillus subtilis* plays a role in RIF resistance and that its N-terminal domain is essential for this function. Furthermore, the regulation occurs already at the transcriptional level. Induction of the *helD* gene expression by RIF is controlled by two convergent promoters employing transcriptional interference where transcription from the stronger promoter (P*anti-helD*, antisense) interferes with transcription from the weaker promoter (P*helD*, sense). In the presence of RIF, transcription from P*anti-helD* is more likely inhibited due to the "higher flux" of RNAP through this promoter, allowing transcription from P*helD* to proceed and leading to the production of the HelD protein. Subsequently, the newly synthesized HelD facilitates the release of stalled RNAP complexes. We identify the same regulation also for the *pps* gene expression; this gene encodes a RIF inactivating enzyme. This dual-promoter regulatory arrangement is present also upstream of various genes in different organisms as identified by bioinformatic analysis. These genes encode potential RIF resistance mediating proteins.

In summary, this thesis reveals a role for Class I HelD in RIF resistance, describes a novel mechanism of gene expression stimulation at the transcriptional level by an inhibitor of transcription, and identifies new genes potentially linked to RIF resistance in various bacteria.

Abstrakt

Bakterie obývají různé environmentální niky a soupeří o přežití. Jednou ze strategií přežití některých bakterií je produkce antibiotik. Selekční tlak antibiotik na jiné druhy bakterií vedl k vývoji mechanismů antibiotické rezistence. Mnoho z těchto mechanismů je stále neznámých.

Adaptace na nehostinné podmínky je nejrychlejší na transkripční úrovni. Centrálním enzymem bakteriální transkripce je RNA polymeráza (RNAP), jejíž aktivita je přesně regulována různými faktory. Jeho nezbytnost pro bakteriální buňky z něj činí ideální cíl pro antimikrobiální látky. Jednou z těchto antimikrobiálních látek je rifampicin (RIF), který zabraňuje syntéze RNA delší než dva až tři nukleotidy a vytváří zastavené komplexy RNAP na DNA, čímž inhibuje transkripci. Bakterie mají různé transkripční faktory (TF), které pomáhají uvolňovat nebo znovu nastartovat zastavené komplexy RNAP. Jedním z těchto TF je protein HelD.

HelD interaguje s RNAP a váže se na ni jak do primárního, tak do sekundárního kanálu. To vede k uvolnění RNAP z DNA a pomáhá znovu nastartovat transkripci. Existují alespoň dvě různé třídy HelD, které se strukturně liší: třída I (např. u Firmicutes) a třída II (např. u Aktinobakterií). Předchozí studie ukázaly, že HelD třídy II zprostředkovává rezistenci vůči RIF. U HelD třídy I však takový efekt nebyl pozorován.

Tato disertační práce se zaměřuje na roli proteinu HelD třídy I v rezistenci vůči RIF a na regulaci jeho exprese jako odpověď na toto antibiotikum. Ukázali jsme, že HelD třídy I u *Bacillus subtilis* hraje roli v resistenci vůči RIF a že jeho N-terminální doména je pro tuto funkci nezbytná. Navíc regulace probíhá již na transkripční úrovni. Indukce exprese genu *helD* pomocí RIF je řízena dvěma konvergentními promotory, které využívají transkripční interferenci, při níž transkripce ze silnějšího promotoru (P*anti-helD*, nekódující vlákno) narušuje transkripci ze slabšího promotoru (P*helD*, kódující vlákno). V přítomnosti RIF je transkripce z P*anti-helD* s větší pravděpodobností inhibována v důsledku „vyššího toku“ RNAP přes tento promotor, což umožňuje transkripci z P*helD* a vede k tvorbě proteinu HelD. Následně nově syntetizovaný HelD usnadňuje uvolnění zablokovaných komplexů RNAP. Stejnou regulaci jsme určili i pro expresi genu *pps*; tento gen kóduje enzym inaktivující RIF. Toto regulační uspořádání s duálními promotory se vyskytuje také před různými geny v různých organismech, jak bylo zjištěno bioinformatickou analýzou. Tyto geny kódují potenciální proteiny zprostředkující rezistenci vůči RIF.

Tato disertační práce odhaluje roli HelD třídy I v rezistenci vůči RIF, popisuje nový mechanismus stimulace genové exprese na transkripční úrovni inhibitorem transkripce a identifikuje nové geny, které mohou být spojeny s rezistencí vůči RIF u různých bakterií.

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List of abbreviations

aa	Amino acid
ATB	Antibiotic
ATP	Adenosine triphosphate
B. subtilis, Bsu	*Bacillus subtilis*
bp	Base pair
C. difficile	*Clostridioides/Clostridium difficile*
CA	Clamp arm/HelD-specific domain
cryo-EM	Cryogenic electron microscopy
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
E. coli	*Escherichia coli*
FDX	Fidaxomicin
Gram-	Gram negative bacteria
Gram+	Gram positive bacteria
KANG	Kanglemycin
kDa	Kilodalton
M. abscessus	*Mycobacterium abscessus*
M. smegmatis, *Msm*	*Mycobacterium smegmatis*
M. tuberculosis	*Mycobacterium tuberculosis*
MIC	Minimum inhibitory concentration
mRNA	Messenger RNA
nt	Nucleotide
NTP	Nucleoside triphosphate
Pch-loop	Primary channel loop
ppGpp	Guanosine tetraphosphate

RAE	Rifampicin associated element
RIF	Rifampicin
RNA	Ribonucleic acid
RNAP	DNA-dependent RNA polymerase
RNAP-σ	RNA polymerase holoenzyme
rNTP	Ribonucleotides
RPc	Closed promoter complex
RPo	Open promoter complex
rRNA	Ribosomal RNA
SAXS	Small angle X-ray scattering
SCA	Secondary channel arm (N-terminal domain of HelD)
sub-MIC	Sub-minimum inhibitory concentration
TF(s)	Transcription factor(s)
TSS	Transcription start site
wt	Wilde type
σ	Sigma factor

Introduction

Bacillus subtilis is a Gram positive (Gram+), non-pathogenic, aerobic, rod-shaped bacterium that belongs to the phylum Firmicutes. It can be found in different environmental niches, mainly in soil. *B. subtilis* is one of the most studied Gram+ bacteria and serves as a model organism for studies various processes such as transcription, translation, replication, sporulation or cell differentiation. *B. subtilis* is extensively used in industrial biotechnology for enzyme production, fermentation processes, and recombinant protein expression (Errington & van der Aa, 2020). Its genome consists of single circular chromosome of approximately 4.2 Mbp encoding around 4 100 proteins (Kunst *et al.*, 1997). Although it is non-pathogenic, this model organism inhabits highly competitive soil ecosystems, making it an ideal system for investigating the mechanisms of antibiotic resistance. We used this model organism for research presented in this thesis.

Antibiotics commonly used to treat bacterial infections are becoming ineffective and increasing numbers of antibiotic resistance pathogens have become a serious worldwide problem. Development of new antimicrobial agents against well-known targets is challenging. Uncovering new mechanisms of antibiotic resistance may lead to the discovery of new promising drug targets.

Bacteria live in an ever changing and competitive environments. Adaptation to these changes and challenges is crucial for survival and is mediated by changes in gene expression. Regulation of gene expression on the transcriptional level is arguably the most effective way of adaptation. The key enzyme necessary for bacterial transcription is RNA polymerase (RNAP). Activity of RNAP can be regulated by various transcription factors. One of these factors is the HelD protein.

HelD is an interacting partner of bacterial RNAP (Delumeau *et al.*, 2011) that facilitates the dissociation of stalled RNAP complexes from DNA, thereby promoting the initiation of new rounds of transcription (Wiedermannová *et al.*, 2014). Gram positive bacteria with low G+C content (*e.g.* Firmicutes, such as *B. subtilis*) possess Class I HelD, while with high G+C content (*e.g.* Actinobacteria, such as *Mycobacterium smegmatis*) possess Class II HelD (Larsen *et al.*, 2021). These classes differ by specific structural features. Class I HelDs contain a relatively long N-terminal domain that penetrates deep into the secondary channel (Newing *et al.*, 2020). On the other hand, Class II HelDs contain shorter N-terminal domains and an additional

structural feature not present in Class I HelDs, the primary channel loop (Pch-loop). The Pch-loop reaches close to vicinity of the active site (Kouba *et al.*, 2020).

Stalled RNAP complexes can be generated by the antibiotic rifampicin, which binds to the β subunit of bacterial RNA polymerase near the DNA/RNA exit channel and sterically blocks RNA synthesis beyond 2–3 nucleotides (Campbell *et al.*, 2001). Class II HelDs have been shown to confer resistance to rifampicin, with the Pch-loop playing a critical role in this process. Due to the absence of the PCh-loop from Class I HelDs, it was proposed that they are likely not involved in rifampicin resistance (Hurst-Hess *et al.*, 2022; Surette *et al.*, 2022).

This doctoral thesis focuses on the potential role of Class I HelDs in rifampicin resistance in the model organism *B. subtilis*. The study also investigates which part of Class I HelD is important for this resistance. Importantly, it demonstrates that expression of the *helD* gene is induced by rifampicin at the transcriptional level, likely through a mechanism involving transcriptional interference.

This thesis consists of a few sections and begins with a Literature review covering bacterial transcription, its main components and stages, transcription inhibitors, and selected transcription factors, with a primary focus on HelD. This is followed by the Aims of the thesis, a List of methods and publications, and a brief Summary of the publications. The final sections include the Discussion and Conclusions, which address various aspects of HelD-mediated rifampicin resistance and outline potential future research directions.

Literary review

1. Bacterial gene expression

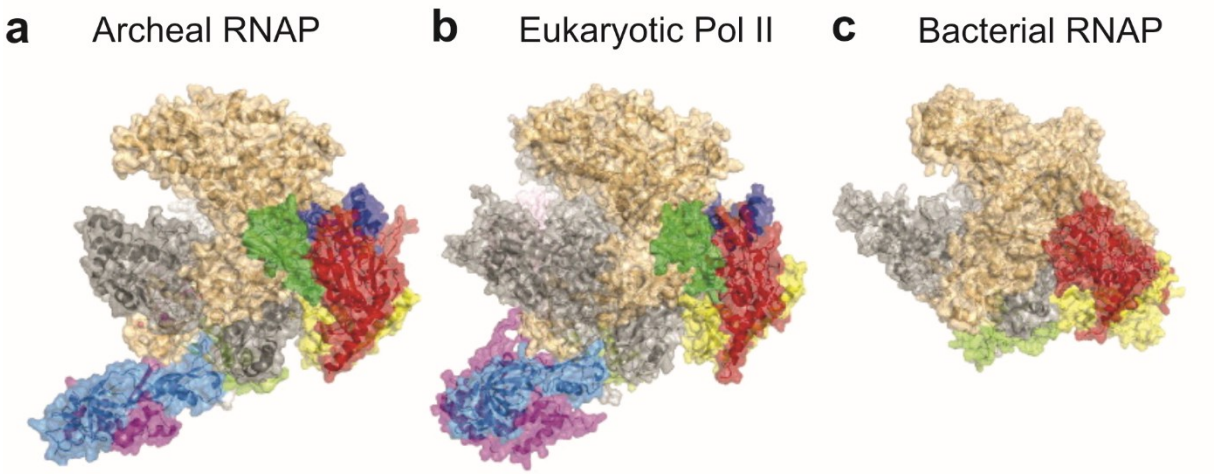
Bacteria inhabit various environments where different species coexist together. Regulation of gene expression is crucial for survival and adaptation in these ever-changing and -competing environments. Gene expression is regulated at transcriptional and translational levels. This Literary review focuses mainly on bacterial transcription and its regulation.

1.1. Transcription

The first step of gene expression is the process called transcription. During this process a copy of RNA is synthetized based on the genomic DNA template by the key enzyme, RNA polymerase (RNAP) (Crick, 1970; Diercks *et al.*, 2021).

RNAP is a multi-subunit enzyme that catalyses transcription and can be found in all domains of life. Bacterial RNAP core consists of five subunits (2α, β, β', ω) and homologues of these subunits were identified in archaea and eukaryotes. However, RNAPs of archaea and eukaryotes contain additional specific subunits (**Figure 1**) (Werner, 2008). The number of RNAPs between the domains of life also differ. Bacteria and archaea use only one type of RNA polymerase to transcribe their entire genome. On the other hand, eukaryotes use at least three types of nuclear RNA polymerases, namely, RNA polymerase I (transcribes rRNA), RNA polymerase II (transcribes mRNA, miRNA, snRNA and snoRNA) and RNA polymerase III (transcribes tRNA and 5S rRNA) (Carter & Drouin, 2009; Werner & Grohmann, 2011). Interestingly plants evolved another two RNA polymerases, Pol IV and Pol V, that play role in DNA methylation and transcriptional silencing (Haag *et al.*, 2012).

In nature, we can also find RNAPs with single-subunit architecture. It should be noted that sequence homology compared to multi-subunit RNAPs is very low. T7 RNAP of the *Escherichia coli* infecting T7 bacteriophage is one of the well-known single-subunit RNAP. Compared to the multi-subunit RNAPs, T7 RNAP can initiate transcription without additional transcription factors. Another example is YonO, which is encoded within the SPβ prophage in the *Bacillus subtilis* chromosome. YonO transcribes the late genes of SPβ. Interestingly YonO is a more error prone RNAP compared to the multi-subunit RNAPs (Forrest, 2018).



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Bacteria	*Archaea*		*Eukarya*	
		RNAP II	RNAP I	RNAP III
β'	A'/A''	RPB1	A190	C160
β	B	RPB2	A135	C128
α	D	RPB3	AC40	AC40
α	L	RPB11	AC19	AC19
ω	K	RPB6	RPB6	RPB6
	H	RPB5	RPB5	RPB5
	G*	RPB8	RPB8	RPB8
	N	RPB10	RPB10	RPB10
	P	RPB12	RPB12	RPB12
	F	RPB4	A14	C17
	E	RPB7	A14	C25
	X/TFS	RPB9	A12	C11

Figure 1: Structural comparison of RNA polymerases in the three domains of life. (a) Archaeal RNAP based on the *Sulfolobus solfataricus* (Protein Data Bank [PDB] code: 2PMZ). **(b)** Eukaryotic RNA polymerase II based on the *Saccharomyces cerevisiae* (PDB code: 1NT9). **(c)** Bacterial RNAP based on the *Thermus aquaticus* (PDB code: 1I6V). **(d)** RNAP subunit composition in bacteria, archaea and eukaryotes. Subunits in the same row are sequence and structural homologues. The lower box contains subunits specific for archaeal and eukaryotic RNAPs. The colored circles indicate the color coding of the RNAP subunits in **(a – c)**. RNAP, RNA polymerase. Adapted from (Werner, 2008).

1.1.1. RNA polymerase

The RNAP core enzyme in bacteria is formed by five subunits: 2α, β, β', and ω.

The α subunit is encoded by the *rpoA* gene, it exists as a homodimer, and each monomer is composed of the N-terminal domain (αNTD) and the C-terminal domain (αCTD) connected by a flexible linker. The subunit α serves as a scaffold for assembling the β and β' subunits. Besides this function it also interacts with multiple transcription factors and regulates gene expression (Sutherland & Murakami, 2018). The β and β' subunits are encoded by the *rpoB* and *rpoC* genes organized in the β operon. Both subunits form the active site. The β' subunit coordinates the

catalytic Mg^{2+} ion required for the nucleotidyl transfer reaction and the β subunit interacts with the incoming nucleoside triphosphate (NTP). These two subunits form the unique crab-claw shape of the RNAP molecule. In some cases, β and β' subunits may contain lineage-specific domain insertions in size from 50-500 amino acid (aa). The inserts are located on the outer surface of RNAP and may serve as interaction interface for regulatory factors and transcription modulation in a lineage-specific manner (Basu *et al.*, 2014; Lane & Darst, 2010; Sutherland & Murakami, 2018). The only non-essential generally occurring subunit of the bacterial RNAP core is ω encoded by the *rpoZ* gene. It consists of three conserved α helices and mainly interacts with the β' subunit, although this interaction varies between bacterial species (Kurkela *et al.*, 2021). Few roles of the ω subunit were described *e.g.* stabilization of the RNAP structure (Weiss *et al.*, 2017), σ factors recruitment (Bhowmik *et al.*, 2017), stringent response (Ross *et al.*, 2013) and biofilm formation (Bhardwaj *et al.*, 2018; Weiss *et al.*, 2017). Interestingly in *Mycobacterium tuberculosis* the ω subunit is essential for RNAP formation (Mao *et al.*, 2018). **Figure 2** shows subunits of *E. coli* RNAP and their assembly.

