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The effect of selected endogenous and exogenous factors on bacterial growth

Vliv vybraných endogenních a exogenních faktorů na bakteriální růst

Doctoral Thesis

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Prohlášení:

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Declaration:

I declare that I am the author of this Thesis. All literary and other sources that I have used in this Thesis are listed in References.

June 2020, Prague

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Ing. Michaela Šiková

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ABSTRACT

The growth of bacteria by binary division is a key characteristic of these organisms. This growth depends on two types of factors: endogenous and exogenous. Endogenous factors make up the molecular apparatus of cells. Among important endogenous factors belong also those involved in gene expression and its regulation. Exogenous factors are external conditions such as nutrient availability, temperature, pH, various stresses or the presence of antibacterial agents.

The main aim of my Thesis was to study the effects of selected endogenous and exogenous factors on bacterial growth. As endogenous factors I studied RNase J1 in *Bacillus subtilis* and a small RNA called Ms1 in *Mycobacterium smegmatis*, which are involved in regulation of gene expression at the transcriptional level. I showed that RNase J1 can, besides its role in RNA degradation, play a role in genome integrity by removing stalled RNA polymerase (RNAP) complexes from DNA. I further showed that Ms1 binds to the RNAP core and affects the level of RNAP in the cell. The results revealed new mechanistic aspects of the transcription apparatus and show how individual components or their combinations affect bacterial growth. As exogenous factors I studied the recently discovered antibacterial compounds, called lipophosphonoxins, their interaction with bacteria, bacterial proteins and their effect on bacterial growth.

In summary, this Thesis advances our understanding of both the factors inside as well as outside the cell on the ability of the bacterium to prosper and grow.

ABSTRAKT

Růst bakteriální kultury binárním dělením je klíčovou charakteristikou těchto organismů. Tento růst závisí na dvou typech faktorů: endogenní a exogenní. Endogenní faktory tvoří molekulární aparát buněk. Mezi důležité endogenní faktory patří rovněž ty, které se podílejí na genové expresi a její regulaci. Mezi exogenní faktory řadíme dostupnost živin, teplota, pH, různé stresy nebo přítomnost antibiotik.

Hlavním cílem mé dizertační práce bylo studium vlivů vybraných endogenních a exogenních faktorů na bakteriální růst. Jako endogenní faktory jsem studovala RNázu J1 u *Bacillus subtilis* a malou RNA zvanou Ms1 u *Mycobacterium smegmatis*, které se podílejí na regulaci genové exprese na úrovni transkripce. Ukazuji zde, že RNáza J1 může, kromě své role v degradaci RNA, hrát roli v genomové integritě odstraněním zastavených komplexů RNA polymerázy (RNAP) z DNA. Dále ukazuji, že Ms1 se váže na jádro RNAP a má vliv na množství RNAP v buňce. Výsledky odhalují nové mechanistické aspekty transkripčního aparátu a ukazují, jak jednotlivé komponenty nebo kombinace ovlivňují bakteriální růst. Jako exogenní faktory jsem studovala nedávno objevené antibakteriální látky zvané lipofosfonoxiny a jejich interakci s bakteriemi, bakteriálními proteiny a vliv na bakteriální růst.

Stručně řečeno, tato práce posouvá naše chápání obou faktorů uvnitř i vně buňky a jejich vliv na bakteriální růst.

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1. INTRODUCTION

Bacteria adapt very quickly to changes in the environment. Based on a large number of factors, the bacterial growth is accordingly regulated. This growth can be schematically represented by the growth curve. This Thesis focuses on how the bacterial growth curve is affected (i) by factors involved in gene expression at the level of transcription/RNA degradation and (ii) by antibacterial compounds. In this Thesis, I used two main model bacterial organisms: *Bacillus subtilis* and *Mycobacterium smegmatis*.

Bacillus subtilis is an aerobic, rod-shaped non-pathogenic gram-positive bacterium found in soil. In harsh conditions it forms spores. Cells divide symmetrically by binary fission or asymmetrically during spore formation (Hong et al., 2009; Kunst et al., 1997). Its original name was *Vibrio subtilis* (Ehrenberg, 1835) and in 1872 it was renamed to *Bacillus subtilis* (Cohn, 1872). *B. subtilis* has on its surface flagella which serve for moving in liquids (Hong et al., 2009). *B. subtilis* is used as a model organism for studying replication, transcription, translation, sporulation and biofilm formation. Its genome contains ~4.3 Mbp and codes for 4,353 genes, of which ~4,100 genes code proteins. The *B. subtilis* chromosome contains a low level of GC sequences (Kunst et al., 1997; Saito et al., 2009; Zhu and Stülke, 2018). *B. subtilis* is also widely used in biotechnology – antibiotics (e.g. polymyxin, subtilin, difficidin, mycobacillin), enzymes (e.g. amylases, proteases, lipases), and metabolites (*e.g.* inosin) (van Dijl and Hecker, 2013; Morikawa, 2006; Asahara et al., 2010; Coulier et al., 2019). *B subtilis* is a fast growing bacterium in rich media. Its doubling time is ~30 minutes (Gray et al., 2019).

Mycobacterium smegmatis is an aerobic, non-pathogenic, rod-shaped Gram-positive bacterium. It was discovered in 1884 by Lustgarten. Its original name was *Bacillus smegmatis*, named by Trevisan in 1889. Ten years later, it was renamed to *Mycobacterium smegmatis* by Lehmann and Neumann (Brown-Elliott and Wallace, 2002). We can find it in water, soil or on plants. Its genome contains 6.9 Mbp and codes for 6,938 genes, of which 6,716 genes encode proteins. The mycobacterial genome is GC rich, containing ~67% of GC. *M. smegmatis* belongs among relatively slowly growing bacteria (Gupta et al., 2011). The doubling time is ~2 hours. Pathogenic mycobacteria have longer doubling times, e.g. *M. tuberculosis* has ~24h, *M. leprae* has ~14 days (Stephan et al., 2005). *M. smegmatis* is also used in biotechnology. It produces sugar Xylitol which is used as a sugar substitute in the diet (Ahmed, 2001). Importantly,

knowledge obtained from studies of this organism advance also our understanding of its pathogenic relatives, such as *M. tuberculosis* (Reyrat and Kahn, 2001).

Gene expression is the process during which the information stored in DNA is transformed into 3-dimensional molecules, proteins. RNA polymerase (RNAP) is the key enzyme of the first step of gene expression, transcription, and its functioning is a critical endogenous factor of the bacterial growth. RNAP itself is then regulated by a number of other factors. One such a factor is RNase J1. I studied cellular roles of RNase J1 in *B. subtilis.* We showed that the absence of RNase J1 from the cell has significant consequences for the cell resulting in dramatic phenotypes. By ChIPseq and RNAseq, we found that RNase J1 mediates transcription termination of incomplete RNAs from stalled transcription elongation complexes by a so-called torpedo mechanism (Publication IV). Further, I studied a small RNA in *M. smegmatis*, called Ms1, and its expression and roles in the cell. Ms1 had been originally discovered as a potential homologue of 6S RNA in *E. coli* (Pánek et al., 2011). The first article about Ms1 (Publication I) addressed whether Ms1 was indeed a homologue of 6S RNA. 6S RNA is a small RNA in many bacterial species that binds to the RNAP holoenzyme in complex with the primary sigma factors and helps the cells survive prolonged stationary phase. The second article about Ms1 (Publication III) was focused on its role in *M. smegmatis* and how Ms1 is synthesized and degraded.

The other factors which regulate bacterial growth are exogenous conditions. I studied selected antibacterial compounds, called lipophosphonoxins (LPPOs) that were discovered relatively recently (Rejman et al., 2011). I searched for potential protein binding targets of LPPOs and investigated their effects on the growth of non-pathogenic *M. smegmatis* (Publication II).

Note: The order of publications (I-IV) reflects when they were finished historically. However, for a more coherent text, the following Literary review describes first RNases and then small RNAs and finishes with LPPOs.

2. LITERARY REVIEW

2.1 Bacterial growth

Bacteria divide asexually by binary fission – two cells are formed from one cell. Cells control timing and position of cell division. The division starts with initiation of DNA replication. Then, the cells are gradually extended and the genetic material is segregated. The last step is cell division. It starts when cells are ~2x longer compared with the original cell size. Then, FtsZ, a key division protein, is assembled into a ring structure in the middle of the cell together with other factors for cell division and cell membrane synthesis. Finally, the septum is formed and the two daughter cells are separated (Angert, 2005). The bacterial growth is affected by both external and internal conditions. The external conditions affecting bacterial growth include *e.g.* nutrients, temperature, pH, aeration, antimicrobial compounds. The internal conditions affecting bacterial growth consist of the cellular components that include the transcription and translation machineries (Klumpp and Hwa, 2014). The number of cells can be quantified by direct methods (microscopy, cytometry) or indirect methods (colony forming units) (Van Heerden et al., 2017). The bacterial growth is divided into four main phases (lag, log, stationary and death phase) and two transition phases between (i) lag and log phase and (ii) log and stationary phase (Monod, 1949).

2.1.1 Lag phase

When bacteria from stationary phase are moved to a fresh medium, the cells have to adapt and first they enter the lag phase (Figure 1, step 1) where they are synthesizing new proteins/RNA/metabolites that are necessary to deal with the new conditions. The length of the lag phase depends on the internal bacterial makeup that, in turn, depends on the previous external conditions (nutrients, temperature etc). The cells inoculated from exponential phase into medium with similar conditions (temperature, nutrients etc) usually do not have lag phase. When all the internal changes are made, the bacteria enter then first transient step between lag and log phases where it is possible to detect changes in optical density (Figure 1, step 2). Bacteria then begin to divide rapidly (Navarro Llorens et al., 2010; Rolfe et al., 2012).

2.1.2 Log (logarithmic) phase

This phase is often called exponential phase (Figure 1, step 3). In this phase, bacteria divide at maximum speed (depending on the media and their genetic makeup) by binary fission. Replication, transcription and translation are at a high activity level. The log phase could be extended by continual cultivation in fermentors. When nutrients are becoming depleted or the environment is becoming unfavourable to bacterial growth, bacteria stop dividing rapidly, and enter the second transient phase between log and stationary phases (Figure 1, step 4) (Monod, 1949).