In Gram + bacteria, RNAP possess two additional auxiliary subunits, δ (*rpoE*) and ε (*rpoY*) (Weiss & Shaw, 2015). It was shown in *Staphylococcus aureus* the δ subunit is involved in transcriptional regulation via its negatively charged C-terminal domain, which interferes with the interaction between DNA and RNAP core (Z. Lin *et al.*, 2021). The exact role of the ε subunit is still under investigation, but it is similar to the Gp2 family of phage proteins, and it may play role in protection from phage infection (Keller *et al.*, 2014).

Topologically, RNAP contains three main channels of RNAP to facilitate bacterial transcription. The primary channel is responsible for accommodation and guidance of the double-stranded (dsDNA) template into the active center. Through the secondary channel building blocks of RNA, NTPs enter in the active center and ensure RNA synthesis. Finally, through the RNA exit channel the nascent RNA leaves the enzyme by separating from the RNA/DNA hybrid strand (Borukhov & Nudler, 2008; Lee & Borukhov, 2016; Sutherland & Murakami, 2018; Ye *et al.*, 2024).

The σ factor is a key part of the RNAP holoenzyme that recognizes specific DNA sequences (promoters). Bacteria possess different types and numbers of σ factors (Feklístov *et al.*, 2014; Sutherland & Murakami, 2018).

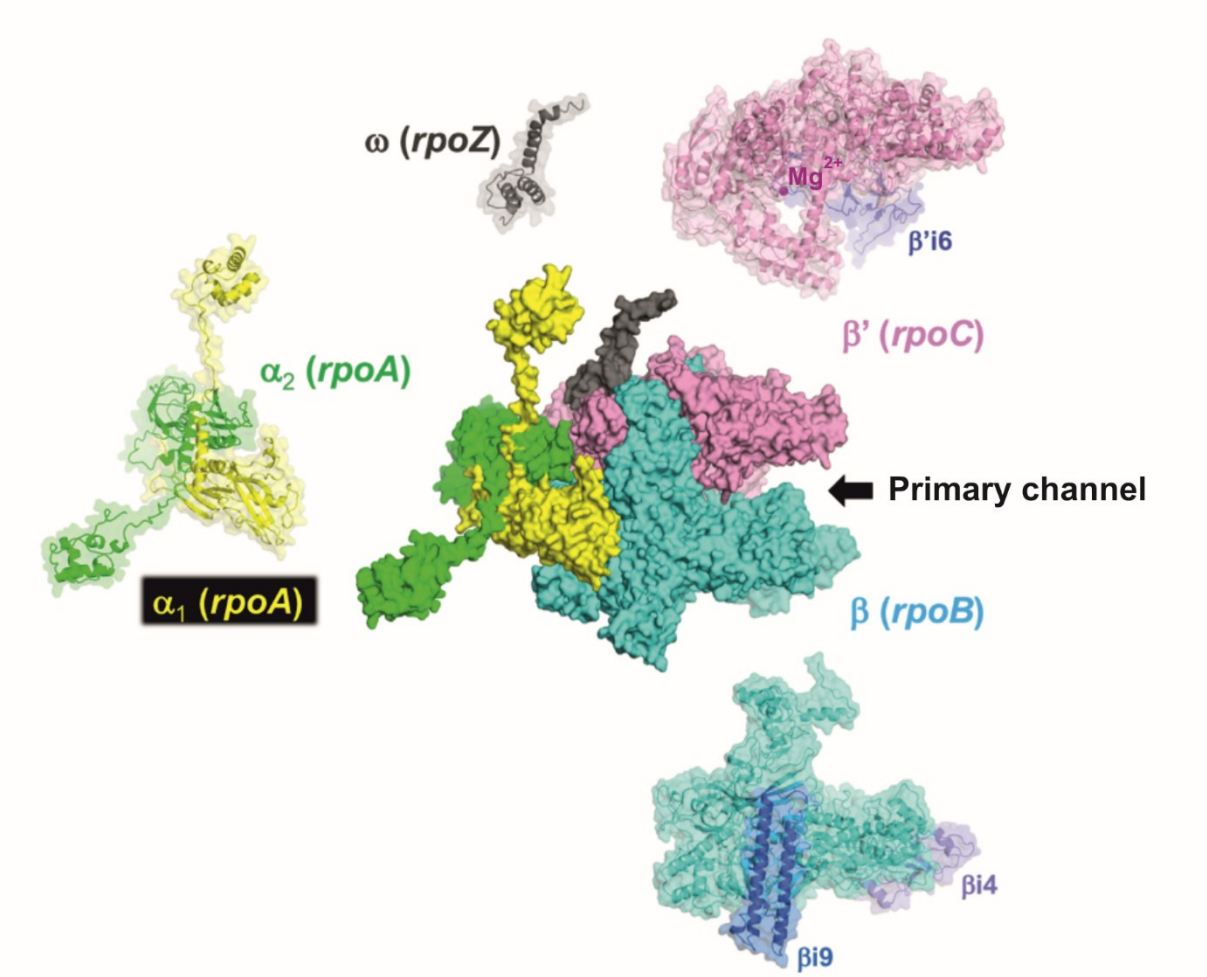

Figure 2: Structure of the *E. coli* RNAP core enzyme. Molecular surface representation color-coded as follows α1: yellow, α2: green, β: cyan, β′: pink, and ω: gray, PDB code: 4YG2. βi4, βi9 (β subunit) and β'i6 (β' subunit), lineage-specific insertions. Magenta sphere, catalytic Mg^{2+} (active site). Black arrow, DNA binding primary channel. Adapted from (Sutherland & Murakami, 2018).

1.1.2. Sigma factors

The presence of sigma factors in the cell is as important as the presence of RNAP. They play important roles in bacterial transcription initiation. Specifically, they are required for (i) formation of the RNAP holoenzyme (**Figure 3**), (ii) melting the duplex promoter DNA, and then stabilizing it in the form of a single-stranded open complex (transcription bubble), (iii) interacting with other DNA-binding transcription factors and modulating gene expression (Davis *et al.*, 2017). After promoter escape by the RNAP holoenzyme, the σ factor dissociates from the complex and can bind to another RNAP (Murakami *et al.*, 2002). In some cases, it was proposed that elongation complex can retain σ factor through transcript elongation (Harden *et al.*, 2016; Tewary *et al.*, 2025).

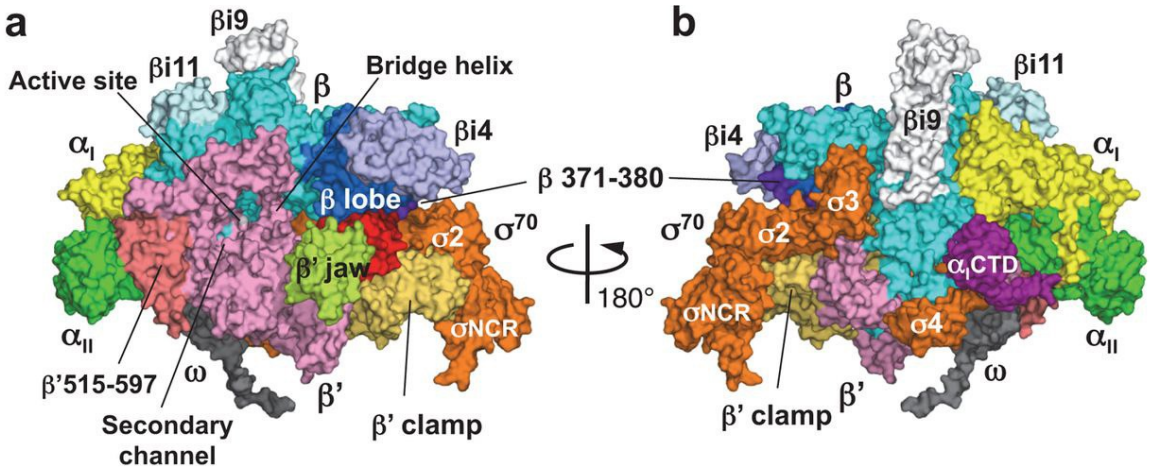

Figure 3: Structure of the *E. coli* RNAP-σ^{70} holoenzyme. (a) RNAP holoenzyme from the view of secondary channel leading to the active site. **(b)** View of RNAP with the σ-binding site. Color coding: α_I, yellow; α_{II}, green; β, cyan; β', pink; ω, grey; σ^{70}, orange. α_ICTD, α_I subunit C-terminal domain. σNCR, σ nonconserved region. βi4, βi9, βi11, lineage-specific insertions of the β subunit. Some of the β and β' residues are indicated (β 371-380, β' 515-597). Adapted from (Murakami, 2013).

σ factors are divided into two families: σ^{70} family and σ^{54} family. These families have distinct sequence homology and different mechanisms of transcription initiation (Feklístov *et al.*, 2014; Paget & Helmann, 2003; Popham *et al.*, 1989; N. Zhang & Buck, 2015). While σ^{70} binds to a conserved -10 and -35 element (in complex with the RNAP core) and is able to open DNA without additional factors (Bae *et al.*, 2015; Feklistov & Darst, 2011), σ^{54} binds to conserved - 12 and -24 elements and to open a double-stranded DNA and initiate transcription requires interaction with ATP dependent activator proteins (Buck *et al.*, 2000; Rappas *et al.*, 2007; Yang *et al.*, 2015).

In bacteria the most abundant family of σ factors is σ^{70}. This family includes the major (housekeeping) σ factors, which are responsible for expression of essential genes and their name vary between bacteria (Gruber & Gross, 2003; Lonetto *et al.*, 1992). In *E. coli*, the primary σ factor is σ^{70} (Sharma & Chatterji, 2010), while in most Gram+ bacteria is called σ^{A} (Jarmer *et al.*, 2001) except for HrdB in *Streptomyces* (Kim *et al.*, 2020). Besides the major σ factors, the σ^{70}-type σ factors also include the so called alternative σ factors. These σ factors usually control genes responsible to response to environmental changes (Chen *et al.*, 2021).

The number of σ factors varies among bacterial species and is also related to the environmental niches where they live. For example, *Streptococcus pyogenes* has only two σ factors (housekeeping σ^{A} and alternative σ^{X}) (Opdyke *et al.*, 2003), while *Sorangium cellulosum* has as many as 109 (Han *et al.*, 2013). This large difference between the number of σ factors is due to the fact that *S. pyogenes* is a Gram+ pathogen with less harsh and fluctuating environment (Henningham *et al.*, 2012), while *S. cellulosum* is a Gram- myxobacteria living in the soil

(Dawid, 2000) constantly exposed to changes and, moreover, generating large amounts of secondary metabolites, which requires complex regulatory network (Gerth *et al.*, 2003). *B. subtilis* as a soil bacterium encodes 18 σ factors on its genome (Gruber & Gross, 2003) and one alternative σ factor (σ^N) on a pBS32 plasmid (Burton *et al.*, 2023).

1.1.3. Bacterial promoters

Promoters are specific DNA sequences located upstream the coding region. They are necessary for transcription initiation and are recognized by specific RNAP holoenzymes. Promoters contain defined regions located upstream from the transcription start site (TSS, +1 position). Namely, the discriminator, -10 region (also called as Pribnow box), the spacer, -35 region and the UP element (found in some bacterial promoters) (Browning & Busby, 2016; Mejía-Almonte *et al.*, 2020). The discriminator can have different sequences, including the G/C-rich sequence, which is mainly found in the rRNA promoters of *E. coli*. It is involved in dsDNA melting during open-complex formation and also in sensing the DNA supercoiling status (Forquet *et al.*, 2021; Haugen *et al.*, 2006; Pemberton *et al.*, 2000). The -35 and -10 regions are hexamers, and their consensus sequences (major σ^{70}-dependent promoters) are as follows, TTGACA and TATAAT, respectively (Siebenlist, 1979). The spacer separates the -10 and -35 regions, and its optimal length is 17 bp ± 1 bp (Harley & Reynolds, 1987). In some cases, the extended -10 element is present (TG-motif). It is found typically in promoters with no or weak -35 region and can serve as its functional substitution (Bae *et al.*, 2015; Kumar *et al.*, 1993; Mitchell *et al.*, 2003; Voskuil & Chambliss, 1998). Lastly, the UP element is an A/T-rich area around 20 bp and with interaction of the α-subunit of RNAP can increases transcription (Estrem *et al.*, 1998; Tidor *et al.*, 1993).

Sigma factors consist of several domains connected by linkers. These domains interact with individual parts of the promoter (**Figure 4**) (Feklístov *et al.*, 2014; Yang *et al.*, 2015). The σ^{70}- type σ factors can be divided into four groups that differ in domain composition. In the case of the major σ^{70} (Group 1), which contains 4 domains the interaction with the promoter is as follows: the σ_4 domain recognizes and interacts with the -35 region, the σ_2 domain interacts with the -10 region and with the discriminator, the σ_3 domain interacts with the extended -10 element and the $\sigma_{1.1}$ domain has no DNA binding feature. Because other groups of σ^{70}-type σ factors (Group 2, 3 and 4 – alternative σ factors) consist of less than 4 domains the two most important domains are σ_4 and σ_2 (reviewed in Feklístov *et al.*, 2014).

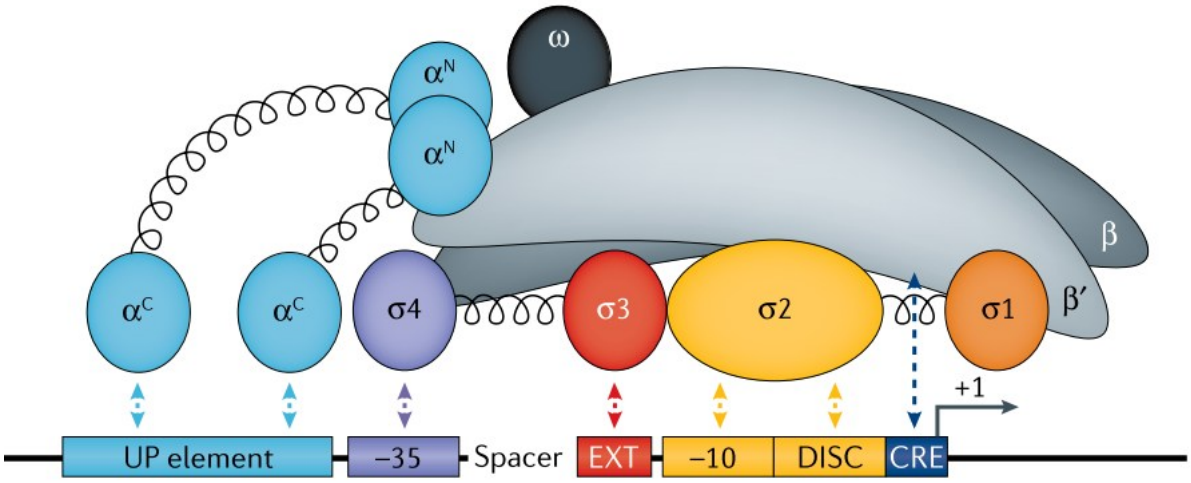

Figure 4: Promoter architecture and RNAPσ^{70}-DNA interactions. DNA promoter regions and their interactions with σ^{70} domains, αCTD and RNAP core. The RNAP αCTD interacts with the UP element of the promoter; σ_4 domain interacts with the -35 region (-35); σ_3 domain interacts with the extended - 10 element (EXT); σ_2 domain interacts with the -10 region (-10) and with the discriminator (DISC); the RNAP core enzyme interacts with the core recognition element (CRE) in a sequence specific manner. The CRE element comprises nucleotides located at positions −3 to +2 on the non-template strand, which specifically interact with ten residues of the RNAP β subunit. Adapted from (Mejía-Almonte *et al.*, 2020).

In bacteria, genes with related functions are often organized into operons and transcribed as a single polycistronic mRNA. Operons can be regulated from a single promoter and associated transcription factors allow coordinated gene expression and enable efficient regulation and rapid adaptation to environmental changes (Ishihama, 2010; Zhao *et al.*, 2019). However, regulation of gene expression can also be achieved by interplay between multiple promoters.

There are three possibilities how two promoters can be oriented: (a) promoters facing opposite direction – divergent promoters, (b) promoters facing each other – convergent promoters and (c) promoters sharing the same orientation – tandem promoters (**Figure 5**) (Shearwin *et al.*, 2005).

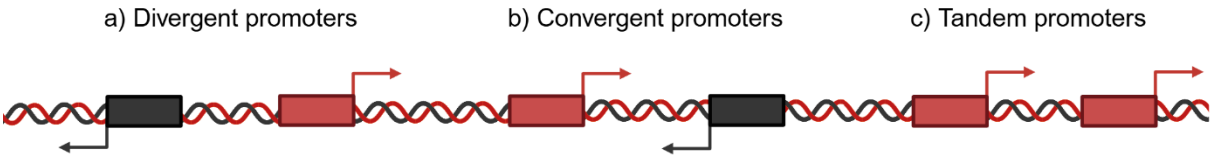

Figure 5: Schematic representation of two-promoter arrangements. (a) Divergent promoters – promoters are in the opposite orientation. **(b)** Convergent promoters – promoters are facing each other. **(c)** Tandem promoters – two promoters share the same orientation. Boxes represent promoters and arrows transcription start sites. Created with BioRender.com

1.1.4. Stages of transcription

Transcription is the first step of gene expression and can be divided into three major parts: initiation, elongation and termination.

1.1.4.1. Initiation

The RNAP holoenzyme (RNAP-σ) recognizes and binds DNA promoter and first forms the closed promoter complex (RPc), where DNA remains double stranded (Saecker *et al.*, 2011). Formation of RPc triggers large conformational changes both in RNAP-σ and DNA, collectively called isomerization that lead to DNA strand separation (melting) of ~12-14 bp from the -10 region extending slightly downstream of the transcription start site and, ultimately, formation of the open complex (RPo) (Chen *et al.*, 2010). The template strand is placed in the polymerase active site ready for nucleotide addition, meanwhile the non-template strand is bound to the σ factor (Y. Zhang *et al.*, 2012). RNAP initiates *de novo* RNA synthesis, creating the initial transcription complex, which in a promoter dependent manner, may revert to RPo and produce short abortive RNA transcripts due to RNA-exit channel blockage in the process called, abortive transcription. During this non-productive process the RNAP is still bound to the promoter and scrunching the DNA (Revyakin *et al.*, 2006). Productive transcription begins when the nascent RNA reaches a critical length (12-15 nt), enabling RNAP to break promoter contacts, partially or fully release σ factor, and escape the promoter to form a highly processive elongation complex (Alhadid *et al.*, 2017).

Figure 6 shows the individual steps involved in transcription initiation.

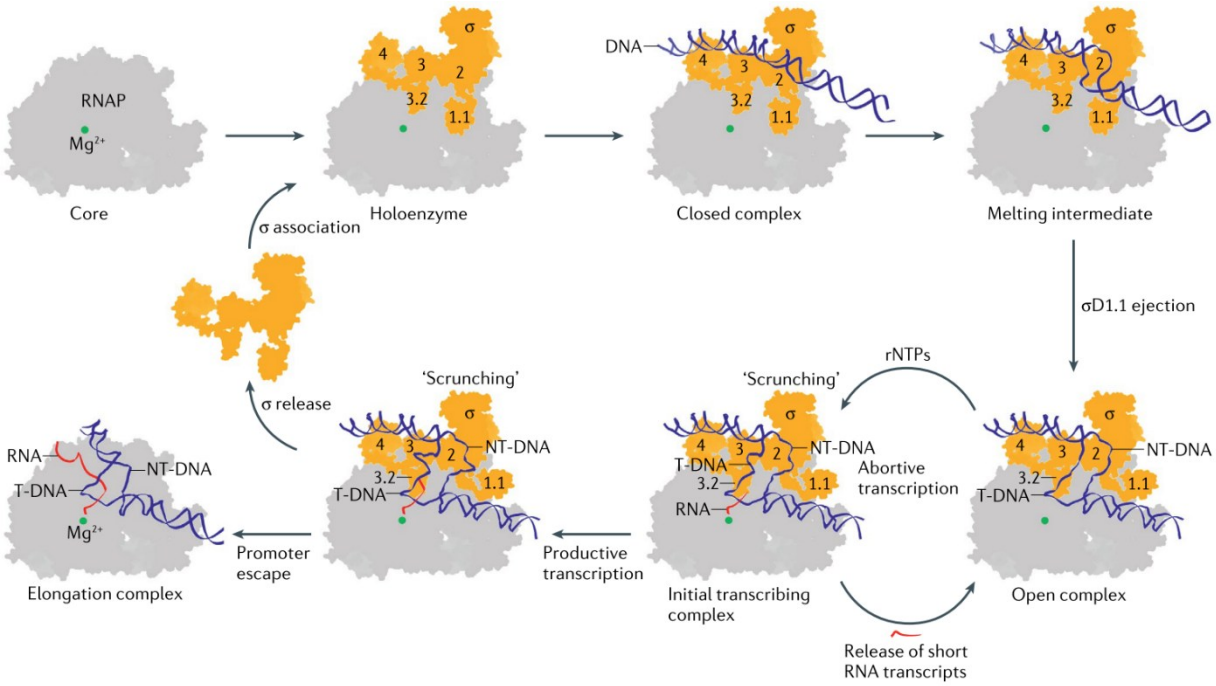

Figure 6: Schematic representation of bacterial transcription initiation steps. The first step is generation of the RNAP holoenzyme. The holoenzyme binds to the promoter DNA, forms RPc (promoter closed complex), melts DNA promoter and creates the RPo (promoter open complex). During abortive transcription many short RNA transcripts produce, through DNA scrunch and ribonucleotides (rNTPs) presence. When the complex reaches a certain transcript length it triggers dissociation of σ from the core RNAP, which leads to promoter escape and formation of elongation complex. Grey, RNAP core; green sphere, Mg^{2+} active site; orange, σ; blue, DNA; red, RNA transcripts; NT-DNA, non-template DNA; T-DNA, template DNA. Adapted from (Chen *et al.*, 2021).

1.1.4.2. Elongation

The stage of transcription elongation can be divided into two phases: active translocation and pausing. During active translocation two translocated states oscillating, consistent with a "Brownian Ratchet" mechanism. As RNAP moves along the DNA template, it synthesizes RNA by incorporating ribonucleotides (rNTP) complementary to the template strand. Throughout this process, RNAP maintains a transcription bubble and 9-10 bp RNA:DNA hybrid (**Figure 7**) (reviewed in Mustaev *et al.*, 2017).

During elongation, variety of factors interact with RNAP influencing its activity and its tendency to pause. Backtracking/pausing of RNA polymerase occurs in response to DNA lesions, or after misincorporating nucleotides (Mohamed *et al.*, 2022). The NusG factor increases the rate of transcription elongation by suppressing the frequency of pauses (Herbert *et al.*, 2010). It also interacts with the ribosome and the transcription termination Rho-factor and coordinates transcription-translation coupling (Bailey *et al.*, 2022; Lawson *et al.*, 2018). Elongation factors GreA and GreB stimulate cleavage of RNA transcripts, whereas DksA

reduces incorrect nucleotide incorporation by RNAP and in both cases help rescue arrested or backtracked RNAP complexes (Abdelkareem *et al.*, 2019; Marr & Roberts, 2000; Roghanian *et al.*, 2015). On the other hand, NusA in *E. coli* increases the pause frequency, which diminishes the overall speed of the elongation process, while NusG inhibits pausing and accelerates transcription (Artsimovitch & Landick, 2000). Interestingly, the role of NusG and NusA in *B. subtilis* are opposite from the above-mentioned NusA and NusG in *E. coli*. While NusG induces pausing, NusA has minor role in pausing stimulation (Jayasinghe *et al.*, 2022).

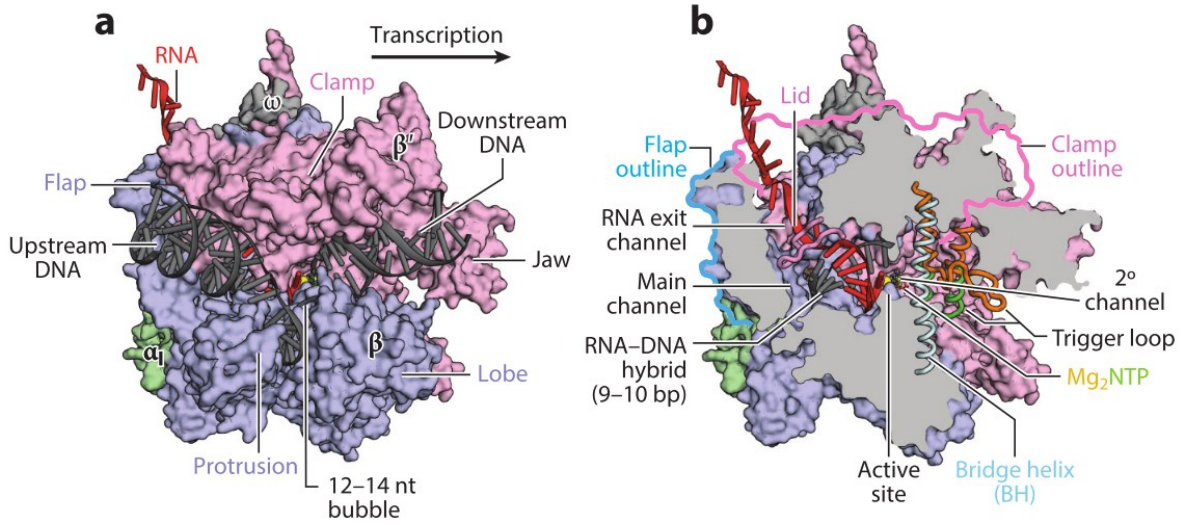

Figure 7: Structure of the transcription elongation complex (EC). (a) Crystal structure of the *Thermus thermophilus* elongation complex (PDB code: 2O5J). The subunits and features of the EC are depicted. **(b)** A sliced view of the EC illustrating the RNA exit channel, main channel, secondary (2°) channel, and the active site. Adapted from (Ray-Soni *et al.*, 2016).

Numerous DNA lesions stall RNA polymerase without dissociation, allowing recruitment of transcription-coupled DNA repair factors. One of these factors is Mfd, which releases the stalled RNAP from the template DNA and recruits a nucleotide excision repair factor UvrA to repair the DNA lesion. UvrD is another factor that induces RNA polymerase backtracking to expose the DNA lesion for repair. This backtracked RNAP could be reactivated by different factors (reviewed in Belogurov & Artsimovitch, 2015).

1.1.4.3. Termination

Termination is the final step of transcription, characterized by the dissociation of RNAP from DNA and release of the newly synthesized RNA transcript. It can be achieved with two major mechanisms: intrinsic termination and Rho-dependent termination (Ray-Soni *et al.*, 2016). Intrinsic termination is template-encoded and does not require additional factors. It involves the formation of a stable RNA hairpin consisting of a G/C-rich sequence followed by a ~8-9 U-rich

sequence (U-stretch). The RNA hairpin forms within the RNAP exit channel and disrupts the weak A/U hybrid bp formed by the U-stretch, which leads to RNA transcript release and dissociation of RNAP from DNA (You *et al.*, 2023).

Rho-dependent termination relies on the activity of the hexameric, ATP-dependent RNA:DNA helicase known as the Rho factor. Rho binds to a Rho utilization (rut) sequences on the nascent RNA via the primary and secondary binding sites, creates the active closed-ring form and moves along the RNA in a 5' → 3' direction, until it catches the stalled RNAP on the pause site. Through ATP hydrolysis, Rho promotes dissociation of RNA polymerase from the DNA template, thereby terminating transcription (Jin *et al.*, 1992; Peters *et al.*, 2011; Thomsen *et al.*, 2016). In addition to this textbook model, an alternative model was proposed and is as follows: the process begins with formation of pre-termination transcription complex, where Rho directly binds RNAP, NusA and NusG before contacting the nascent RNA (Z. Hao *et al.*, 2021).

The commensal bacterium *Bacteroides thetaiotaomicron* harbors an unusual Rho factor with a large, intrinsically disordered domain (IDR). Under nutrient starvation, the IDR promotes liquid-liquid phase separation (biomolecular condensates) of Rho. This enhances Rho-mediated transcription termination and modulates the expression of numerous genes, including several those required for gut colonization, thereby promoting bacterial fitness in the gut (Krypotou *et al.*, 2023).

Harden *et al.* proposed an alternative termination cycle in which RNAP remains associated with DNA following RNA release, undergoes diffusional sliding, reorients on the DNA, forms a holoenzyme through σ-factor binding, and reinitiates transcription at promoter-like sequences to generate either sense or antisense transcripts (Harden *et al.*, 2020).

1.1.5. Inhibitors of bacterial transcription

Bacterial RNAP and its associated transcription factors (TFs) are essential for transcription and are highly conserved among bacteria. They lack sequence homology with eukaryotic RNAP and TFs, making them excellent targets for antibiotic (ATB) development. Transcription-inhibiting compounds can bind to different regions of RNAP, including the primary and secondary channels and the switch regions, and may also target associated TFs (*e.g.* Rho factor, σ factor). Although numerous ATBs have been isolated from microorganisms and prepared synthetically, only rifamycins (*e.g.* rifampin, rifabutin) and fidaxomicin/lipiarmycin are used clinically (Ma *et al.*, 2016).

1.1.5.1. Rifampicin

Rifampicin (RIF, also known as rifampin) is a semisynthetic derivative of the natural product rifamycin, which was originally isolated from the soil bacterium *Amycolatopsis rifamycinica*, and it belongs to the ansamycin family of natural products. It exhibits broad-spectrum activity against mycobacteria and Gram+ bacteria but shows little to no efficacy against Gram- bacteria because of their low membrane permeability and active efflux mechanisms (Surette *et al.*, 2021).

RIF binds to the β subunit of RNAP (encoded by *rpoB*) deep within the DNA/RNA exit channel (**Figure 8a**), thereby sterically blocking the synthesis of new RNA strands beyond two to three nucleotides and causing premature transcription termination (**Figure 8b**) (Campbell *et al.*, 2001). It is a clinically important ATB and a key component of anti-tuberculosis therapy. As bacteria develop resistance to RIF at a high frequency, it is generally used in combination with other antibiotics (Surette *et al.*, 2021, 2022).

Resistance to RIF is most commonly caused by point mutations in the *rpoB* gene, typically resulting in single amino acid substitutions within the rifampicin resistance–determining region (RRDR). This region comprises four clusters: the N-terminal cluster, cluster I, II, and III (Mosaei & Zenkin, 2020). Approximately 95 % of clinically observed RIF resistance mutations occur within the 81-bp segment of cluster I (Mosaei & Zenkin, 2020; Surette *et al.*, 2022). These mutations reduce the affinity of RIF for the RNA exit channel of RNAP (Surette *et al.*, 2022).

An opportunistic pathogen, *Nocardia farcinica*, evolved a different type of RIF resistance. Its genome consists of two different RNAP β subunit genes, *rpoB* and *rpoB2*. The RpoB2 protein contains aa substitutions within clusters I and II of the RRDR, which are likely to confer resistance to RIF (Ishikawa *et al.*, 2006; Tupin *et al.*, 2010).

Environmental actinobacteria and actinomycetes can also enzymatically inactivate RIF by ADP ribosyltransferases (Arr), glycosyltransferases (Rgt), phosphotransferases (Rph) and monooxygenases (Rox) (Surette *et al.*, 2021) .