2.1.3 Stationary phase

In stationary phase, the amount of bacteria is approximately constant (Figure 1, step 5). The amount of new cells equals the amount of cells that die (Monod, 1949). In this phase, secondary metabolites are produced (e.g. antibiotics, toxins) (Lazzarini et al., 2000).

2.1.4 Death

When nutrients run out or toxic/waste substances are at a high level, bacteria start dying (Figure 1, step 6). The dying occurs also in exponential rate but it is negligible relative to the number of newly formed cells (Monod, 1949).

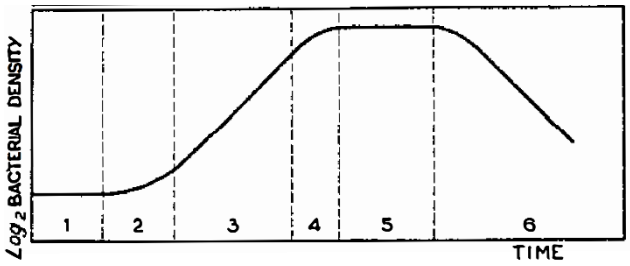

Figure 1. Bacterial growth curve. 1. Lag phase, **2.** Transition between lag and log phases, **3.** Log (exponential) phase, **4.** Transition between log and stationary phases, **5.** Stationary phase, **6.** Death. X-axis – time with growth phases indicated, y-axis shows optical density in log scale (Monod, 1949).

2.2 Endogenous factors

Bacterial growth can be regulated by the molecular machinery of the cell (transcription, translation and its regulation). Activity of RNA polymerase (RNAP, the key transcription enzyme) and amount of ribosomes (key translation units) affect bacterial growth. Activity of RNAP is regulated by factors that bind to RNAP and stimulate or inhibit its activity and affect growth (Klumpp et al., 2009; Sarkar et al., 2017). The growth rate is dependent also on the rate of protein synthesis and it depends on the number of ribosomes in the cell (Bosdriesz et al., 2015). Here, in this Review part, I describe bacterial transcription, starting with RNAP and its interactions with DNA, then I focus on proteins involved in RNA degradation, and finish by describing how small RNAs bind to, and regulate transcription.

2.2.1 Bacterial transcription

One possibility how to control bacterial growth is to control transcription (Klumpp and Hwa, 2014). Transcription is a highly regulated process where DNA is converted into RNA by RNAP. Transcription is divided into three main steps: initiation, elongation and termination. Bacteria have one type of RNAP whereas eukaryotes have three types of RNAPs – RNAPI, RNAPII and RNAPIII (Sekine et al., 2012).

2.2.1.1 RNA polymerase

Bacterial RNAP is a multi-subunit enzyme which catalyses transcription of genetic information from DNA into RNA. The RNAP core consist of five subunits: two αs, and then one of each β, β' and ω (Figure 2) (Browning and Busby, 2016). There are other subunits of RNAP depending on the bacterial species, *e.g.* δ and ε subunits in *Bacillus subtilis* (Keller et al., 2014; Rabatinová et al., 2013). Further, other factors, e.g. HelD in *Bacillus subtilis* (Wiedermannová et al., 2014),, CarD and RbpA in *Mycobacterium smegmatis* (Bae et al., 2015; Hu et al., 2014) bind to RNAP and /affect transcription. They may have both, stimulatory or inhibitory effects, depending on the promoter or phase of transcription where they act. Some factors (*e. i.* antibiotics) specifically inhibit the activity of RNAP and transcription.

Each α subunit consists of an N-terminal domain (NTD) and a C-terminal domain (CTD). Two α subunit then form a homodimer via their NTDs. The NTD homodimer allows the assembly β and β'subunits. CTDs of α subunits interact with transcription factors (Lawson et al., 2004) and/or bind to UP-elements of some bacterial promoters. These UP elements are A-T rich sequences positioned upstream of the -35 element [for definition see the next chapter] (Ross et al., 1993). β and β' subunits form the catalytic core of RNAP and its structure resembles the crab claw. The β' subunit binds to DNA and the β subunit is responsible for RNA synthesis (Paget, 2015). Between these two subunits lies a large cleft containing (i) the primary channel, where DNA enters for transcription process, (ii) the secondary channel, where NTPs enter the active site, and (iii) the RNA exit channel, where RNA separates from DNA and leaves RNAP (Lee and Borukhov, 2016). The ω subunit is the smallest subunit of RNAP and binds to the β' subunit and plays role as a chaperon in RNAP folding (Browning and Busby, 2016). The RNAP core itself cannot initiate transcription and needs another factor. This factor is the sigma factor that recognises the bacterial promoter and helps RNAP initiate transcription (Paget, 2015).

The structure of RNAP has been solved from various organisms, such as *E. coli* (Murakami, 2013), *M. smegmatis* (Hubin et al., 2017; Kouba et al., 2019), *M. tuberculosis* (Boyaci et al., 2018) and *B. subtilis* (MacDougall et al., 2005). The mycobacterial RNAP has a characteristic shape, different from that of *E. coli* in its structure of the β'subunit which forms an additional "finger" structure (Figure 2B) (Boyaci et al., 2018; Hubin et al., 2017; Kouba et al., 2019).

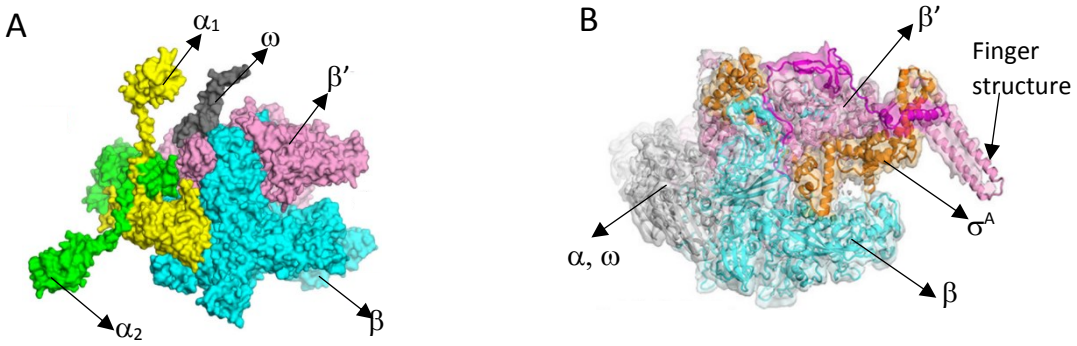

Figure 2. Structure of RNAP. **A.** *E. coli* RNAP. α_1 is yellow, α_2 is green, β is cyan, β' is pink, and ω is grey (Sutherland and Murakami, 2018). **B.** *M. tuberculosis* RNAP. α and ω are grey, β is cyan, β' is pink, σ^A is orange (Boyaci et al., 2018).

2.2.1.2 Bacterial promoter

Bacterial promoters are specific sequences where transcription starts. Here, I will describe bacterial promoters in the model organism *E. coli*, which is recognized by the housekeeping sigma factor (σ^{70}). This information is also applicable to *B. subtilis* or *M. smegmatis*. *E. coli* promoters contain the transcription start site (+1), -10 element, extended -10 element, spacer, -35 element and UP-element (Figure 3) (Browning and Busby, 2016). Transcription start site is at +1 position. Preferred initiation nucleotidtriphosphate (iNTP): ATP ≥ GTP > UTP >> CTP (Liu and Turnbough, 1994). The iNTPs are important for the transcription initiation efficiency. This region between +1 and -10 (sometimes referred to as "discriminator") plays a role in regulation of open complex stability (Bird et al., 2016; Browning and Busby, 2016; Ruff et al., 2015). The -10 element is AT-rich (consensus 5'-TATAAT-3') and typically positioned from –12 to –7. DNA unwinding (transcription bubble formation) starts in this region. The extended -10 element (TGn) at position –17 to –14 from transcription start site increases promoter activity. Spacer separates -10 and -35 sequences and its length is typically 17 bp ± 1 bp (Aoyama et al., 1983; Browning and Busby, 2016). The consensus -35 element sequence is 5'-TTGACA-3' and located from –35 to –30. The TTG sequence is highly conserved. The UP-element is an AT-rich region at position −40 to −60 from transcription start that are recognized by CTDs of α subunits and bind there (Browning and Busby, 2016; Ruff et al., 2015).

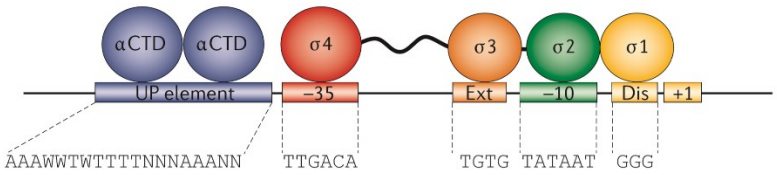

Figure 3. Structure of the primary bacterial promoter and its interaction with RNAP holoenzyme. Important bacterial promoter elements, their typical sequence and binding domain of σ factor. +1 is transcription start site, discriminator (Dis, yellow), -10 element (green), extended -10 element (Ext, orange) , -35 element (red) and UP element (blue) is located -37 to -58 from transcription start site and C-terminal domain of α subunits (αCTD) bind here (Browning and Busby, 2016).

2.2.1.3 σ-factors

RNAP requires a sigma (σ) factor to recognize the promoter. RNAP with a σ factor is called the RNAP holoenzyme. Each bacterium has one housekeeping σ factor (σ^{70} in *E. coli*, σ^{A} in *B. subtilis*, *M. smegmatis* and most other species) and varied numbers of alternative σ factors. Housekeeping σ factors are responsible for the main bulk of transcription, especially in exponential phase; alternative sigma factors are activated during changes in the environment and by stresses (Browning and Busby, 2016; Paget, 2015).

The housekeeping σ factor of *E. coli* contains four main domains (σ_1, σ_2, σ_3, σ_4). Domains σ_2, σ_3, σ_4 bind specifically to sequence elements in promoter DNA (Figure 4). Domain σ_4 and domain σ_3 are involved in the initial localisation of RNAP on DNA promoter. Domains σ_2 and $\sigma_{1.1}$ then play roles in the formation of the open complex. Alternative sigma factors lack the $\sigma_{1.1}$ domain and the presence of the σ_3 domain is variable (Figure 4A). Each sigma factor recognizes its own consensus promoter sequence (Paget, 2015).