1.1.5.2. Other transcription inhibitors

Fidaxomicin (FDX) is an antimicrobial agent that is clinically used for the treatment of diarrhea associated with *Clostridioides difficile* infection and inhibits transcription initiation earlier than rifampicin. FDX interacts with the β and β′ subunits, as well as the σ subunit, within the switch region of RNA polymerase (**Figure 8a**). The switch region is positioned at the base of the RNA

polymerase clamp and plays a critical role in mediating its conformational changes. Binding FDX to this region stabilizes the clamp in an open conformation, thereby preventing the σ subunit from simultaneously recognizing the -10 and -35 promoter elements and blocking formation of the transcription bubble (**Figure 8b**) (Dorst *et al.*, 2020; W. Lin *et al.*, 2018).

Other reported transcription inhibitors include sorangicin and GE23077, which act as primary channel inhibitors. Streptolydigin, salinamide, and CBR703 target mobile elements within the primary channel. Tagetitoxin and microcin J25 function as secondary channel inhibitors, whereas myxopyronins and squaramides inhibit transcription by targeting the switch region of RNA polymerase. However, none of these antibiotics are currently used in clinical practice (Ma *et al.*, 2016; Ye *et al.*, 2024).

Figure 8 illustrates different transcription inhibitors and their binding sites on RNAP.

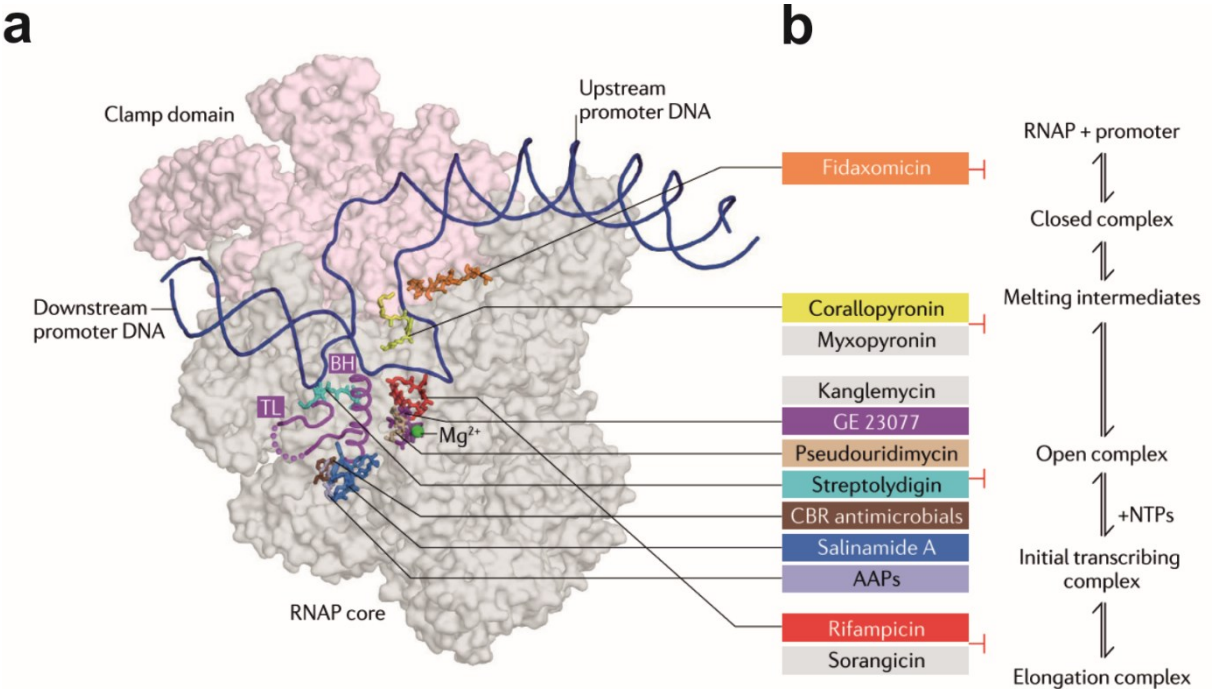

Figure 8: Transcription inhibitors and their binding sites, as well as the steps of the transcription cycle that they affect. (a) Antimicrobial compounds and their corresponding binding sites on RNA polymerase. The structure of myxopyronin is not displayed due to its overlap with corallopyronin. Similarly, the structures of kanglemycin and sorangicin are not shown because they overlap with rifampicin. AAPs, N^{α}-aroyl-*N*-arylphenylalaninamides; BH, bridge helix; TL, trigger loop; Green sphere, active site **(b)** A schematic representation of transcription initiation highlighting the specific steps that are targeted and inhibited by each antibiotic. NTPs, nucleotides. Adapted from (Chen *et al.*, 2021).

1.1.6. Transcription factors

Transcription factors (TFs) are regulatory proteins that fine tune bacterial gene expression. They bind directly to the RNAP or specific DNA sequences and either activate (activators) or repress (repressors) targeted genes. The crucial role of TFs is controlling transcription processes in response to various environmental changes (Browning *et al.*, 2019; Browning & Busby, 2016). They are involved in all steps of bacterial transcription (initiation, elongation, termination).

During transcription elongation RNAP can be restrained by a static protein roadblock (DNA bound protein), DNA lesions or rNTPs misincorporation, which leads to RNAP pausing, backtracking and creation of stalled RNAP complexes (Belogurov & Artsimovitch, 2015; N. Hao *et al.*, 2023). The formation of these stalled complexes can also be caused by certain antibiotics (*e.g.* RIF). The bacterial cell possesses factors responsible for the release or reactivation of stalled RNAP complexes once they have formed.

The following paragraphs outline several TFs that bind to the RNAP secondary channel and can release or reactivate stalled RNAP complexes, with a main focus on the protein HelD.

1.1.6.1. Gre factors

RNAP can backtrack during transcription elongation and undergo reverse translocation, creating inactivated transcription machinery. In this state, the 3′ end of the RNA transcript is no longer associated with the active site; it cannot be further extended and is instead extruded through the secondary channel. RNA synthesis can be restored only if the enzyme moves forward or if the extruded nucleotides are removed by nucleolytic cleavage (Abdelkareem *et al.*, 2019; Opalka *et al.*, 2003).

Bacteria encode cleavage factors, such as Gre factors – GreA and GreB in *E. coli* – which bind to the RNAP secondary channel reaching the active site, recruit Mg^{2+} and catalyze RNA hydrolysis. The Gre factors stimulate the intrinsic activity of the transcription elongation complex (Laptenko *et al.*, 2003; Sosunova *et al.*, 2003). Despite being sequence homologues, GreA and GreB differ in how they stimulate transcript cleavage. GreA promotes remove of short 3′ RNA segments (2–3 nt) and acts mainly to prevent arrest, whereas GreB facilitates cleavage of much longer RNA fragments, up to 18 nt, allowing it to reactivate already arrested complexes (Borukhov *et al.*, 1993; Toulmé *et al.*, 2000).

1.1.6.2. DksA

Another TF binding to the RNAP secondary channel is the protein DksA. Together with the alarmone ppGpp are involved in regulation of gene expression of many genes during stringent response in *E. coli*. They mainly inhibit ribosomal transcription and stimulate transcription of some genes engaged in *de novo* amino acid biosynthesis during starvation. The regulation occurs during transcription initiation in a step of open promoter formation (Gourse *et al.*, 2018; Zenkin & Yuzenkova, 2015). In many cases DksA and ppGpp act synergistically (Paul *et al.*, 2004), but they can also act independently (Magnusson *et al.*, 2007) or even antagonistically (Åberg *et al.*, 2008; Łyzén *et al.*, 2016). DksA contains a globular domain and coiled-coil domain similarly to Gre factors (Gourse *et al.*, 2018).

In addition to its role in transcription initiation, it also functions during transcription elongation, where it enhances transcription fidelity and helps prevent transcription–replication collisions (Roghanian *et al.*, 2015; Tehranchi *et al.*, 2010). DksA, in concert with ppGpp, reduces erroneous nucleotide incorporation by RNAP and inhibits the formation of backtracked complexes arising from misincorporation (Roghanian *et al.*, 2015). While DksA and ppGpp prevent the formation of stalled misincorporated complexes, Gre factors resolve such complexes once they form.

1.1.6.3. HelD

The first mention of HelD as an interacting partner of RNAP in *B. subtilis* dates back to 2011 (Delumeau *et al.*, 2011). At that time, the function and structure were unknown and were revealed over time. HelD is a ~ 90 kDa large protein, encoded by the *helD* (previously known as *yvgS*) gene and based on sequence homology belongs to the superfamily I (SF1) helicases, more precisely it is an UvrD-like helicase, however without helicase activity. *B. subtilis* HelD binds to RNAP core enzyme (**Figure 9a**) instead of holoenzyme. It was shown that HelD releases stalled RNAP complexes from DNA in an ATP-dependent manner, thereby enabling restarting new cycle of transcription (**Figure 9b**). The stimulatory effect on transcription was even more pronounced in the presence of the δ subunit of RNAP (Wiedermannová *et al.*, 2014).

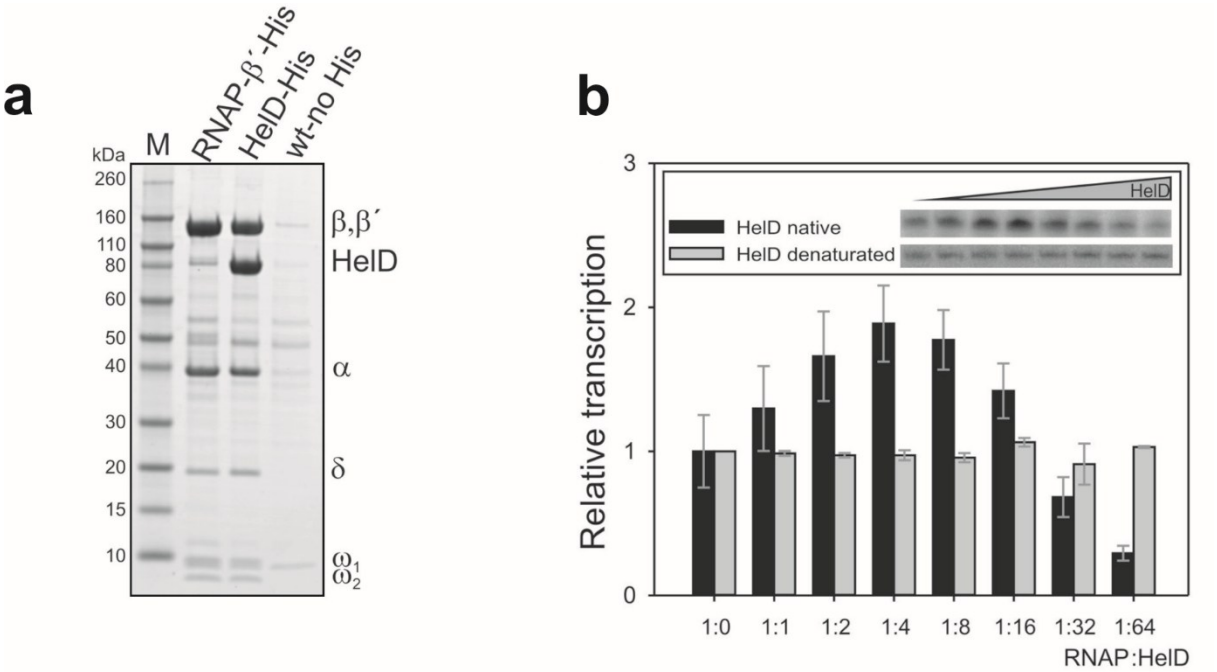

Figure 9: HelD interacts with RNAP core and induces transcription by RNAP recycling. (a) Coomassie-stained gel demonstrating the interaction between RNAP and HelD. RNAP carrying a His-tag on the β′ subunit (RNAP-β'-His) efficiently pulls down HelD, and reciprocally, His-tagged HelD (HelD-His) pulls down RNAP. A wild-type strain lacking a His-tag (wt-no His) was included as a negative control. **(b)** Effect of HelD on *in vitro* transcription. RNAPΔHelD with saturating concentrations of σ^A was reconstituted to create holoenzyme. Afterwards, it was incubated with native HelD (black bars) or heat-denaturated HelD (gray bars) at molar ratios 1:0 – 1:64 and serve for transcription initiation. The representative primary data are shown above the graph in the box. The 1:0 ratio was set as 1. The bars are averages from three independent experiments, and the error bars show ±SD. Adapted from (Wiedermannová *et al.*, 2014).

The first characterization of the shape, domain structure and ATP-driven conformation changes of *B. subtilis* HelD was reported in 2019 and it was determined by small angle X-ray scattering (SAXS) (Koval' *et al.*, 2019). One year later, in 2020, two different groups solved the RNAP-HelD structure from *B. subtilis* and described in more detail the transcription recycling by HelD (Newing *et al.*, 2020; Pei *et al.*, 2020). In the same year, Kouba *et al.* obtained the structure of the *Mycobacterium smegmatis* RNAP core-HelD complex using cryo-EM (Kouba *et al.*, 2020).

B. subtillis HelD (*Bsu*_HelD) is composed of four domains (**Figure 10, upper panel**), namely from the following: N-terminal, putative 1A-part 1, HelD-specific, putative 1A-part 2 and C- terminal. Several domains have alternative names, including the secondary channel arm (SCA; N-terminal), the clamp arm (CA; HelD-specific), and putative 2A domain (C-terminal). The unique N-terminal domain is joined to a putative 1A-part 1. The putative 1A domain is divided into two parts by the HelD specific domain and continuous with the C-terminal domain (Koval' *et al.*, 2019; Newing *et al.*, 2020). The ATPase unit is formed by the putative 1A domain and the C-terminal domain. Interestingly, the N-terminal domain is not essential for ATPase

activity, DNA binding or RNAP binding, but it is required for the stimulatory effect of HelD (Kovaľ *et al.*, 2019).

*Bsu*_HelD binds to RNAP via its N-terminal and HelD-specific domains. The N-terminal domain penetrates deep into the secondary channel, beyond the active site leading to displacement of the transcript and template DNA while preventing catalytic Mg^{2+} and NTP access. The HelD-specific domain forces the primary channel to open, causing a conformational change that displaces nucleic acids from the active site. *Bsu*_HelD dissociates from RNAP when ATP binds and undergoes hydrolysis (Newing *et al.*, 2020; Pei *et al.*, 2020). It is noteworthy that δ subunit of RNAP supports *Bsu*_HelD in its recycling activity by recruiting *Bsu*_HelD, guiding the HelD-specific domain into the primary channel and assisting opening it (Pei *et al.*, 2020).

M. smegmatis HelD (*Msm*_HelD) possesses a distinct structural feature compared to *Bsu*_HelD, which results in a different mechanism of its function. One of the *Msm*_HelD characteristics is a shorter N-terminal domain, which binds less deep into the RNAP secondary channel compared to *Bsu*_HelD, but it still interacts with the trigger loop and interferes with the nucleotide addition. The other characteristic is the primary channel loop (Pch-loop) located in the HelD-specific domain, and it is completely missing from the *Bsu*_HelD. The Pch-loop penetrates deep into the primary channel reaching the active site and sterically interferes with its content (Kouba *et al.*, 2020). For a domain composition of *Msm*_HelD see **Figure 10, bottom panel**. Another notable observation is that *Msm*_HelD can bind the RNAP holoenzyme (RNAP-σ^A-RbpA-HelD), whereas *Bsu*_HelD interacts only with the RNAP core (Kouba *et al.*, 2020; Wiedermannová *et al.*, 2014). Further studies indicate that *Msm*_HelD is present during transcription initiation. HelD associates with the RNAP holoenzyme to form the closed complex and is subsequently released during open complex formation. The dissociation of HelD is stimulated by ATP binding, and ATP hydrolysis further enhances this process. Additionally, the promoter DNA itself plays an active role in facilitating the dissociation of HelD (Kovaľ *et al.*, 2024).

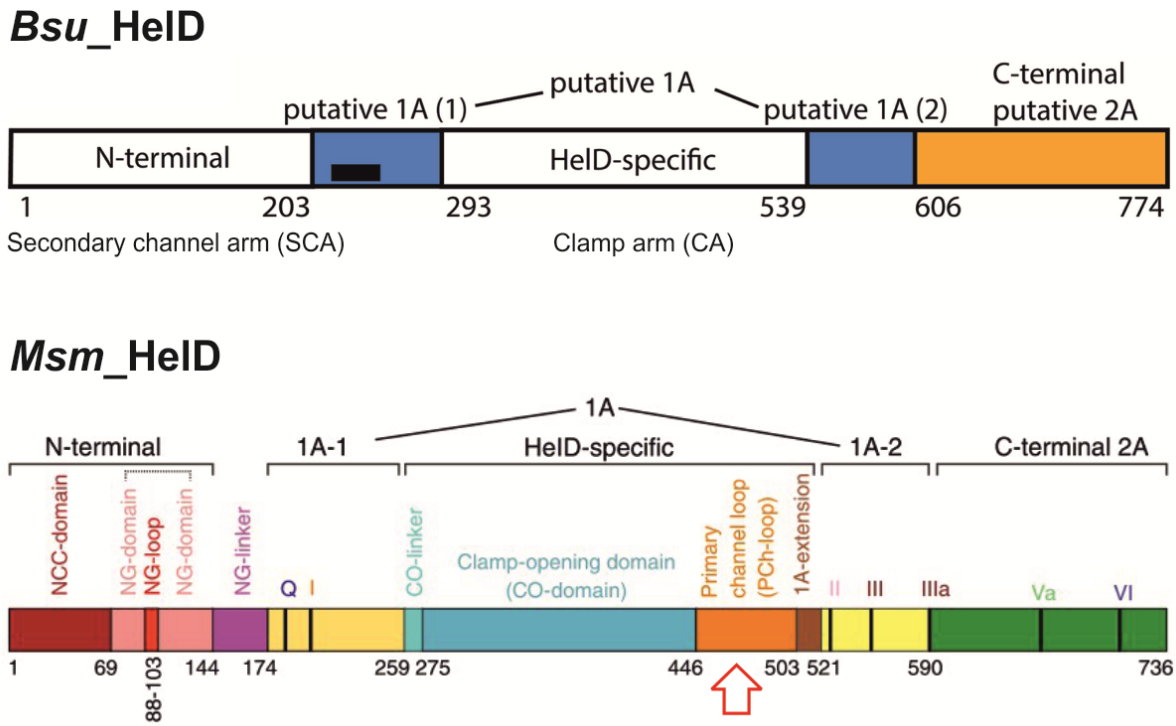

Figure 10: Schematic representation of the domain structures of *B. subtilis* and *M. smegmatis* HelDs. Upper panel: Domain structure of *B. subtilis* HelD (*Bsu*_HelD). The 1A domain is divided into two parts with the HelD-specific insertion. An alternative names of the N-terminal domain, as secondary channel arm (SCA) and HelD specific domain, as clamp arm (CA) are indicated. The ATP-binding box is represented by the black bar. Residue numbers defining the boundaries of the selected domains are indicated below the scheme. **Bottom panel:** Domain structure of *M. smegmatis* HelD (*Msm*_HelD). The N-terminal domain consists of α-helical coiled-coil (NCC) domain and β-globular (NG) domain. The 1A domain as in *B. subtilis* is divided with the insertion of the HelD-specific domain. This domain contains two regions, the clamp-opening domain (CO-domain) and the primary channel loop (Pch-loop). Q, I, II, III, IIIa, Va and V are indicated the conserved residues. Amino acid numbering is shown below the scheme. The red arrow indicates the Pch-loop, which is specific for the *Msm*_HelD and missing from the *Bsu*_HelD. The upper panel was adapted from (Koval' *et al.*, 2019) and the bottom panel from (Kouba *et al.*, 2020).

Structural differences lead to the classification of HelDs into the following categories: (i) Class I HelDs are found in low G+C Gram+ bacteria (*e.g.* Firmicutes, such as *B. subtilis*) and Gram- *Bacteroidia*, (ii) Class II HelDs are found in high G+C Gram+ bacteria (*e.g.* Actinobacteria, such as *M. smegmatis*), (iii) Class III HelDs were identified bioinformatically in Gram- *Deltaproteobacteria* (*e.g. Myxococcus xanthus*). **Figure 11** shows a comparison of the three classes. The third class can be distinguished from the other two by an additional aa insertion in the N-terminal domain and by the intermediate length of this domain, which is longer than that of *Msm*_HelD but shorter than that of *Bsu*_HelD (Larsen *et al.*, 2021).

Notably, some organisms possess multiple *helD* genes (ranging from 1 to 5, *e.g. B. subtilis* possesses only one *helD* gene, whereas *Streptomyces venezuelae* harbors up to five *helD* genes),

and these genes can be differentially expressed depending on the growth phase or environmental conditions (Larsen *et al.*, 2021).

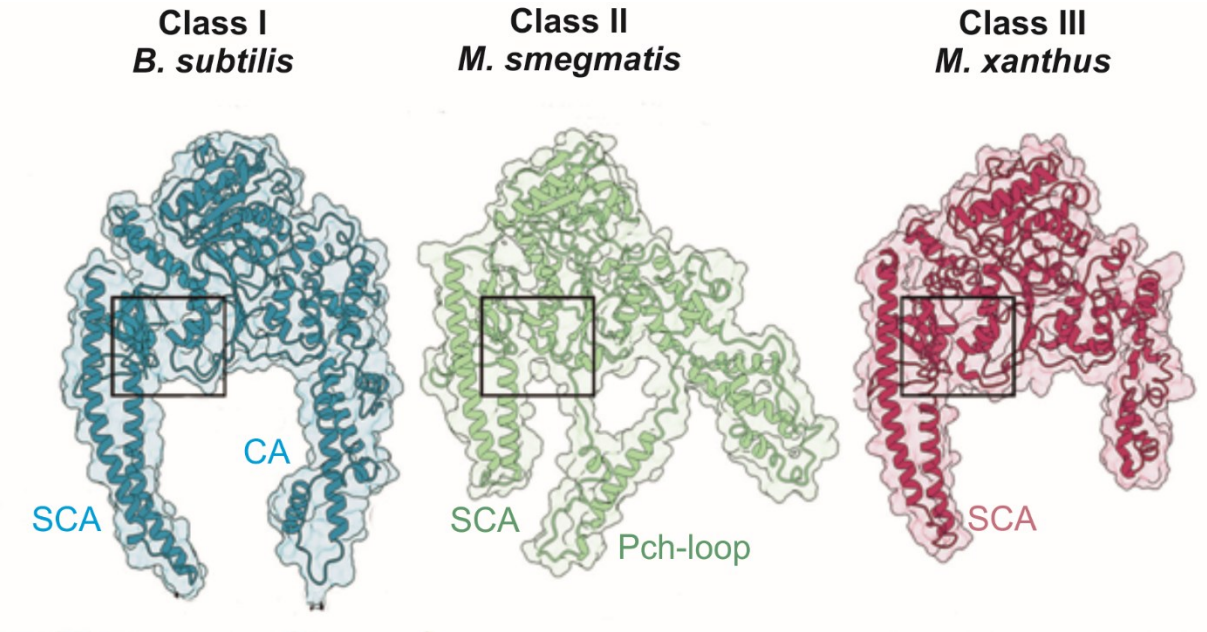

Figure 11: Comparison of three classes of the protein HelD. Class I HelD is represented in low G+C Gram+ and Gram- bacteria and possess a long secondary channel arm (SCA; N-terminal domain). Class II HelD is represented in high G+C Gram+ bacteria and their SCA is shorter compared to the Class I HelD. The characteristic feature of this class is the primary channel loop (Pch-loop). Class III HelD is represented in Gram- Deltaproteobacteria and its SCA has an intermediate length. The black brackets show the position of the conserved DWR sequence motif in the SCA. SCA, secondary channel arm (N- terminal domain); CA, clamp arm (HelD-specific domain); Pch-loop, primary channel loop. Adapted from (Larsen *et al.*, 2021).

In 2022, two research groups showed that HelD confers RIF resistance in *Mycobacterium abscessus* (**Figure 12a**) and *S. venezuelae*, which belong to the phylum Actinobacteria. In both cases they renamed HelD to HelR, which more reflects its role in RIF resistance (Hurst-Hess *et al.*, 2022; Surette *et al.*, 2022). Two years later, in 2024, Koval' et al. demonstrated that *Msm*_HelD also has a protective effect on RNAP against RIF (**Figure 12b**) (Koval' *et al.*, 2024).

Alongside *M. abscessus*, HelR can be found in other pathogenic species as well, such as *Nocardia farcinica* and *Rhodococcus equi* (Surette *et al.*, 2022). Interestingly, HelR is absent from the clinically important pathogen *Mycobacterium tuberculosis* (Hurst-Hess *et al.*, 2022; Surette *et al.*, 2022). However, heterologous expression of HelR from *M. abscessus* enhanced rifampicin tolerance in *M. tuberculosis* (**Figure 12c**) (Hurst-Hess *et al.*, 2022).

These studies demonstrated that Class II HelD/HelR provides resistance to RIF through the Pch-loop, a structural feature that is absent in Class I HelD, thereby raising the question of whether Class I HelD also contributes to RIF resistance. Furthermore, an old study by Hutter *et al.* revealed that exposure to sub-inhibitory concentrations of RIF resulted in increased

expression of a reporter gene fused to the Class I *helD* promoter (Hutter *et al.*, 2004), indicating that RIF exerts a regulatory effect although the underlying mechanism remained to be elucidated.

Thus, our research was dedicated to investigating the possible role of Class I HelD in RIF resistance, and to elucidating the mechanism by which RIF regulates HelD expression.

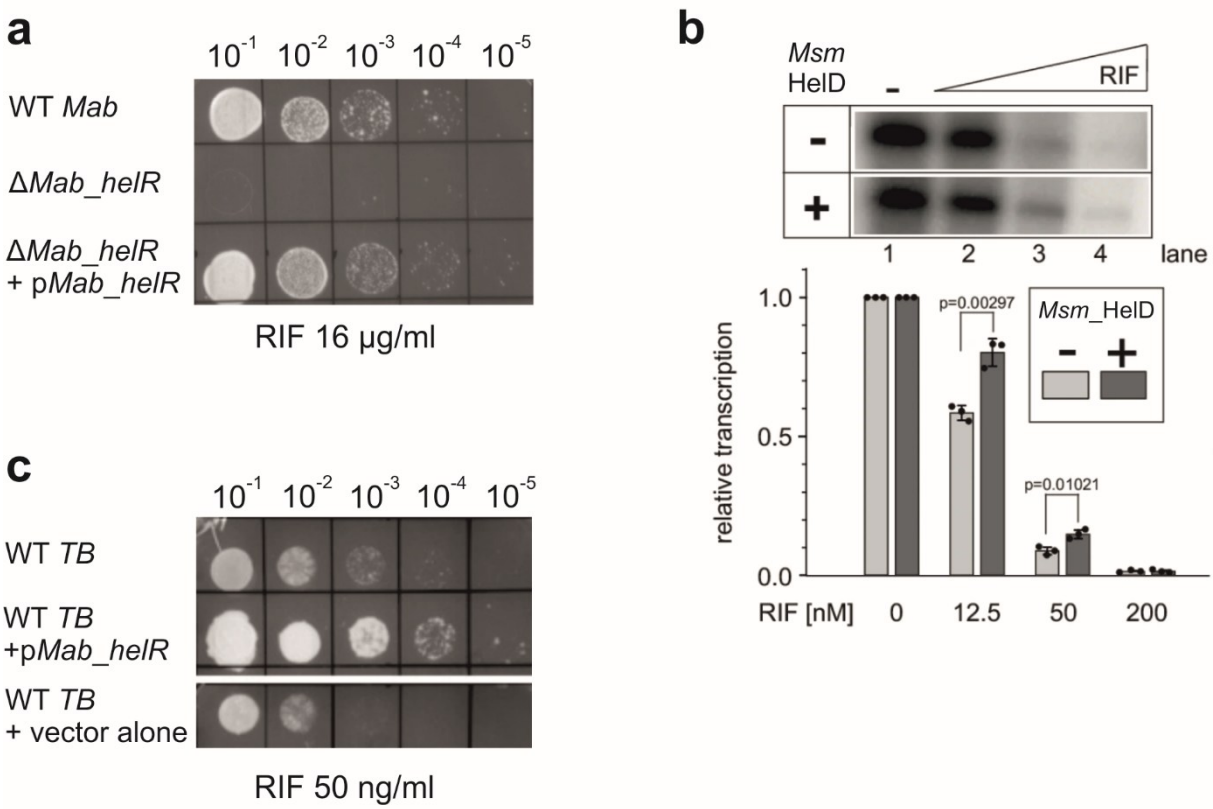

Figure 12: HelR/HelD provides RIF resistance in *M. abscessus* and *M. smegmatis*. (a) Growth of *M. abscessus* strains on solid medium in the presence of sub-inhibitory concentration of rifampicin (RIF). Deletion of *helR* rendered *M. abscessus* hypersensitive, and complementation restored the wild-type level of resistance. WT *Mab*, wild-type *M. abscessus*; Δ*Mab_helR*, *helR* deletion strain; Δ*Mab_helR* + p*Mab_helR*, complementation strain (*helR* deletion strain with a chromosomally integrated plasmid that expresses *helR*). **(b)** Multiple round transcriptions in the presence or absence of *Msm*_ HelD with increasing amounts of rifampicin (RIF) were performed on the *Msm* P*rrnAPCL1* promoter. For protein reconstitution the 1:1 RNAP:HelD ratio was used. Transcription at zero RIF was set as 1 for both -/+ HelD to facilitate visualization of the changes. The bars show averages of three independent experiments, the error bars show ± SD, and the dots represent individual experimental data. *p*-values indicated in the graph. Primary data are shown above the graph. **(c)** Growth of *M. tuberculosis* strains on solid medium in the presence of sub-inhibitory concentration of rifampicin (RIF). Heterologous expression of HelR from *M. abscessus* increased the RIF tolerance of *M. tuberculosis*. WT *TB,* wild-type *M. tuberculosis;* WT *TB* + p*Mab_helR*, complementation strain (wild-type *M. tuberculosis* strain with a chromosomally integrated plasmid expressing *M. abscessus helR*); WT *TB* + vector alone, wild-type *M. tuberculosis* strain with a chromosomally integrated empty vector serves as a control. Panel **(a)** and **(c)** were adapted from (Hurst-Hess *et al.*, 2022) and panel **(b)** from (Koval' *et al.*, 2024).

Aims

The main goal of this Doctoral thesis was to study the potential role of Class I HelD in rifampicin resistance, and to investigate the regulation of Class I HelD expression in response to the antibiotic rifampicin in *B. subtilis*.

Specific aims:

a) Investigate whether *B. subtilis* HelD is involved in rifampicin resistance.

b) Identify the part of HelD that is responsible for rifampicin resistance.

c) Determine which part of the *helD* gene upstream region is responsible for regulation of its expression by rifampicin.

d) Elucidate the mechanism of expression control of *helD* by rifampicin.

List of methods

Bacillus subtilis strain BaSysBio 1 (BSB1) 168 trp+ was used as a model organism for the purpose of this project. Plasmids were purified from *E. coli* DH5α cells and proteins were expressed and purified from *E. coli* DE3 (BL21) cells.