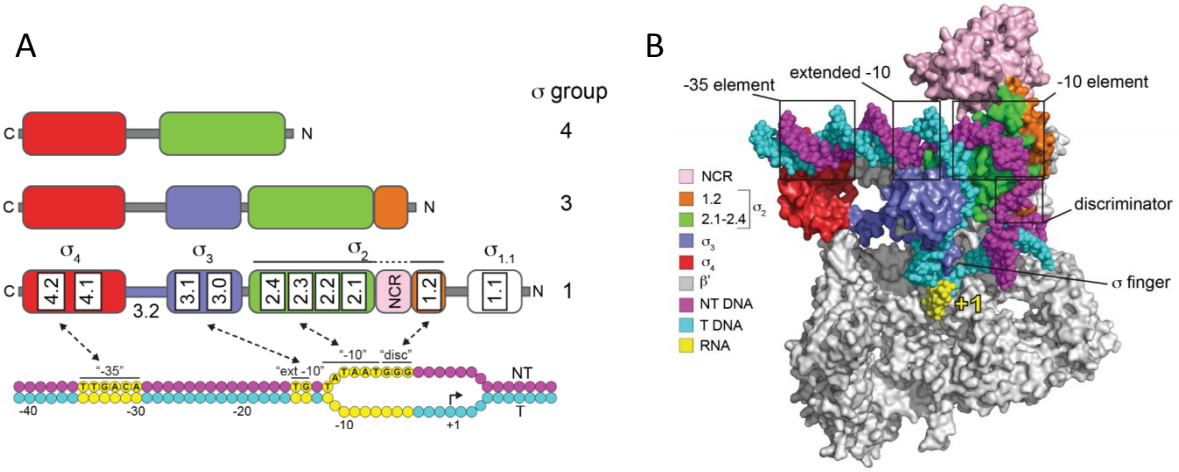

Figure 4. Domains of σ factors. A. Organization of σ factor groups (1, 3 and 4) and their domains. Conserved subdomains are shown for group 1. Domain $\sigma_{1.1}$ is white, σ_2 is green and orange, σ_3 is blue, σ_4 is red. NCR (non-conserved region) is pink; NT (non-templated DNA strand) is magenta; T (template strand) is cyan. Key promoter elements are yellow. **B.** Organization of RNAP and σ factor from *E. coli*. Domains of σ factor are colored as in A. (Paget, 2015)

2.2.1.4 Transcription initiation

Before transcription is initiated, RNAP subunits are assembled to form the RNAP core. Subsequently, a σ factor reversibly binds to the RNAP core to form an RNAP holoenzyme (Figure 5, step 1). The transcription begins by binding of RNAP holoenzyme to DNA promoter sequences. First, DNA:RNAP forms the closed promoter complex (RP_c) where DNA is still double stranded (Figure 5, step 2). Thereafter, DNA is bent around RNAP and at the -10 promoter region the transcription bubble is formed (~13 bp), creating the open promoter complex – RP_o (Figure 5, step 3) and transcription can start (Ruff et al., 2015). Transcription starts at the +1 position on template DNA by binding initiation nucleoside triphosphate (iNTP) or 2-5nt short RNA (nanoRNA), see Figure 5, step 4 (Barvík et al., 2017; Nickels and Dove, 2011). During initiation of transcription, 2-15 nt short abortive RNA can be formed *in vitro* and *in vivo* (Figure 5, step 5a). It is a process where RNAP starts with transcription, but RNA is released from the complex before RNAP leaves the promoter and reverts back to the promoter open complex state (Goldman et al., 2009; Hsu, 2008). When RNAP leaves the promoter, the σ factor is released from the complex and transcription enters its elongation phase (Figure 5, step 5b).

2.2.1.5 Transcription elongation

During elongation, the transcription bubble moves along DNA base by base, NTPs enter the active site and RNAP adds nucleotides in a template-dependent manner to the 3' end of nascent RNA. Within the transcription bubble, an RNA:DNA (8 – 9 bp) hybrid is formed. The nascent RNA is around positions -9/-10 separated from DNA by loops in RNAP. RNA then leaves RNAP through the exit channel. Finally, DNA is restored to its original double-stranded structure (Figure 5, step 6) (Vassylyev, 2009). During elongation, RNAP can encounter obstacles on DNA, e.g. DNA-binding proteins, thymine dimers or hairpins. These obstacles lead to RNAP pausing, stalling, reverse translocation or backtracking. Pausing of RNAP could be either short or longer followed by RNAP stalling. Reverse translocation and backtracking of RNAP result in backward sliding of RNAP along DNA and extra factors are required to resolve such RNAPs (Belogurov and Artsimovitch, 2015; Kireeva and Kashlev, 2009; Tornaletti and Hanawalt, 1999).

Elongation factors (Nus, Gre factors) play important roles in transcription elongation (Borukhov et al., 2005). Gre factor is one of the elongation factors. Gre factor binds to RNAP and resolves backtracked RNAP by cleavage of nascent RNA. Gre factor inserts its coiled-coil domain into the secondary channel and contacts the active site of RNAP. The conformation of the active site of RNAP is consequently changed and RNAP performs hydrolysis of RNA, creating a new 3' end of RNA positioned now in the active site (Kusuya et al., 2011; Yuzenkova and Zenkin, 2010). *E. coli* has two homologues of Gre factor, GreA and GreB. The action of the GreA factor produces 2-3nt short RNAs whereas in the case of the GreB factor the cleaved products can be 2-18nt long (Toulmé et al., 2000). NusA is an essential elongation transcription factor, which stimulates pausing and termination of transcription. NusG is another important Nus transcription elongation factor, which also stimulates pausing (Ma et al., 2015; Yakhnin et al., 2016) and mediates coupling between transcription and translation through interaction with 70S ribosomes (Saxena et al., 2018).

2.2.1.6 Transcription termination

The last step of transcription is termination (Figure 5, step 7). In bacteria, transcription termination is either Rho dependent or Rho independent (Peters et al., 2011). Rho independent termination causes dissociation of elongation complexes without the assistance of any transcription factor. It is based on a GC-rich sequence followed by a U-rich sequence (Carafa et al., 1990). The GC rich sequences in RNA forms hairpins (stem loops) followed by Us-track (7-9nt long), causing slowdown/pause of RNAP and, finally, RNA is released from the transcription complex. Approximately 50% of transcripts terminate with this mechanism (Larson et al., 2008).

The second type of termination requires the presence of the Rho protein. The Rho protein forms homohexameric ring protein and it is conserved in most bacterial species. The Rho protein binds to C-rich unstructured RNA, called *rut* (**R**ho **UT**ilization) sites, and translocates towards the elongation complex. Transcription termination occurs after the Rho protein makes contact with the RNA exit channel of RNAP. Finally, the DNA:RNA hybrid is unwound and RNA is released (Mitra et al., 2017).

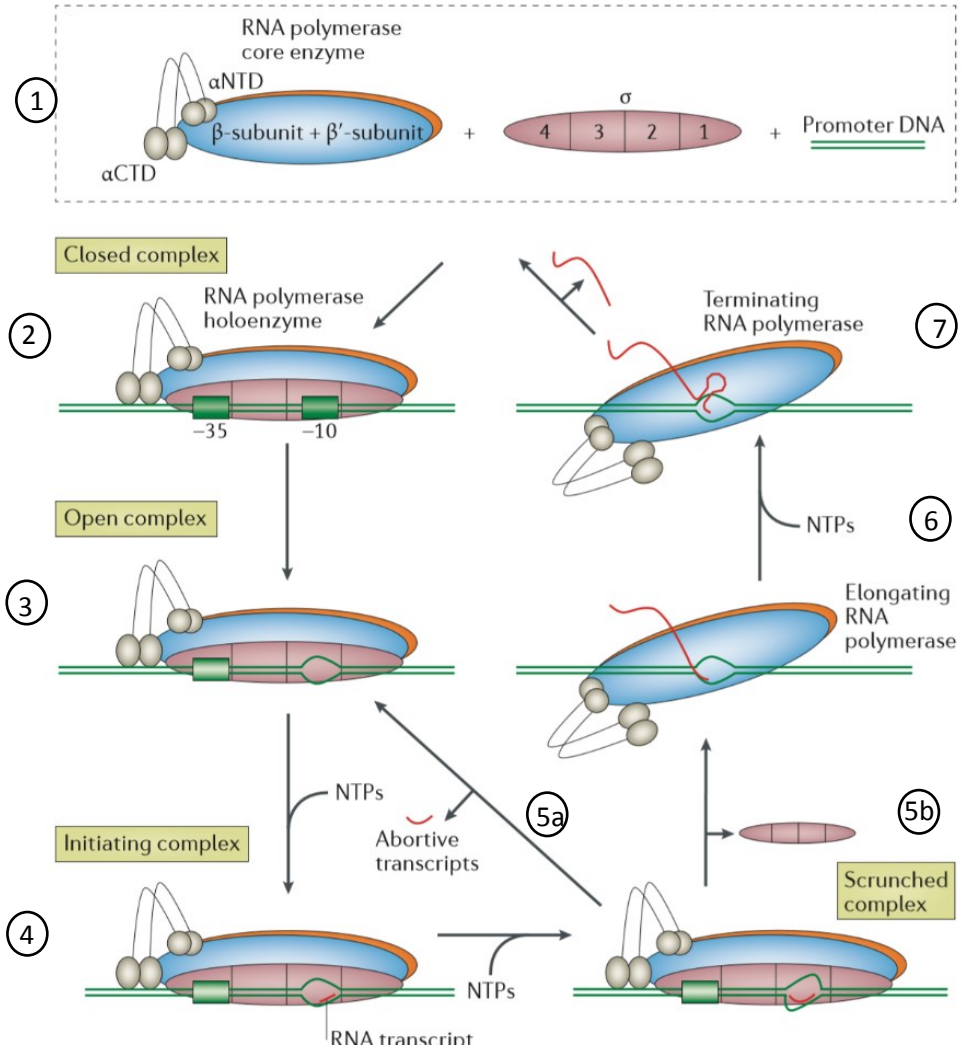

Figure 5. Overview of bacterial transcription. (1) σ factor binds to RNAP and forms RNAP holoenzyme. (2) RNAP holoenzyme recognizes and forms the closed complex. (3) -10 element is then unwound and the open complex is formed. (4) In the presence of NTPs, transcription starts. During transcription initiation, (5a) abortive transcripts can be formed (the transcription complex is held at the promoter) or (5b) RNAP escapes from the promoter, σ factor is released and (6) RNAP enters into elongation phase. RNAP transcribes until it reaches the terminator. (7) Finally, RNAP is released from DNA and is available for another transcription cycle (Browning and Busby, 2016).

2.2.2 RNA degradation

Posttranscriptional regulation of RNA plays important roles in gene expression. In bacteria, the life-time of mRNAs is typically short (from seconds to one hour); rRNA and tRNA are more stable. This low stability of mRNA helps bacteria adapt to environmental changes and control the correct amount of the respective protein. Degradation of RNA is highly regulated. Cells also use a variety mechanisms to protect RNAs against to RNases (Deutscher, 2015; Laalami et al., 2014).

RNA is degraded by enzymes called ribonucleases (RNases). RNases cleave phosphodiester linkages in RNA. Based on the mechanism of action, RNases are divided into two main classes (i) endoribonucleases (cleavage within RNA) and (ii) exoribonucleases (cleavage from the 3' or 5' end of RNA). Each bacterium species contains many RNases with various modes of action and different bacterial species contains different sets of RNases. An example are *B. subtilis* and *E. coli* that each contain ~40 RNases but only nine RNases are found in both organisms. Except for mRNA degradation, RNases play roles also in tRNA, rRNA and sRNA processing (Durand and Condon, 2018; Trinquier et al., 2020). Here, I focus on major bacterial RNases in model microorganisms – *E. coli* and *B. subtilis*.

2.2.2.1 Endoribonucleases

Endonucleases are enzymes that cleave phosphodiester bonds inside the RNA chain. The result are two shorter fragments of RNA. The cleavage creates a new 3'OH end on one fragment and 5'monophosphate on the other fragment. The first fragment with the free -OH group at the 3'end can be degraded by an exoribonuclease with a 3'→5' activity (*e. g.* PNPase, RNase R). It is assumed that endonucleases are major RNases that start the degradation of RNA. The "job" is then finished by exonucleases. Moreover, most bacterial mRNAs contain at their 5'ends the triphosphate group or another type of cap – *e. g.* NAD (Bird et al., 2016; Cahová et al., 2015), Np4N (Hudeček et al., 2020; Luciano and Belasco, 2020; Luciano et al., 2019), Co-enzyme A (Kowtoniuk et al., 2009) and on 3'end hairpins (stem-loops) (Trinquier et al., 2020) and these molecules/structures affect the stability of RNA.

RNase E

RNase E is the main RNase involved in degradation of mRNA in *E. coli*. It is an essential RNase, encoded by the *rne* gene, discovered in 5S rRNA processing (Apirion and Lassar, 1978). RNase E binds to the inner membrane and thus is protected against degradation (Hadjeras et al., 2019). RNase E forms a tetramer. The monomer of RNase E (1061 aminoacids long) contains two main domain – N-terminal domain (aminoacids 1–529) with catalytic function and C-terminal domain (aminoacids 530-1061), which forms a scaffold for RNA degradosome assembly. N-terminal domain contains large (first 400 aminoacids) and small subdomains (aminoacids 415-529) that are connected via Zn^{2+} ions (Figure 6AB). The large domain contains the active site and a 5'phosphate binding pocket (5'PBP), which lies at the entrance to the RNA-binding channel near the active site (Callaghan et al., 2005). Generally, RNase E prefers 5'monophosphorylated RNA and single stranded AU rich sequences to initiate mRNA decay (McDowall et al., 1994).

RNase E cleaves mRNA by two mechanisms. The first mechanism of cleavage is based on binding of the 5'monophosphorylated end of mRNA into the 5'PBP within one large domain and the cleavage is in the active site of the second large domain (Figure 6C) (Laalami et al., 2014; Mackie, 2013). mRNAs usually contain triphosphates at their 5'ends. The monophosphate at the 5'end is formed by cleavage with RppH. RppH is an RNA 5´ pyrophosphohydrolase that removes pyrophosphate from the 5´ end of triphosphorylated RNA (Deana et al., 2008). The second mechanism of cleavage is based on direct entry. In this case, RNase E cleaves mRNA without binding to the 5'PBP (Figure 6D). The second mechanism is probably the main way for initiation of mRNA degradation (Kime et al., 2010; Mackie, 2013). This mechanism is used also for tRNA processing (Li et al., 1999) and 16S rRNA maturation (Mudd and Higgins, 1993). The mRNA stability increases five-fold upon depletion of RNase E (from 2.5 to 10 minutes) (Babitzke and Kushner, 1991).

RNase E also degrades sRNAs such as CsrB and CsrC (Suzuki et al., 2006). Besides the mRNA degradation function, RNase E possesses autoregulatory function. If cells contain high level of the rne mRNA, the mRNA is degraded by RNase E itself (Mudd and Higgins, 1993).

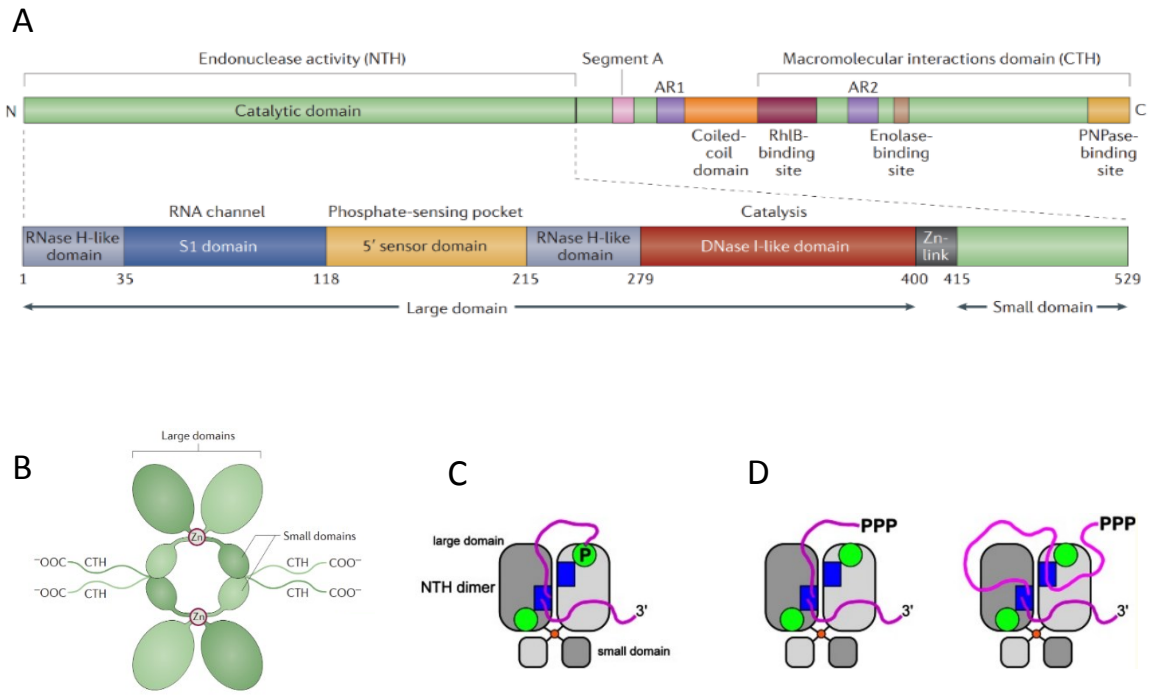

Figure 6. Structure and function of RNase E. A. Structure of RNase E monomer domain. **B.** Tetramer of RNase E composed of two dimers (Mackie, 2013). **C**. and **D.** represent mechanism of action. **C.** Cleavage based on binding of 5'monophosphorylated end. The 5'monophosphorylated end of mRNA (violet) binds to the 5'PBP (P, green circle) of one large domain and the cleavage is in the active site (blue rectangle) of the second domain. **D.** Direct cleavage. mRNA is recognized by RNase E base on sequence and binds to active sites (blue rectangle) of large domain (Laalami et al., 2014).

RNase Y

RNase Y is the main endoribonuclease in *B. subtilis* (Shahbabian et al., 2009) and is encoded by the *rny* gene. Similarly to RNase E, RNase Y binds to the membrane but it is not an essential gene. It contains five domains – transmembrane domain, coiled-coil domain, KH domain, HD domain and C-terminal domain (Figure 7). Transmembrane domain is the N-terminal domain that binds RNase Y to the membrane. This part of RNase Y is the essential part for its activity. The coiled-coil domain mediates interaction between RNase Y and other proteins. These two domains play a key role in dimerization of RNase Y. The KH domain binds RNA and the HD domain contains the catalytic site. RNase Y cleaves single-stranded AU rich sequences and its activity is stimulated by 5'monophosphorylated RNA ends but this feature is not essential for

degradation by RNase Y (Durand and Condon, 2018; Lehnik-Habrink et al., 2011; Shahbabian et al., 2009).

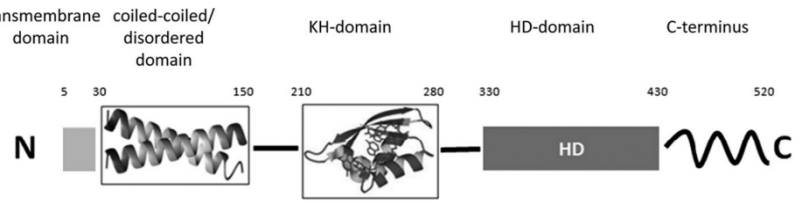

Figure 7. Structure of RNase Y. (Lehnik-Habrink et al., 2011)

Based on transcriptomic data, RNase Y affects ~1100 genes (Durand et al., 2012). The mutant strain grows more slowly, displays reduced sporulation and competence and moderately altered cell morphology (Figure 8) (Figaro et al., 2013). RNase Y is also involved in RNase P RNA and small cytoplasmic RNA (scRNA) maturation (Gilet et al., 2015). In *Staphylococcus aureus*, *Streptococcus pyogenes* and *Clostridium perfringens*, RNase Y has effects on virulence (Chen et al., 2013; Marincola et al., 2012; Obana et al., 2017).