Work with bacteria

Bacterial cells cultivation (solid, liquid media)

Transformation (*E. coli*, *B. subtilis*)

Characterization of bacterial phenotypes (growth curve analysis and spotting assay in the absence/presence of rifampicin)

β-galactosidase assay

Determination of minimum inhibitory concentration (MIC)

Work with nucleic acids

Cloning, sequencing

DNA isolation (column-based kit), polymerase chain reaction (PCR)

RNA isolation (column-based kit), reverse transcription, quantitative polymerase chain reaction (qPCR)

Northern blot analysis

Work with proteins

Protein purification (affinity chromatography)

Size-exclusion chromatography

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

Native polyacrylamide gel electrophoresis (native PAGE)

Electrophoretic mobility shift assay (EMSA)

In vitro transcription assays (single- and multiple- round)

List of publications

Publication I

Bacteria sense the antibiotic rifampicin through a widespread dual-promoter based alarm system

Petra Sudzinová[1,†], Tamara Knežová Balgová[1,5,†], Marek Schwarz[1], Klára Juříková Mikesková[1], Karolína Hegrová[1], Dragana Vítovská[1], Priyanka Rawat[1], Šárka Bobková[1], Veronika Kočárková[1,5], Saran Natarajan[1], Debora Pospíšilová[1], Alena Křenková[2], Martin Hubálek[2], Petr Halada[1], Ivan Barvík[3], Tomáš Kovaľ[4], Jan Dohnálek[4], Jana Wiedermannová[1], Hana Šanderová[1] and Libor Krásný[1,*]

†The first two authors contributed equally to this work

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Type: Research article

Contribution of the author: 45 %. I prepared numerous constructs (CORE extensions, FULL P*anti-helD* MUT-35, P*anti-pps* CORE) for *in vivo* β-galactosidase experiments. For whole proteome analysis, I grew cells and prepared samples for mass spectrometry from three of the six biological replicates. I performed RT-qPCR to determine the relative levels of mRNAs for the selected genes (*helD*, *pps*, *veg*) before and after rifampicin (RIF) treatment. I carried out part of the β-galactosidase experiments, primarily those for identifying the regulatory region and element of the *helD* gene important for RIF regulation. I conducted the majority of the *in vitro* transcription experiments, which demonstrated the importance of the HelD N-terminal domain in RIF resistance, characterized key parameters of the core P*helD* and P*anti-helD* promoters (including strength, open complex formation, and promoter escape), and revealed how these convergently oriented promoters respond differently in the presence of RIF. I also contributed to the analysis of data and the preparation of figures. Additionally, I participated in the writing process, working on the manuscript from its initial draft to its final version.

Publication II

Dual-promoter control of gene expression: from transcriptional fine-tuning to antibiotic resistance

Petra Sudzinová[1], Tamara Knežová Balgová[1], Šárka Bobková[1], Marek Schwarz[1,2] and Libor Krásný[1,*]

Published in Current Opinion in Microbiology in 2026

DOI: https://doi.org/10.1016/j.mib.2026.102747

PMID: 41903510

Impact factor (2025): 7.5

Type: Review article

Contribution of the author: 15 %. I gathered relevant articles related to the manuscript's topic and created the figures. Moreover, I contributed to the preparation of the manuscript.

I confirm that this Doctoral thesis was written by Tamara Knežová Balgová and that it is based on published data collected and generated during her Ph.D. study in between 2022-2026. I hereby declare that her contribution to the publications described above is based on the truth.

……..

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

Summary of publications

Publication I

Bacteria sense the antibiotic rifampicin through a widespread dual-promoter based alarm system

Previous reports have shown that Class II HelDs in Actinobacteria confer rifampicin (RIF) resistance through RNAP protection (target-protected mechanism). This article reveals that Class I HelD likewise protects RNAP against RIF in *B. subtilis*. It further uncovers the region of HelD that is critical for RIF resistance and elucidates the regulation of *helD* gene expression by convergent promoters in response to RIF.

The study began with a mass spectrometry analysis of the RIF resistome of *B. subtilis*, in which HelD was identified as one of the most highly enriched proteins following treatment with sub-minimum inhibitory (sub-MIC) concentrations of RIF. Phenotypic experiments revealed growth defects in the Δ*helD* strain in both liquid (prolonged lag-phase) and solid (slow growth) medium, suggesting a link between HelD and RIF resistance.

Based on the literature and *in silico* modelling we speculated that the part important for RIF resistance might be the HelD N-terminal domain that reaches to the vicinity of the RIF binding pocket. Indeed, the HelDΔN strain had even more prolonged lag-phase in liquid media than the Δ*helD* strain. Furthermore, *in vitro* transcription assays revealed that intact HelD confers a protective effect, whereas no such effect was observed with HelD lacking the N-terminal domain. This protection is reflected by a less pronounced inhibition of transcription in the presence of RIF. These results demonstrate that the N-terminal domain is essential for RIF resistance.

RT-qPCR then revealed that induction of HelD by RIF is already at the transcriptional level. To pinpoint the region important for the *helD* gene regulation, the *helD* promoter was initially identified by 5' RACE (rapid amplification of cDNA ends) method. Subsequently, various constructs with the *lacZ* fusion were created, and β-galactosidase assays were performed. The results showed that the *helD* 5' untranslated region (5' UTR) contains a RIF responsive sequence. Further studies than revealed that this region contains a putative promoter in the antisense direction that plays an important regulatory role. To obtain more information about the antisense transcript in the absence/presence of RIF we performed Northern blot analysis.

Interestingly the antisense transcript decreased in the presence of RIF whereas the *helD* mRNA increased. Next, we decided to perform transcriptions *in vitro* to elucidate the mechanism. The results corresponded to the *in vivo* data, suggesting that the regulation is based on an interplay between two promoters facing each other, sense – P*helD* and antisense – P*anti-helD*, that react differently to RIF. To gain more insight into the properties of the promoters and their interplay we performed both β-galactosidase and *in vitro* transcription assays. The experiments demonstrated that P*anti-helD* is a stronger promoter than P*helD*, primarily due to its faster promoter escape. Additionally, swapping P*helD* and P*anti-helD* promoters disrupted regulatory control, indicating that promoter arrangement is important. A similar loss of regulation was observed when the P*helD* promoter was replaced with the stronger promoter, suggesting that the promoter strength also plays a role in RIF-inducible regulation.

Based on the data collected so far, we proposed the following model: In the absence of RIF, transcription occurs predominantly from the P*anti-helD* promoter due to its higher RNAP promoter escape efficiency. This interferes with RNAP binding and initiation at the P*helD* promoter, thereby limiting transcription from P*helD* and allowing *helD* to be transcribed only occasionally. The situation changes in the presence of low levels of RIF. Because transcription from P*anti-helD* occurs more frequently, RNAP bound by RIF is more likely to bind to this promoter and become stalled. When RNAP is stalled at P*anti-helD*, RNAP initiating from P*helD* has sufficient time to begin transcription and, during elongation, can displace the stalled complex at P*anti-helD*, thereby enabling transcription of the *helD* gene (**Figure 13**). Consistent with the model, when we examined a *B. subtilis* RIF resistant strain carrying an RNAP mutation that prevents stalling at the P*anti-helD* promoter, the regulatory mechanism was disrupted.

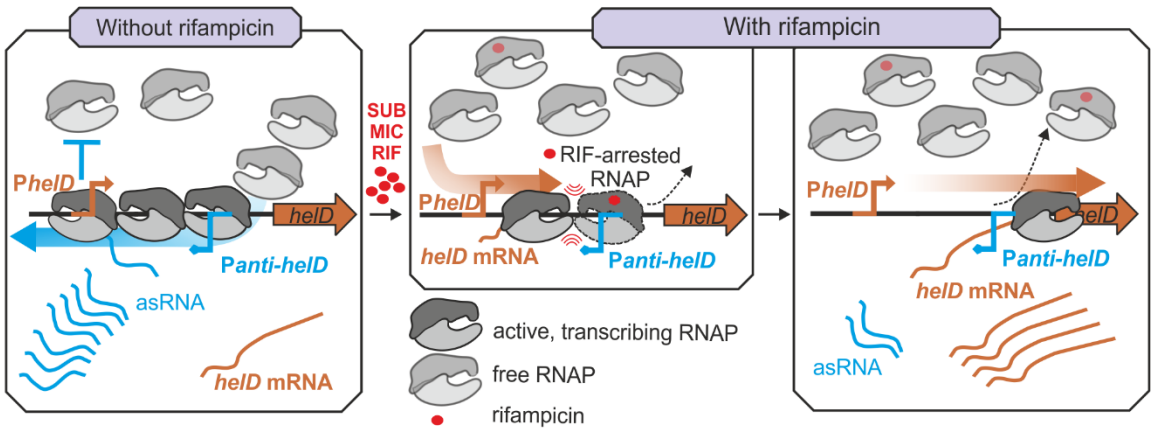

Figure 13: Model of regulation of *helD* gene expression by RIF. In the absence of RIF (left panel), transcription mainly initiates from the stronger P*anti-helD* promoter. This activity interferes with the weaker P*helD* promoter, resulting in only occasional transcription from P*helD*. At sub-MIC concentrations of RIF, RIF-bound RNAP is preferentially arrested at P*anti-helD*, thereby preventing transcriptional interference. This allows RNAP molecules not bound by RIF to bind to P*helD*, initiate

transcription, and escape the promoter. Once elongation begins, the advancing RNAP collides with and displaces the RIF-arrested RNAP at P*anti-helD* (middle panel) and then proceeds to transcribe the *helD* gene (right panel). Adapted from (Sudzinová *et al.*, 2026).

Finally, bioinformatic analyses revealed that this two-promoter arrangement is present upstream of various genes (*e.g* hydrolases, transporters) across different organisms. In *B. subtilis*, a similar promoter arrangement was identified upstream of the *pps* gene, which encodes a RIF phosphotransferase. Phenotypic analysis, RT-qPCR and β-galactosidase assays demonstrated the same RIF-dependent regulation mediated by convergent promoters.

In summary, this study demonstrates that Class I HelD confers protection against the antibiotic RIF through its N-terminal domain. Moreover, it elucidates the mechanism underlying RIF-dependent regulation of *helD* expression and identifies, through bioinformatic analysis, additional candidate genes that may contribute to RIF resistance.

Publication II

Dual-promoter control of gene expression: from transcriptional fine-tuning to antibiotic resistance

This review focuses on transcriptional regulation mediated by pairs of promoters. It discusses their possible combinations and orientations, as well as the factors that influence their interplay. In addition, it highlights examples of antibiotic resistance genes controlled by dual promoters. Finally, it presents a prospective bioinformatic analysis and explores potential applications of such promoter arrangements in synthetic biology.

Regulation of gene expression by two promoters is observed across all domains of life. There are three possible orientations: (i) tandem promoters, which share the same direction; (ii) divergent promoters, which face away from each other; and (iii) convergent promoters, which face one another. The interplay between two promoters can be influenced by various factors, including σ factors, DNA supercoiling, transcription factors (TFs, activators and repressors), and promoter strength.

Dual-promoters are often located upstream of antibiotic resistance genes, where they regulate transcription. This regulation can be further modulated by the antibiotic itself or by TFs. An intriguing case occurs in Actinobacteria, where RIF resistance-associated genes are controlled by a 19 bp palindromic sequence known as the rifampicin-associated element (RAE), though the underlying mechanism remains unclear. It has been proposed that a putative transcription factor binds to the RAE and, together with tandem promoters, modulates gene expression.

We sought to determine whether the RAE, together with a potential transcription factor, could function in combination with convergent promoters. Our bioinformatic analysis revealed such combinations, namely genes encoding transporters, transcriptional regulators, and transferases with potential RIF resistance activity exhibited this arrangement. Finally, dual-promoter configurations can be used in synthetic biology to enhance, fine-tune, and coordinate gene expression for protein production and metabolic pathway optimization.

Discussion

This thesis focuses on the new antibiotic resistance function of *B. subtilis* Class I HelD (Sudzinová *et al.*, 2026). In this section of my doctoral thesis, I will discuss the role of Class I HelD in rifampicin (RIF) resistance and the regulation of *helD* gene expression by RIF. I will also discuss other RNAP inhibitors as potential targets of HelD, as well as the regulation of gene expression by dual promoters.

Rifampicin resistance mediated by HelD

An approach to identify proteins associated with antibiotic resistance is to analyze the proteome (resistome) of bacteria exposed to a specific antibiotic. Methods for protein identification have advanced remarkably over time, with significant improvements in sensitivity. However, the results of such analyses can vary depending on the method of detection, antibiotic concentration used and the growth conditions.

Such discrepancy is evident when comparing our proteomic data with previously published results from 2002, which reported induction of σ^B- dependent general stress response proteins following RIF treatment (Bandow *et al.*, 2002), in contrast to our findings. This discrepancy may be explained by differences in experimental conditions, including the use of a basal limitation medium in the previous study compared with LB medium in our work, as well as differences in protein identification methods. The earlier study used pulse-labeling followed by 2D PAGE separation, resulting in partial protein identification, whereas our analysis was based on mass spectrometry. It cannot be excluded that the unidentified proteins from the 2002 study included HelD and Pps, which would be consistent with our findings.

Class I and Class II HelDs confer RIF resistance through distinct mechanisms that reflect their structural differences. Class II HelD employs its Pch-loop to distort the rifampicin-binding pocket and promote the release of RIF from RNAP (**Figure 14a**) (Hurst-Hess *et al.*, 2022; Surette *et al.*, 2022). In contrast, Class I HelD utilizes its extended N-terminal coiled-coil domain to achieve a similar outcome (**Figure 14b**) (Sudzinová *et al.*, 2026).

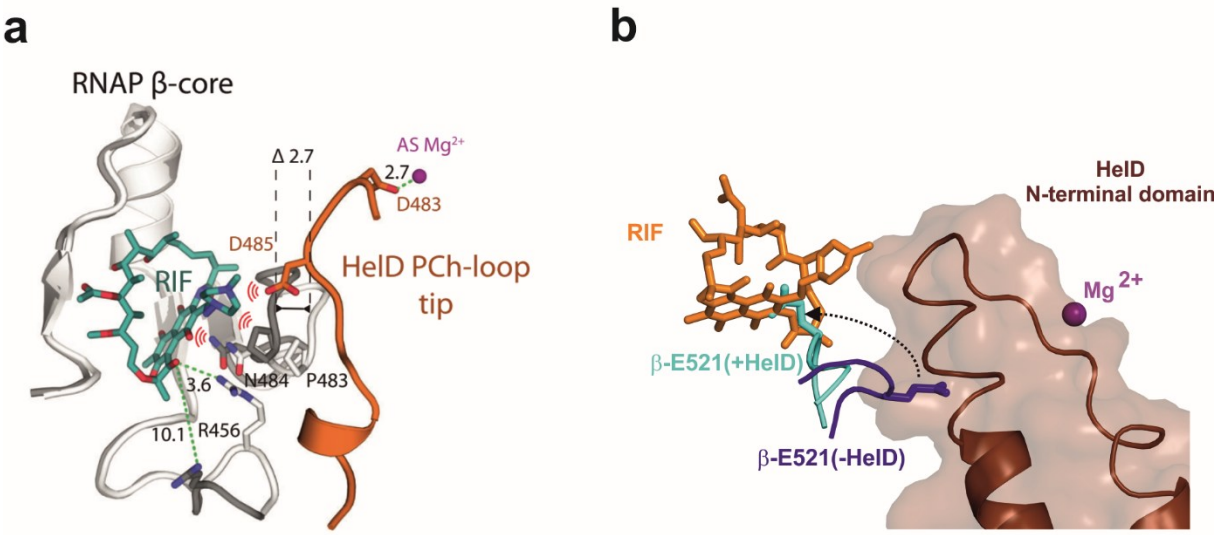

Figure 14: Deformation of RIF binding pocket by the HelD Pch-loop (Class II) and N-terminal domain (Class I). (a) Class II HelD Pch-loop binding remodels the RIF binding pocket. The β-core P483–N484 loop is displaced toward RIF by 2.7 Å, D485 within the HelD PCh-loop creates a steric clash with RIF, and β-core R456—normally responsible for coordinating RIF—is shifted away from its native position. The model is based on the superposition of the *M. tuberculosis* RNAP–rifampicin complex (light gray, RNAP β-core; PDB code: 5UHC) and *M. smegmatis* us-fork–HelD–RPc-II complex (dark gray). Teal, RIF; Pink sphere, Mg^{2+} active site; Orange, Pch-loop; Red waves, putative atomic clashes between RIF, the distorted pocket, and HelD; Green dashed lines, distances (Å). **(b)** Class I HelD N-terminal domain binding deforms the expected rifampicin binding pocket. The black arrow highlights the movement of β-E521 caused by the HelD N-terminal domain. Dark blue, RNAP β-E521 (-HelD); Teal, RNAP β-E521 (+HelD); Ruby brown, HelD N-terminal peptide backbone within the semitransparent surface; Orange, RIF; Magenta sphere, Mg^{2+} active site. The model is based on the superposition of the *B. subtilis* RNAP elongation complex (PDB code: 6WVJ) and *B. subtilis* RNAP with HelD (PDB code: 6ZFB). The anticipated position of rifampicin is taken from the *M. tuberculosis* RNAP–rifampicin complex (PDB code: 5UHC). Panel **(a)** was adapted from (Koval' *et al.*, 2024) and panel **(b)** was adapted from (Sudzinová *et al.*, 2026).