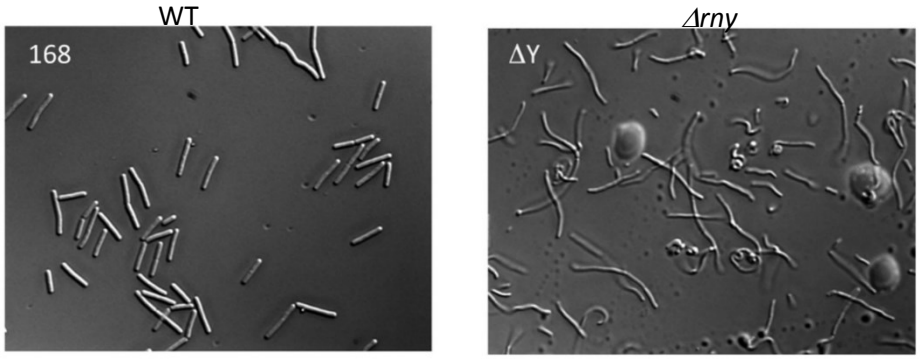

Figure 8. Cell morphology of RNase Y. Light microscopy images of the wild type (WT, left panel) and Δ*rny* (right panel) strain (Figaro et al., 2013).

2.2.2.2 Exoribonucleases

Generally, all bacteria have exoribonucleases with 3' → 5' activities such as PNPase, RNase R. For a long time, exoribonuclease with a 5'→3' activity was unknown in bacteria. First 5'→3' exoribonuclease was discovered in *B. subtilis* (Even et al., 2005; Mathy et al., 2007). Recently, a 5'→3' exoribonuclease was discovered also in *E. coli* (Ghodge and Raushel, 2015; Jain, 2020). In the following text I will describe RNase J1 in from *B. subtilis*.

RNase J1

RNase J1 was the first discovered RNase with the 5' to 3' exoribonuclease activity in *B. subtilis*. Moreover, RNase J1 has also an endoribonuclease activity although this activity was so far detected only *in vitro*. GC rich gram-positive bacteria have usually one RNase J whereas low-GC gram-positive bacteria have usually two paralogues (Durand and Condon, 2018; Even et al., 2005; Mathy et al., 2007). RNase J1 is encoded by the *rnjA* gene. RNase J1 contains three domains: β-lactamase, β-CASP and C-terminal domain. The active site is located between the β-lactamase and β-CASP domains and contains two Zn^{2+} ions (Figure 9) (De La Sierra-Gallay et al., 2008).

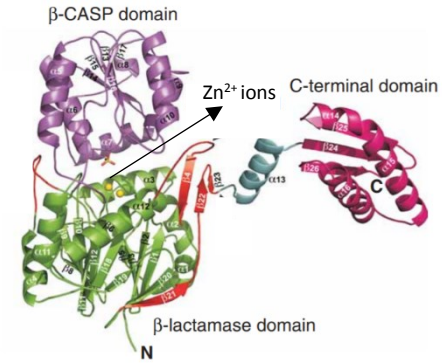

Figure 9. Structure of RNase J monomer. β-CASP domain is violet, β-lactamase domain is green, C-terminal domain is magenta. The active site composed of two Zn^{2+} ions are shown by yellow spheres (De La Sierra-Gallay et al., 2008).

RNase J1 forms a stable heterotetramer with its paralogue RNase J2. In contrast to RNase J1, RNase J2 alone has a weaker exonuclease activity (Mathy et al., 2010). RNase J1 also interacts with RNase Y, PnpA (polynucleotide phosphorylase) and PfkA (phosphofructokinase) (Commichau et al., 2009; Lehnik-Habrink et al., 2011). Previously it was though that RNase J1 is essential, and the first experiments that addressed its effect on

the trancriptome were done with a depleted-RNase J1 strain. Depletion of RNase J1 had an effect on ~21% of mRNAs (Durand et al., 2012). In 2013, it was published that both RNases (RNase J1 and J2) are not essential in *B. subtilis*. A strain with lacking RNase J1 has an altered cell morphology (Figure 10), grows slower, is cold sensitive and has problems to sporulate. Deletion of RNase J2 does not have an effect on growth rate (Figaro et al., 2013).

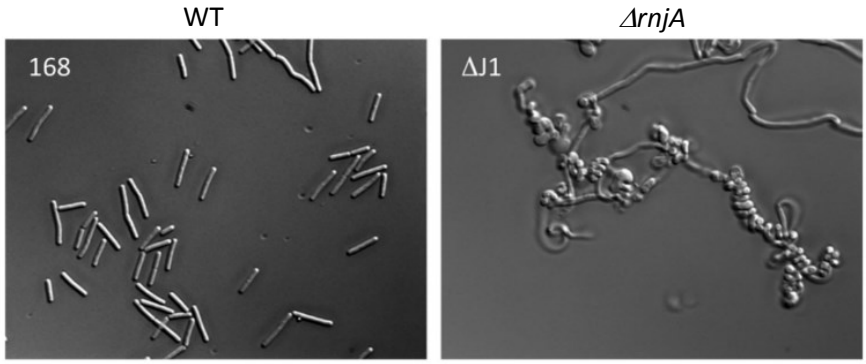

Figure 10. Cell morphology of RNase J1. Light microscopy images of the wild type (WT, left panel) and Δ*rnjA* (right panel) strain (Figaro et al., 2013).

RNase J1 has a 5'monophosphate-binding pocket for binding RNA with monophosphate or -OH group at the 5'end. Triphosphate at the 5'end blocks RNase J1-dependent degradation of RNA (Mathy et al., 2007). RNase J1 can degrade mRNA via two different mechanisms. The first possible mechanism is based on mRNA degradation after mRNA cleavage with an endonuclease RNase Y. The cleaved mRNA with RNase Y has one mRNA fragment with 3'OH end which is degraded by 3'→5' exoribonuclease (e.g. PNPase), and one mRNA fragment with 5'monophosphate end that could be cleaved by RNase J1 with 5' to 3' direction (Figure 11) (Daou-Chabo et al., 2009; Yao and Bechhofer, 2010). The second possible mechanism is direct degradation of mRNA. RNase J1 prefers a 5' monophosphorylated end in mRNA for its activity. The 5' triphosphate is cleaved with RppH (Deana et al., 2008). Then, RNase J1 is bound to 5'monophosphate end of mRNA and cleaves mRNA with its 5' to 3' direction (Figure 11) (Richards et al., 2011). RNase J1 has processive cleavage on long RNAs and distributive cleavage on RNA ≤5 nucleotides (Dorléans et al.,

2011). In addition, RNase J1 also affects the 5’ end of 16S rRNA during its maturation (Britton et al., 2007).

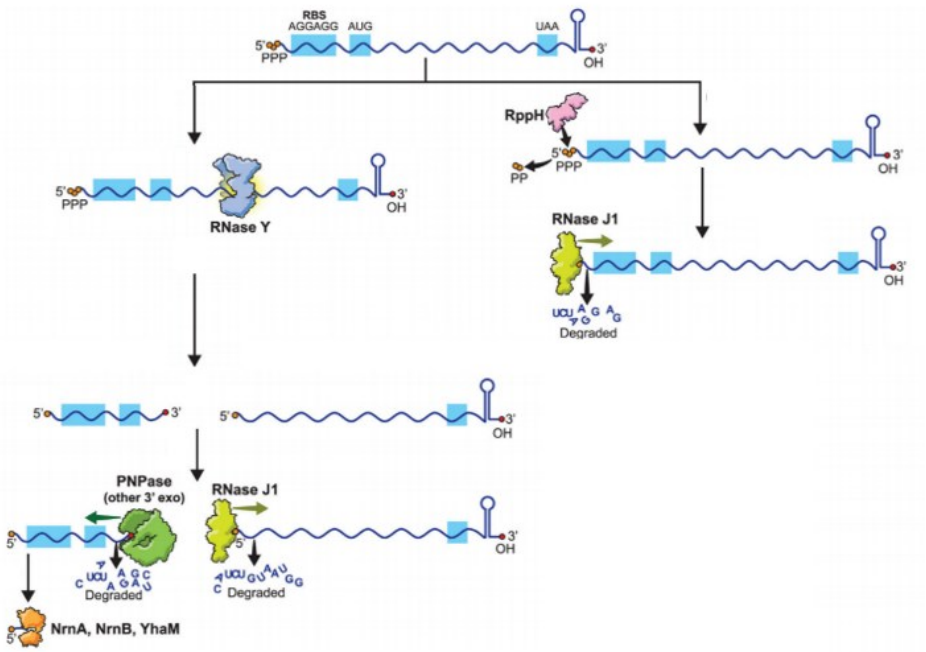

Figure 11. Mechanism of mRNA degradation by RNase J1. A. mRNA is endonucleotically cleaved by RNase Y. Two mRNA fragments are degraded by exonuclease with either 3’ to 5’ direction or 5’ to 3’ direction (RNase J1). **B.** 5’triphosphate is cleaved by RppH and RNase J1 is bound to 5’monophosphate and degrade mRNA with 5’ to 3’ direction (Bechhofer and Deutscher, 2019).

RNase J in other bacteria

RNase J homologs were found also in *e.g. S. aureus*, *S. pyogenes*, *S. mutans*, *E. faecalis*, *M. smegmatis*, archea. In *S. aureus*, RNase J1 and J2 are also not essential but deletion of RNases has an effect on the growth rate (Linder et al., 2014). In contrast to *B. subtilis* RNase J1, *S. aureus* RNase J1 degrades RNA with a 5’triphosphate (Hausmann et al., 2017). *S. pyogenes* RNase J1 and RNase J2 are essential. It was shown that RNase J2 *in S. pyogenes i*s more expressed than RNase J1 and depletion of RNase J2 had an effect on mRNA stability (Bugrysheva and Scott, 2010).