Distinct pathways of RNAP-RIF complex rescue by HelD

There are several possibilities in which stalled RNAP-RIF complexes can be rescued by Class II HelD (also known as HelR): (i) HelD can bind to stalled RNAP-RIF complex, dissociate it from DNA and with addition of ATP the HelD and RIF release from the complex, which lead to RNAP recycling (Hurst-Hess *et al.*, 2022). (ii) Alternatively, HelD binds to the free RNAP occupied by RIF. In the presence of ATP, HelD and RIF release from the RNAP, restarting a new transcription cycle (Hurst-Hess *et al.*, 2022; Surette *et al.*, 2022). (iii) At the end, HelD can eject RIF as well as the DNA/RNA hybrid from a stalled RNAP complex at the early stage of transcription initiation without releasing RNAP from DNA promoter. It can revert the complex to a closed complex–like state, thereby enabling the initiation of a new round of transcription (Koval' *et al.*, 2024). The first two scenarios could also apply to Class I HelD. However, the

third scenario is unlikely, as Class I HelD binds to the RNAP core rather than the holoenzyme (Wiedermannová *et al.*, 2014) and is therefore not present during transcription initiation.

Gene expression regulated by dual-promoters

Regulation of gene expression by dual-promoter arrangement is common phenomenon and can be found across all domains of life (Warman *et al.*, 2021; Wiechens *et al.*, 2025). Antibiotic resistance genes are often controlled by dual-promoter architectures that function in coordination with transcription factors. For example, in *E. coli*, expression of *marA* is regulated by a divergent promoter arrangement together with the repressor MarR (Beggs *et al.*, 2020). Similarly, in *Pseudomonas aeruginosa*, *ampC* expression is governed by a divergent promoter configuration and the transcriptional regulator AmpR (Caille *et al.*, 2014; Demeester *et al.*, 2024).

Dual-promoter architectures are not limited to antibiotic resistance genes; they also govern the expression of genes involved in a wide range of cellular processes. These include genes involved in carbohydrate and amino acid metabolism (Jaishankar *et al.*, 2022), flagellar assembly (Rivera-Osorio *et al.*, 2018), virulence factor production (Jia *et al.*, 2017), and ribosomal RNA synthesis (Murray & Gourse, 2004).

Regulation of *helD* gene expression by RIF and dual-promoters

In Actinobacteria, some genes associated with RIF resistance (*e.g.* RIF inactivating enzymes) are associated to a 19 nt long palindromic sequence (GGG(G/C)CTTGCGGCAAG(C/G)CCC) located upstream of the genes. This sequence is referred to as the rifampicin-associated element (RAE) (Spanogiannopoulos *et al.*, 2014; Surette *et al.*, 2022). The details of the mechanism of induction associated with RAE remain unknown. However, inverted repeat DNA motifs frequently serve as binding sites for regulatory proteins and thereby influence gene expression, which could likewise apply to RAE (Spanogiannopoulos *et al.*, 2014). It is worth noting that RAE is also located upstream of genes with no known role in resistance (Spanogiannopoulos *et al.*, 2014; Surette *et al.*, 2022).

In *M. smegmatis,* the *rpoB-rpoC* operon (encodes the β and β' subunits of RNAP, respectively) is regulated by two tandem promoters, Promoter 1 and Promoter 2. In the absence of RIF, transcription from Promoter 1 suppresses transcription from Promoter 2, resulting in sub-maximal *rpoB* expression. However, at low concentrations of RIF, transcription from

Promoter 1 is preferentially inhibited. This alleviates its repressive effect on Promoter 2, leading to enhanced overall *rpoB* expression (Zhu *et al.*, 2018). Regulation of Class II HelD expression in *M. smegmatis,* and *M. abscessus* is similarly controlled by two tandem promoters (**Figure 15a**). This regulation involves the RAE element, which overlaps with the -35 region of Promoter 2, indicating a potential regulatory interplay between these elements (Sudzinová *et al.*, 2023). However, further investigation is required to elucidate the precise molecular mechanism underlying this regulatory interaction.

In *B. subtilis*, Class I HelD expression is regulated through a distinct mechanism. It relies on a convergent promoter arrangement in which two promoters are oriented toward each other (**Figure 15b**), and the RAE element is not required (Sudzinová *et al.*, 2026). In this configuration, transcription initiated from the stronger promoter interferes with transcription from the weaker promoter – a phenomenon known as transcriptional interference (Callen *et al.*, 2004; Shearwin *et al.*, 2005). In the presence of RIF, transcription from the stronger promoter is attenuated, thereby allowing *helD* transcription to proceed from the weaker promoter (Sudzinová *et al.*, 2026).

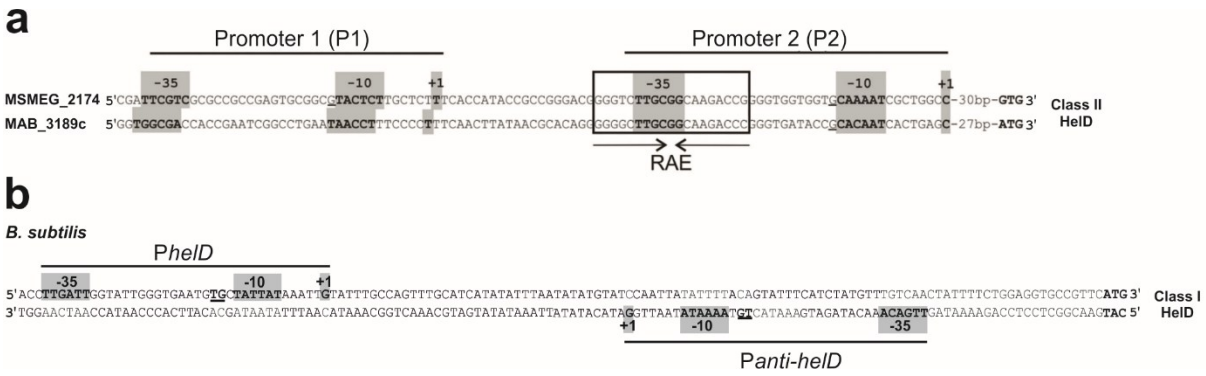

Figure 15: Promoter region architectures of Class I and Class II HelDs. (a) *M. smegmatis* (MSMEG_2174) and *M. abscessus* (MAB_3189c) Class II HelD promoter region. The region contains two promoters with tandem architecture, Promoter 1 (P1) and Promoter 2 (P2). The -35, -10 elements and TSSs (+1) are indicated and highlighted with grey background. The bracket indicates RAE (rifampicin-associated element), which overlaps with the -35 element of the Promoter 2. The arrows indicate the palindrome. Conserved Gs upstream of the -10 elements are underlined. The scheme shows the nontemplate strand (5' → 3'). **(b)** *B. subtilis* Class I HelD promoter region. The region contains two promoters with convergent architecture, P*helD* (sense strand) and P*anti-helD* (antisense strand). The - 35, -10 elements and TSSs (+1) are indicated and highlighted with grey background. The extended - 10 element is underlined. Panel **(a)** was adapted from (Sudzinová *et al.*, 2023) and panel **(b)** was adapted from (Sudzinová *et al.*, 2026).

Different properties of Class I HelD promoters mediate RIF induction

Bacteria can regulate transcription through various mechanisms. One such mechanism is through a core promoter, which consists of multiple elements (-35, spacer, extended -10, -10, discriminator elements) that vary in length and sequence composition (Kuo *et al.*, 2025).

In *B. subtilis*, the convergent promoters, P*helD* (sense) and P*anti-helD* (antisense), which regulate *helD* gene expression via RIF, contain -35, spacer, extended -10 and -10 promoter elements (**Figure 16a**) (Sudzinová *et al.*, 2026). These elements likely determine the functional properties of these promoters.

P*anti-helD* is a stronger promoter which exhibits faster promoter escape compared to P*helD*. Moreover, it contains an unusually long 19 bp spacer compared with the 18 bp long spacer of P*helD* (**Figure 16a**) (Sudzinová *et al.*, 2026). Previous studies have demonstrated that spacer length and sequence play a role in determining promoter strength. The highest promoter strength is typically achieved with an optimal 17 bp spacer, followed by an 18 bp spacer (**Figure 16b**). In addition, the number of -TG- motifs within the extended -10 element (part of the spacer) also influence promoter activity (**Figure 16c**). Tandem repeats of this motif decrease promoter activity by increasing RNAP binding affinity, which can result in RNAP trapping at the promoter (Klein *et al.*, 2021). This may be the case for P*helD*, whose spacer contains - TGTG - tandem repeat motifs (**Figure 16a**) that could contribute to weaker promoter activity and slower promoter escape.

An interesting example of gene regulation involving a 19 bp spacer is found in *B. subtilis*, where BmrR, a member of the MerR-TFs, activates transcription of the *bmr* gene (bacterial multidrug resistance), which encodes an efflux pump. RNAP and BmrR together induce four kinks in the upstream promoter dsDNA, thereby shortening the non-optimal spacer to facilitate efficient promoter unwinding. Interestingly during this process, RNAP and BmrR barely interact with each other (Fang *et al.*, 2020). It is a possibility that a TF also binds to a P*anti-helD* spacer, induces DNA distortion and thus modulates its transcriptional activity. Additionally, interaction between RNAP and P*anti-helD* may be less stable due to a longer spacer, potentially facilitating RNAP displacement by transcription initiated from the opposite direction.

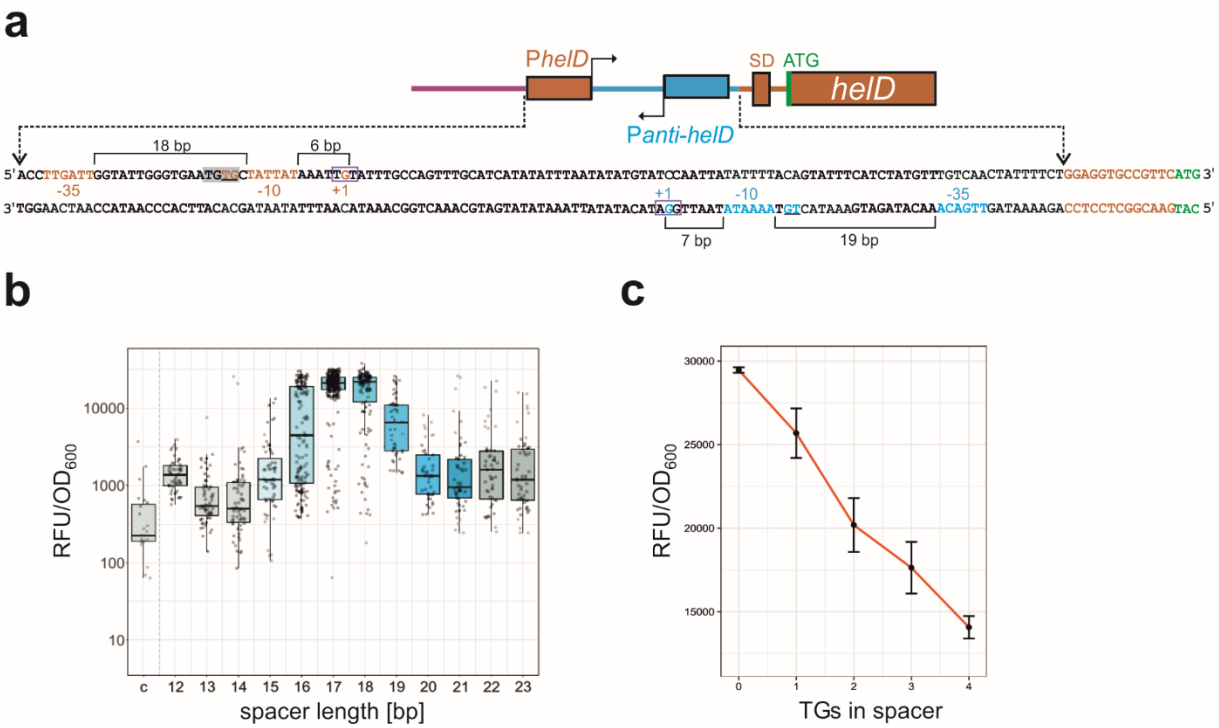

Figure 16: Spacer length and sequence affect promoter strength. (a) Scheme and sequence of the *helD* upstream region with indicated P*helD* and P*anti-helD* promoters, Shine-Dalgarno (SD), and start codon (ATG, green). P*helD* (sense strand) is highlighted with dark orange, P*anti-helD* (antisense strand) in blue, extended -10 elements (-TG-) are underlined and the -TGTG- tandem repeat motif within the P*helD* spacer is highlighted with grey background. The purple brackets indicate the "start" element (conserved 3 bp motif centered on TSS). Spacer length between the -35 and -10 elements of P*helD* (18 bp) and P*anti-helD* (19 bp) are indicated. The location of the TSSs downstream from the -10 elements are also indicated (6 bp for P*helD* and 7 bp for P*anti-helD*). **(b)** Spacer length dictates promoter strength. A synthetic promoter library was fused to the mVenus reporter gene, and promoter activity was measured using fluorescence signals. Fluorescence signal was normalized by OD_{600}. High levels of gene expression were observed for spacer lengths ranging from 15 bp to 19 bp, with the highest expression occurring at 17 bp. Blue bars indicate the native spacer range, gray bars represent spacer lengths outside the native range, and column c shows the background signal from a random DNA sequence library. **(c)** Number of -TG- motifs influence promoter activity. Increasing number of -TG- motifs decrease promoter activity. A synthetic promoter library was fused to the mVenus reporter gene, and promoter activity was measured using fluorescence signals. Fluorescence signal was normalized by OD_{600}. Panel **(a)** was adapted from (Sudzinová *et al.*, 2026) and panels **(b)** and **(c)** from (Klein *et al.*, 2021).

Recently, Kuo *et al.* described a novel promoter element termed, "start". This element included a 3 bp region with a conserved motif, pyrimidine – purine – pyrimidine (Y^{-1} – R^{+1} – Y^{+2}), centered on TSS and plays a key role in TSS selection and enhances transcription. When the TSS is located 7 or 8 bp downstream of the -10 element promoter strength significantly enhanced (Kuo *et al.*, 2025). The TSS of the P*anti-helD* is located 7 bp downstream of the -10 element (**Figure 16a**) and may also be responsible for the strength of the promoter.

Rifampicin resistance through rifampicin inactivating enzymes

Bacteria have evolved various mechanisms to develop resistance to RIF (Tupin *et al.*, 2010). One such mechanism involves chemical modification of the drug, resulting in its inactivation. Four types of rifampicin modifications are known so far: ADP-ribosylation, glycosylation, phosphorylation, and hydroxylation (Surette *et al.*, 2021). Certain bacteria are equipped with not only an inactivating enzyme but also with another RIF resistance mechanism.

The primary mechanism of RIF resistance in *Nocardia farcinica* is a duplication of the *rpoB* gene in the genome, where *rpoB2* encodes a RIF resistant protein (Ishikawa *et al.*, 2006; Tupin *et al.*, 2010). The secondary supportive mechanism relies on rifampicin monooxygenase, which is encoded by *rox*. This enzyme oxidizes the C3 side chain of RIF by introducing an oxygen atom, thereby converting it into a derivative with diminished antimicrobial activity (Abdelwahab *et al.*, 2016; Hoshino *et al.*, 2010).

In addition to Class II HelD mediated protection against RIF, *M. abscessus* and *S. venezuelae* also produce RIF inactivating enzymes (Hurst-Hess *et al.*, 2022; Surette *et al.*, 2022). *M. abscessus* produces ADP-ribosyltransferase, encoded by *arr*, which catalyzes the transfer of ADP-ribose to the C23 hydroxyl group of RIF, causing steric hindrance that prevents binding to RpoB (Hurst-Hess *et al.*, 2022). *S. venezuelae* express rifampicin monooxygenase, encoded by *rox* (Surette *et al.*, 2022). The regulation of the *helD*, *arr*, and *rox* genes is controlled by RAE (Hurst-Hess *et al.*, 2022; Surette *et al.*, 2022), which may help the cell combat RIF more efficiently.

B. subtilis also possess another rifampicin resistance protein in addition to Class I HelD, Pps (Sudzinová *et al.*, 2026). Pps functions as a rifampicin phosphotransferase that enzymatically inactivates RIF (Bobková *et al.*, 2026). It catalyzes the transfer of the β-phosphate group from ATP to the C21 hydroxyl group of RIF, thereby blocking RIF binding to the rifampicin-binding pocket of RNAP (Surette *et al.*, 2021, 2022). Like Class I HelD, Pps expression is controlled by convergent promoters (Sudzinová *et al.*, 2026). It was proposed that *pps* be renamed to *rphT* to better reflect its function, particularly since *B. subtilis* already contains the *rph* gene, which encodes RNase PH, a 3'-5' exoribonuclease. Notably, proteins of the bacterial phosphotransferase system (PTS) display structural similarity to rifampicin phosphotransferase domains supporting the hypothesis that the PTS may constitute an evolutionary ancestor of rifampicin phosphotransferases (Bobková *et al.*, 2026).

Both Class I and Class II HelDs promote the release of RIF from RNAP, thereby facilitating the resumption of transcription, whereas RIF inactivating enzymes chemically modify the antibiotic. The combined action of these mechanisms reduces the pool of active RIF and contributes to resistance. It's worth noting that in *B. subtilis* additional mechanisms are likely to contribute to RIF resistance, as Δ*helD* and Δ*pps* strains remain capable of growth in the presence of sub-MIC levels of RIF, albeit with a prolonged lag-phase.

RNAP inhibitors exhibiting properties different from rifampicin

Fidaxomicin (FDX) is another clinically important antibiotic used in the treatment of *C. difficile* infections (Le *et al.*, 2024). Its binding site on RNAP and its mechanism of action differ from those of RIF, as described previously in Chapter – 1.1.5.2. Other transcription inhibitors. Notably, FDX exhibits a lower frequency of resistance-conferring mutations compared with RIF. However, its antimicrobial spectrum is relatively narrow, largely due to poor systemic absorption and its specific target site (Chen *et al.*, 2021; Zhanel *et al.*, 2015). Despite the comparatively low mutation rate, resistance to FDX can still emerge in *C. difficile*. The primary molecular mechanism of resistance is associated with single point mutations in either *rpoB* (encoding the β subunit), or *rpoC* (encoding the β′ subunit). Less frequently, resistance-associated mutations occur in the *marR*/CD22120 gene, which encodes the MarR transcription factor responsible for repressing genes involved in efflux pump expression (Le *et al.*, 2024; Zhanel *et al.*, 2015).

Another promising antimicrobial compound not yet used clinically is kanglemycin (KANG). An important advantage of KANG is its activity against rifampicin resistant Gram+ bacteria and multidrug resistant *M. tuberculosis*. Kanglemycin, a rifamycin congener, has been shown to bind to the same position on RNAP as RIF, and it contains additional structural moieties: a sugar group (β-*O*-3,4-*O,O′*-methylene digitoxose) at position C27 and an acid moiety (2,2-dimethylsuccinic acid) at position C20 (**Figure 17a**). These groups interact with residues of the RNAP β subunit that are not contacted by RIF, thereby stabilizing KANG binding and compensating for interaction losses caused by resistance-conferring mutations. Mechanistically, KANG acts at an earlier stage of transcription inhibition than RIF (**Figure 17b**). It interferes with the binding of the initiating NTP, preventing formation of the first phosphodiester bond, and subsequently blocks RNA chain elongation (Mosaei *et al.*, 2018; Peek *et al.*, 2018). In addition, KANG is not susceptible to inactivation by the ADP-

ribosyltransferase Arr from *M. smegmatis*, although it can be inactivated by the Arr enzyme from *M. abscessus* (Harbottle *et al.*, 2021).

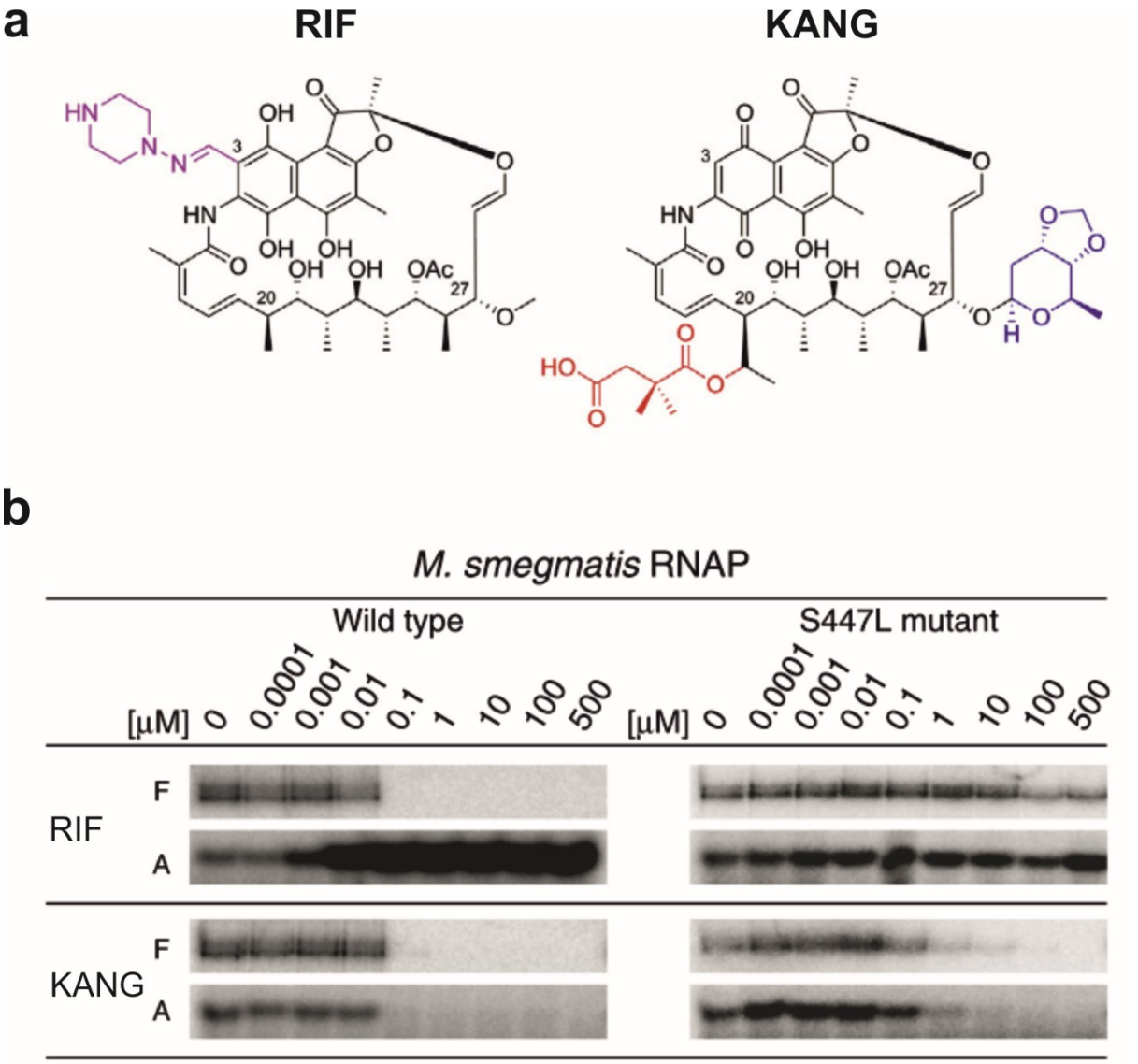

Figure 17: Structure and activity of kanglemycin. (a) Comparison of the chemical structures of rifampicin (RIF) and kanglemycin (KANG). The semisynthetic group of RIF is represented by a purple circle. In KANG, a sugar group ansa chain modification at position C27 is highlighted in dark purple, and an acid group modification at position C20 is highlighted in red. **(b)** Activity of RIF and KANG against *M. smegmatis* wt and RIF resistance (βS447L) RNAP was performed by *in vitro* transcription. KANG inhibits the synthesis of both full-length (F) and abortive (A) transcripts, indicating that it interferes with transcription at an earlier stage than RIF. Furthermore, it inhibits transcription even in mutant strain compared to RIF. Panel **(a)** was adapted from (Mosaei *et al.*, 2018) and panel **(b)** from (Peek *et al.*, 2018).

The following question is hereby posed for consideration: does Class I HelD also contribute to resistance against FDX and KANG, despite their different mechanisms of action, or in the case of FDX even distinct binding site? It can be hypothesized that RNAP inhibited by either FDX or KANG, forming a RNAP stalled complex, can be released from DNA by HelD. In the case of FDX, it remains uncertain whether HelD is capable of removing FDX from RNAP, as its

binding site differs from that of RIF. Moreover, structural analysis of the *B. subtilis* RNAP-HelD-FDX complex indicated that HelD does not reach the FDX binding site (**Figure 18**). However, our structural biology collaborators proposed HelD induced conformational opening of RNAP may alter the FDX binding site, thereby reducing or potentially abolishing FDX affinity. In contrast, HelD may be more likely to remove KANG because it binds to the same site as RIF. However, the additional interactions formed between KANG and the β subunit of RNAP could potentially reduce the efficiency of this process.

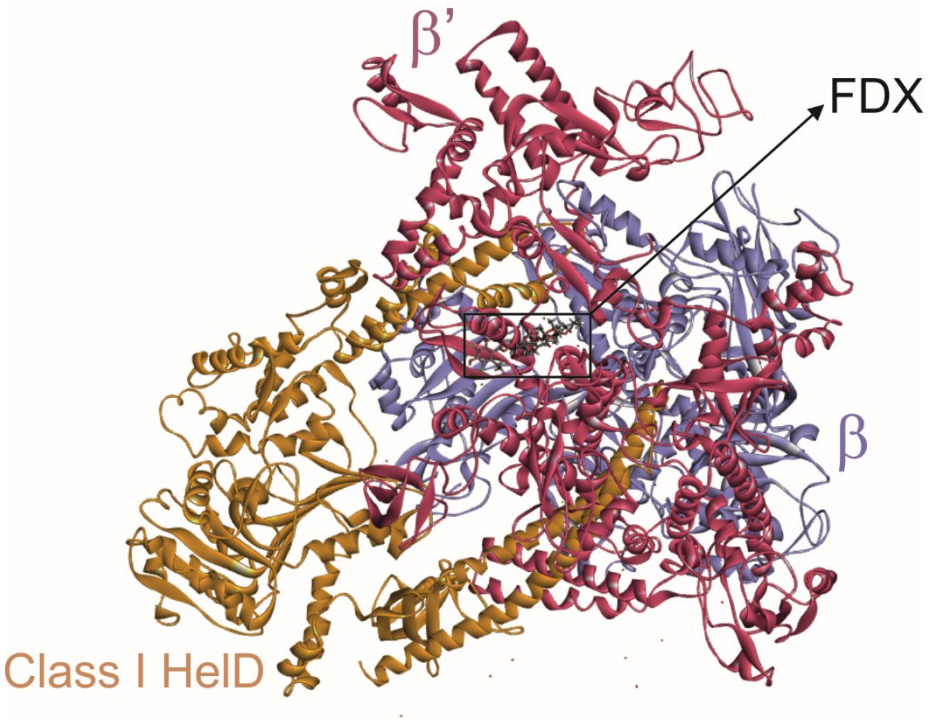

Figure 18: *B. subtilis* RNAP-HelD-FDX complex. Class I HelD does not interact with the antibiotic fidaxomicin (FDX). Purple, RNAP β; Magenta, RNAP β'; Brown, Class I HelD; Black square with an arrow indicates FDX. The model is based on the superposition of the *B. subtilis* RNAP with HelD [PDB code: 6WVK; (Newing *et al.*, 2020)] and *C. difficile* RNAP with FDX [PDB code: 7L7B; (Cao *et al.*, 2022)]. The anticipated position of fidaxomicin is taken from the *C. difficile* RNAP–FDX complex [PDB code: 7L7B; (Cao *et al.*, 2022)]. Created with BIOVIA Discovery Studio Visualizer.

Future directions

Protein HelD has been extensively studied for more than a decade and continues to surprise with unexpected results, such as its role in RIF resistance. However, there are still unanswered questions.

The regulation of *helD* gene expression by RAE (Class II) or dual-promoters (Class I) may still be missing some pieces. RAE has the potential to bind an unknown transcription factor (TF), which can regulate transcription. In the case of dual-promoters, the current model can reproduce the effect even in a minimal *in vitro* system. However, the effect is not as prominent as *in vivo*,

suggesting that another factor may be needed for regulation. Identifying these TFs can provide a more detailed explanation of how the *helD* gene is regulated by RIF. We attempted to identify a TF for Class I HelD, but we were unsuccessful due to initial difficulties in the experiment. We need to consider a different approach.

Next, we are interested in the effect of HelD against FDX and KANG. We are already working on potential role of HelD in FDX resistance. We also plan to investigate the potential of HelD in RNAP protection against KANG.

Expression of *helD* among the bacterial population can be, in principle, homogenous or heterogenous (in other words identical in all cells within the population or differing between different cells). We would like to distinguish between these two scenarios. For this purpose, we have already prepared the construct where the *helD* upstream region was fused with green fluorescent protein (GFP) to detect the expression level of *helD* in the presence or absence of RIF in *B. subtilis*. We would like to prepare a construct similar to the one used for the *pps* gene, but with a different marker. We would also like to create a dual reporter system that would allow us to observe expression in both the *helD* and *pps* genes. Future experiments are planned to address the mode of expression of these genes across the bacterial population.

A bioinformatic analysis was performed to search for convergent promoter regulation, similar to those observed for *helD* and *pps* in different organisms. This analysis revealed a number of genes that have the potential to play a role in RIF resistance. It will be interesting to observe how some high-potential candidates, possibly involved in RIF resistance, will be tested, and what kind of effect, if any, will be seen. This could also serve as a good basis for future research.

Conclusions

- Class I HelD, similarly to Class II HelD, contributes to rifampicin (RIF) resistance.
- This protection is mediated by its N-terminal domain, representing a mechanistically new mode of RNAP protection against RIF.
- Regulation of *helD* gene expression in response to RIF is governed by convergent promoters with different kinetic properties.
- An identical promoter arrangement and regulatory pattern was identified upstream of the *pps* gene (alternative name *rphT*, encoding a rifampicin phosphotransferase).
- Similar convergent promoter architecture has also been found upstream of various genes across different organisms.

Collectively, RIF resistance facilitated by Class I and Class II HelDs constitutes a distinctive and unprecedented mechanism of antibiotic resistance. The insights from this study could potentially pave the way for the development of promising new antimicrobial agents that focus not only on RNAP but also on its TFs.

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Appendix

The Appendix attached to the thesis contains two published articles, along with their respective supplementary information.

Publication I:

Sudzinová, P. †, **Knežová Balgová, T.** †, Schwarz, M., Juříková Mikesková, K., Hegrová, K., Vítovská, D., Rawat, P., Bobková, Š., Kočárková, V., Natarajan, S., Pospíšilová, D., Křenková, A., Hubálek, M., Halada, P., Barvík, I., Koval', T., Dohnálek, J., Wiedermannová, J., Šanderová, H., & Krásný, L. (2026). Bacteria sense the antibiotic rifampicin through a widespread dual-promoter based alarm system. *Nucleic Acids Research*, *54*(2). https://doi.org/10.1093/nar/gkaf1407

†The first two authors contributed equally to this work

Due to their size, Supplementary Tables S3, S4 and S5 are not included in the Appendix. They are available online on the publisher's website (https://doi.org/10.1093/nar/gkaf1407 – Nucleic Acids Research).

Publication II:

Sudzinová, P., **Knežová Balgová, T.**, Bobková, Š., Schwarz, M., & Krásný, L. (2026). Dual-promoter control of gene expression: from transcriptional fine-tuning to antibiotic resistance. *Current Opinion in Microbiology*, *91*, 102747. https://doi.org/10.1016/j.mib.2026.102747