2.2.3 Small RNAs

Previously, RNA was considered only as an intermediate between DNA and proteins. During the past ~20 years, it was discovered that RNA has also regulatory, structural and catalytic roles (Gimpel and Brantl, 2017; Morris and Mattick, 2014; Raina et al., 2018). This group of RNA includes small RNAs (sRNAs). sRNAs play a key role in gene expression. sRNAs regulate cellular processes, *e.g.* virulence, biofilm formation and also generally adaptation to changes in environment. sRNAs are typically ~50 - 500 nucleotides long. They are transcribed by RNAP and processed by endoribonucleases (Kim and Lee, 2004). Based on the mechanism of their action, sRNAs could be classified into three main classes: (i) sRNA interacting with RNA, (ii) sRNA interacting with protein and (iii) sRNA with intrinsic function *e.g.* catalytic activity (Desgranges et al., 2020; Wassarman, 2018).

2.2.3.1 sRNA interacting with RNA

These types of sRNAs are pairing with their target mRNAs and regulate translation or stability of the target mRNA. Frequently, proteins are also involved in this process. Examples of these proteins are Hfq or ProQ that function as chaperones involved in sRNA stability (Desgranges et al., 2020). This group of sRNAs is divided into two classes: *cis*- and *trans*- encoded sRNAs.

Cis encoded sRNA

Cis encoded sRNAs are RNAs which bind fully complementarily to their target mRNAs. They are encoded within the same region of DNA but transcribed from the opposite strand (Figure 12A). Such a sRNA was discovered in the ColE1 plasmid in *E. coli* (Brantl, 2007; Tomizawa et al., 1981). We can find them also on transposons, phages and bacterial chromosomes. *Cis* encoded sRNAs control replication by inhibition of replication primers (e.g. ColE1), transcription by premature transcription attenuation by forming terminator hairpins (e.g. pT181), mRNA degradation (e.g. antitoxin/toxin RatA/*txpA*) and translation by blocking of ribosome binding sites (e.g. pLS1, antitoxin/toxin SymR/*symE* or Sof/*gef*). Most *cis*-encoded sRNAs are expressed constitutively (Brantl, 2007; Brantl, 2012).

Trans encoded sRNA

The second type of sRNA interacting with RNA are *trans* encoded sRNAs. In contrast to cis encoded sRNA, trans encoded sRNAs have partial complementarity to their target mRNAs (Figure 12B). The first *trans*-encoded sRNA was discovered in *E. coli* by Mizuno et al., 1984. It regulates translation by binding to ribosome binding sites (RBS) or near to RBS and inhibits translation or activates translation by unwinding the double stranded structure containing the RBS. Thus, RBS is then available for binding of small ribosomal subunit. *Trans* encoded sRNA has also an effect on mRNA stability. One *trans* encoded sRNA is usually targets more than one specific mRNA. Base pairing between a sRNA and its target mRNA is usually 10-25 nucleotides long. RNA chaperons, such as Hfq or ProQ, are required for binding sRNA to mRNA and protect sRNA from degradation. Most trans encoded sRNAs are expressed under specific growth conditions, *e.g.* under oxidative stress (OxyR/OxyS), outer membrane stress (σE/MicA and RybB) or changes in glucose concentration (CRP/CyaR) in *E. coli* (Brantl, 2007).

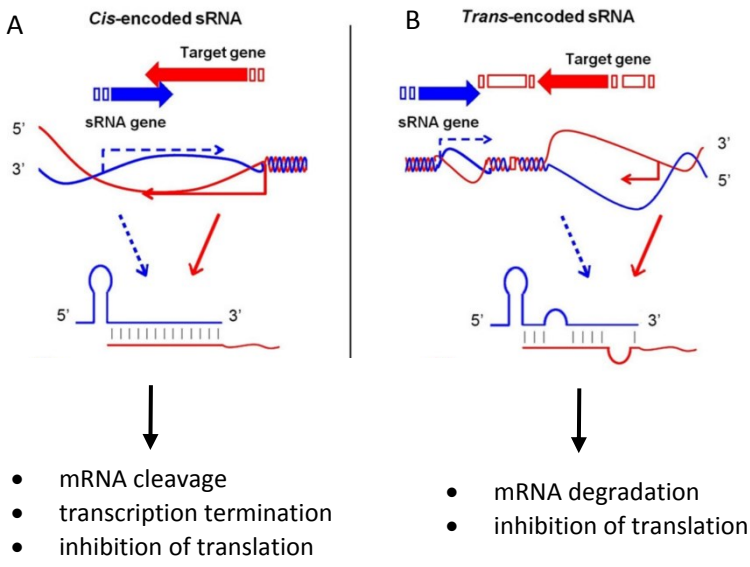

Figure 12. *Cis*- and *trans*-encoded sRNAs. Illustration of **A.** *cis*- and **B.** *trans*-encoded sRNA and their interaction with target mRNA (Li et al., 2012).

2.2.3.2 sRNA interacting with protein

6S RNA

In the 1960s, the first sRNA was discovered in *E. coli*, named 6S RNA (Hindley, 1967). It is a highly abundant and stable RNA. 6S RNA is a ~190 nt long RNA. It is expressed throughout growth but its expression is especially high in stationary phase (~10,000 copies per cell in stationary phase, ~1000 copies per cell in exponential phase, Figure 13) (Wassarman and Storz, 2000).

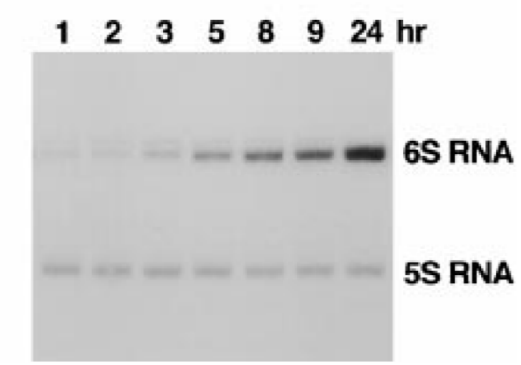

Figure 13. Expression of 6S RNA. Total RNA was isolated from *E. coli* at different time points and analysed by Northern blotting using primers against 6S RNA or 5S RNA (Wassarman and Storz, 2000).

The function of 6S RNA had been unknown for a long time. Thirty years after the discovery of 6S RNA, it was found that 6S RNA binds to the RNAP holoenzyme with the housekeeping σ factor and regulates transcription. 6S RNA inhibits many σ^{70} dependent genes whereas transcription of some σ^{S} dependent genes is stimulated (Chen et al., 2017; Wassarman and Storz, 2000).

6S RNA is transcribed from two promoters. One promoter is σ^{70} dependent (P1), the second promoter is σ^{70} and σ^{S} dependent (P2). Interestingly, the subsequent 6S RNA processing depends also on the promoter from which it is transcribed. If 6S RNA is transcribed from the P1 promoter, it is processed by RNase E and RNase G. In contrast to the P1 promoter, 6S RNA transcribed from the P2 promoter is processed by RNase E (Kim and Lee, 2004).

The reason why 6S RNA plays a role in the regulation of transcription is its structure. 6S RNA is a double stranded RNA with internal an ~15nt single stranded bubble (Figure 14A). This internal bubble imitates the transcription bubble, reminiscent of the transcription open

complex on B-DNA where the RNAP holoenzyme binds and initiates transcription (Figure 14BCD) (Chen et al., 2017; Wassarman and Storz, 2000). Region 4.2 of the σ^{70} factor is important for binding RNAP-σ to 6S RNA (Cavanagh et al., 2008). In *E. coli*, 6S RNA increases cell survival in late stationary phase. After 3 days of growth, cells with deletion of the 6S RNA gene decrease the number of cells by ~70% compared to wild type (wt) strain (Trotochaud and Wassarman, 2004). RNA-seq data showed that 6S RNA has an effect on more than 400 genes in the whole growth. Surprisingly, 6S RNA has an effect also on the genes encoding the main subunits of RNAP (Lal et al., 2018).

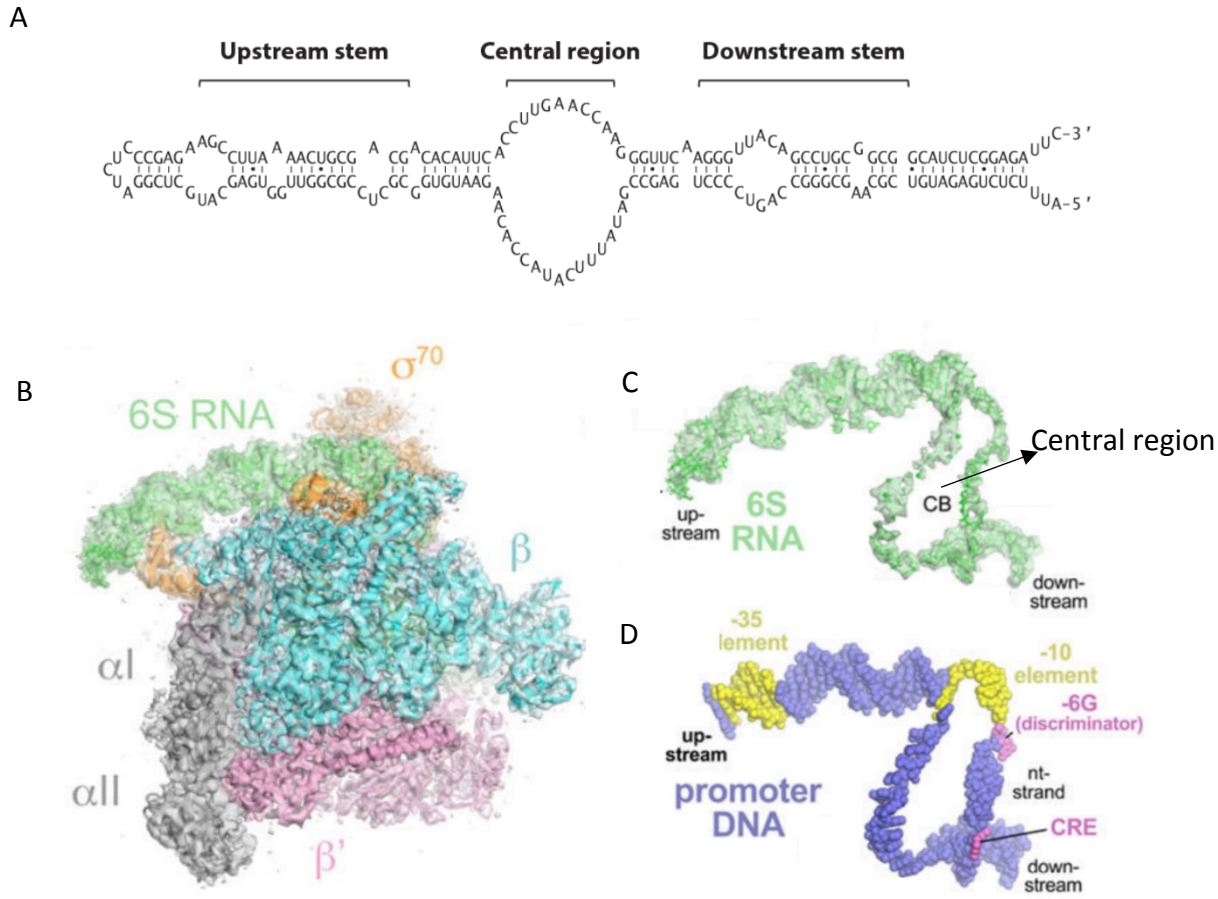

Figure 14. Structure of 6S RNA in *E. coli*. A. Secondary structure of 6S RNA (Cavanagh and Wassarman, 2014). **B.** The 3.8 Å resolution cryoEM density map of 6S RNA with RNAP holoenzyme. **C.** The 3.8-Å resolution cryoEM density map of 6S RNA and **D.** promoter DNA. αI, αII, β and β' are main subunits of RNAP; blue color represents promoter DNA, yellow color represents -35 and -10 elements, violet color represents 6G of the discriminator and Core Recognition Element (CRE) (Chen et al., 2017).

6S RNA as a template for RNAP

In addition, 6S RNA is used as a template for 'product' RNA (pRNA) synthesis (Figure 15). During transition from exponential phase to stationary phase, the 6S RNA level is raised, free RNAP holoenzyme is bound to 6S RNA and transcription of most σ^{70} dependent genes is inhibited. During outgrowth (transition from stationary phase to exponential phase), when nutrients are plentiful again and concentrations of NTPs in the cell are increased, RNAP transcribes ~12-14 nt short pRNA from 6S RNA. Synthesis of pRNA leads to a conformational change in the secondary structure of 6S RNA – the central bubble is extended and a hairpain is formed within 3'-portion of the central bubble. This conformational change leads to the release of the σ factor and subsequently the RNAP core. The 6S RNA-pRNA complex could play a role in degradation of 6S RNA. The pRNA level is the highest level during three to four minutes after reinoculation into fresh medium (Nitzan et al., 2014; Panchapakesan and Unrau, 2012; Wassarman and Saecker, 2006).

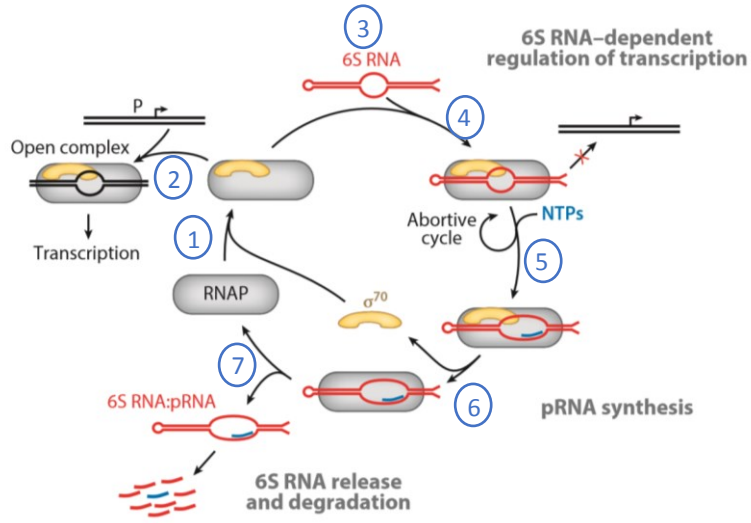

Figure 15. Model of 6S RNA and pRNA synthesis. (1) formation of RNAP-σ complex, (2) transcription of genes in exponential phase, (3) expression of 6S RNA in transition into stationary phase, (4) RNAP-σ complex binds to 6S RNA, (5) in outgrowth, pRNA is synthesized, (6) σ-factor is released from complex because of secondary structure changes, (7) RNAP is released for a new transcription (Cavanagh and Wassarman, 2014).

6S RNA in other bacteria

The secondary structure of 6S RNA is conserved among bacteria (Trotochaud and Wassarman, 2005). Based on this conserved structure, homologues of 6S RNA were found in other pathogenic and non-pathogenic bacteria (Barrick et al., 2005; Pánek et al., 2011), e.g. *Bordetella pertussis* (Trotochaud and Wassarman, 2005), *Streptomyces coelicolor* (Pánek et al., 2011), *Helicobacter pilori* (Sharma et al., 2010), *Yersinia pestis* (Yan et al., 2013), *Borelia burgdoferi* (Drecktrah et al., 2020). Some bacteria have two 6S RNAs, e.g. *B. subtilis* (Trotochaud and Wassarman, 2005), *Clostridium acetobutylicum*, *Bacillus halodurans* (Barrick et al., 2005), *Legionella pneumophila* (Weissenmayer et al., 2011). In contrast to 6S RNA accumulation in non-pathogenic bacteria, 6S RNA in pathogenic bacteria accumulates under stress and could play roles in pathogenesis or help the pathogens survive in host cells (Wassarman, 2018).

In *B. subtilis* contains two types of 6S RNA. Both 6S RNAs (6S-1 and 6S-2 RNA) bind to the RNAP holoenzyme and are templates for pRNAs like 6S RNA in *E. coli*. However, there are differences in pRNA synthesis between *E. coli* and *B. subtilis*. In *E. coli*, pRNA synthesis does not have a preference for its iNTP. In *B. subtilis*, pRNA synthesis needs specific iNTPs. 6S-1 RNA prefers iGTP whereas 6S-2 RNA prefers iATP (Cabrera-Ostertag et al., 2013). In addition, rearrangement of the central bubble of 6S RNA is also different during pRNA synthesis. *B. subtilis* RNAP is released from 6S RNA by a central bubble collapse (Beckmann et al., 2012; Steuten et al., 2014) whereas *E.coli* RNAP is released from 6S RNA by a hairpin formation in the 3'-portion of the central bubble (Panchapakesan and Unrau, 2012). Furthermore, 6S-1 and 6S-2 RNA are expressed in different growth phases (Trotochaud and Wassarman, 2005). 6S-1 RNA is expressed mainly in stationary phase, whereas 6S-2 RNA is expressed over the whole growth but its level is increased in early and mid-exponential growth (Burenina et al., 2014). 6S-1 RNA regulates timing of sporulation. Cells with deletion of 6S-1 RNA sporulate earlier compare to wt strain – after 20h of growth wt strain had ~15% fewer spores compared to strains that lack 6S-1 RNA (Cavanagh and Wassarman, 2013). Several proteins were deregulated in the single (6S-1 or 6S-22 RNA) or double 6S RNA mutant in *B. subtilis* PY79. It suggests that 6S RNAs are have effect on metabolic function and stress response (Hoch et al., 2015).

Ms1

Ms1 was originally discovered as a potential homologue of 6S RNA by *in silico* modelling in *M. smegmatis*. Its predicted secondary structure is similar to 6S RNA – double stranded RNA with a single stranded internal bubble, which mimics the open transcription complex (Pánek et al., 2011). Ms1 was also found in *M. tuberculosis* (named MTS2823) where it is expressed especially in stationary phase (Figure 16). Overexpression of MTS2823 in exponential phase has moderate effect on cell growth rate and affects 304 genes. Importantly, MTS2823 was found to be expressed in *M. tuberculosis* in chronically infected lung tissue (Arnvig et al., 2011).

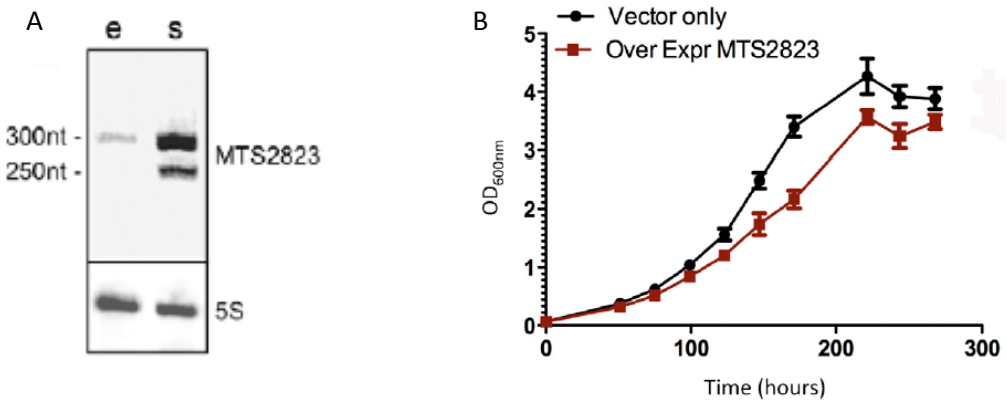

Figure 16. Characterization of MTS2823. A. Expression of MTS2823. **B.** growth rate of overexpressed MTS2823 in exponential phase and strain with empty vector. e – exponential phase, s – stationary phase (Arnvig et al., 2011).

CsrB

Carbon storage regulator A (CsrA) is an RNA binding protein first discovered in *E. coli* (Liu et al., 1997) and its level increases during transition into stationary phase. CsrA regulates carbohydrate metabolism, biofilm formation, motility and virulence (Potts et al., 2018). CsrA binds to GGA sequences at 5' untranslated region (5'UTR) of target mRNA and (i) inhibits translation by binding to Shine Dalgarno sequences which are blocked for binding to the small ribosomal subunit; (ii) activates translation by changing RNA structure; (iii) support Rho-dependent transcription termination where CsrA binding prevents hairpin formation; or (iv) protects RNA from degradation by RNase E. Purification of recombinant CsrA protein via

affinity chromatography showed that it was pulled down with 350 nt long CsrB RNA. CsrB is a sRNA which regulates carbon utilization by controlling the activity of CsrA. CsrB contains 22 GGA motifs (Figure 17) where CsrA proteins are bound and forms ribonucleoprotein (Liu et al., 1997; Potts et al., 2018).

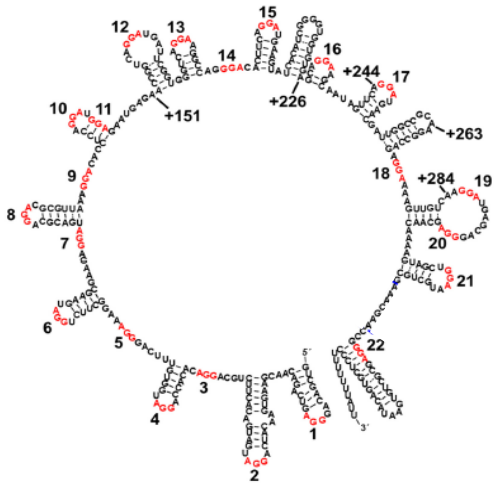

Figure 17. Structure of CsrB. Predicted structure of CsrB. Binding sites for CsrA are marked with red color (Potts et al., 2018).

tmRNA

Transfer messenger RNA (tmRNA, originally called 10S RNA) is a small RNA (~325 – 400nt long) with mRNA and tRNA functions. tmRNA controls protein synthesis and rescues stalled ribosomes (Gueneau de Novoa, 2004). Similarly to tRNA, tmRNA is processed by RNase P and has typical base modifications (Gimple and Schoön, 2001). In contrast to tRNA, tmRNA does not have an anticodon. tmRNA is aminoacylated with alanine and it binds to elongation factor Tu (EF-Tu) (Keiler and Ramadoss, 2011). This complex then binds to the A site of the stalled ribosome, subsequently peptidyl -Ala-tmRNA is translocated to the P-site. Finally, 11 alanines are added to the C-terminus of the truncated protein (Figure 18). The poly-Alanine end of mRNA is a signal for proteases to degrade the tagged protein (Janssen and Hayes, 2012).

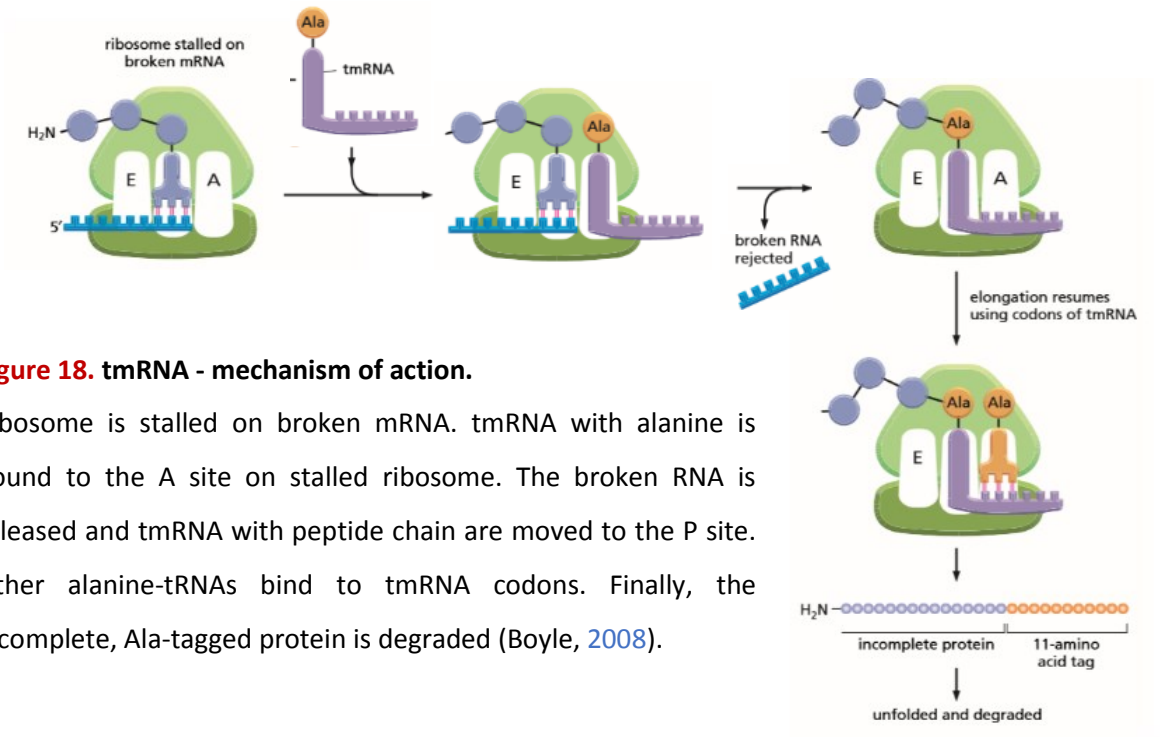

Figure 18. tmRNA - mechanism of action.

Ribosome is stalled on broken mRNA. tmRNA with alanine is bound to the A site on stalled ribosome. The broken RNA is released and tmRNA with peptide chain are moved to the P site. Other alanine-tRNAs bind to tmRNA codons. Finally, the incomplete, Ala-tagged protein is degraded (Boyle, 2008).

2.3 Exogenous factors

Exogenous factors, such as nutrients, temperature, aeration, nutrients, antibacterial compounds have profound effects on bacterial growth. Different bacteria need different conditions for their optimal growth. The most important nutrients are carbon, hydrogen, nitrogen and oxygen. These compounds are parts of macromolecules, such as nucleotides, amino acids and sugars. Temperature also plays a role in bacterial growth. Many bacteria grow ideally at 37°C. However, there are bacteria growing well under 20°C and other bacteria thriving at temperatures vastly exceeding 40 °C and some archea prospering even at temperatures around 100 °C (Farrell and Rose, 1967). Generally, lower temperature than the ideal slow growth, higher temperature can ultimately destroy bacteria. An important group of exogenous factors affecting bacterial growth are antibacterial compounds that I will briefly describe in the following text.

2.3.1 Antibacterial compounds

Bacteria can be beneficial to human health but they can also cause diseases. In 1928, Sir Alexander Fleming discovered the first antibacterial agents, penicillin. Subsequently, other antibiotics were discovered – either from natural sources or man-made. Antibiotics could be bacteriostatic or bactericidal. Bactericidal antibiotics target the cell wall (e.g. Bacitracin, β-lactams such as penicillin, ampicillin) or membrane (e.g. polymixins) and destroy/kill bacteria. Bacteriostatic antibiotics inhibit bacterial growth. This group includes antibiotics targeting protein synthesis (30S subunit inhibition – tetracylines, aminoglycosides; 50S subunit inhibition – e.g. chloramphenicol, macrolides), nucleic acid synthesis (DNA inhibitors - quinolones; RNA inhibitors – rifampicin, ripostatins, pseudouridimycin) or various bacterial metabolic pathways (e.g. sulfonamides) (Kapoor et al., 2017; Maffioli et al., 2019; Tang et al., 2014; Ullah and Ali, 2017). Figure 19 shows effects of selected antibiotics on bacterial growth in *E. coli*.

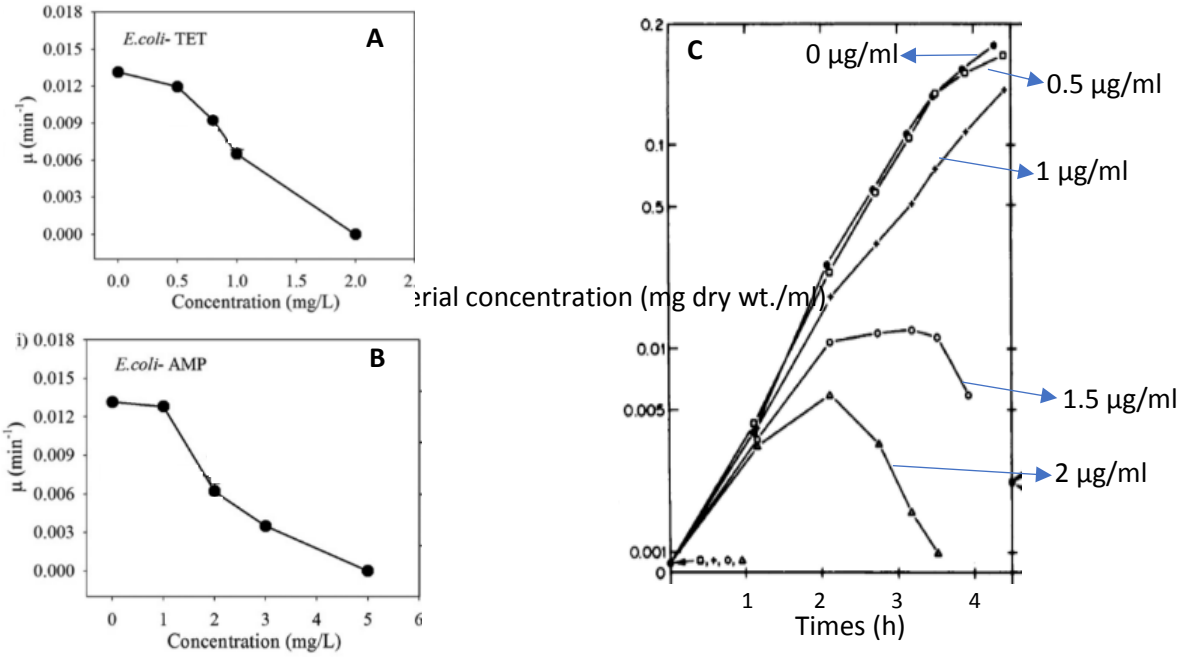

Figure 19. Growth of *E. coli* in the presence of selected antibiotics. A. Cells treated with tetracycline (TET) at different concentration (x-axis). μ - growth rate. **B.** Cells treated with ampicillin (AMP) at different concentration (x-axis) (Li et al., 2016). **C.** Batch culture of *E. coli* treated with rifampicin. The concentration is described in graph (Koch and Gross, 1979).

2.3.1.1 Antimicrobial peptides

In the last years, bacteria are becoming more resistant to antibiotics. Therefore, it is necessary to discover/design new antibacterial compounds and scientists have already begun in earnest this quest. An example are antimicrobial peptides. Antimicrobial peptides (AMPs) are produced in all organism and they are active against bacteria, viruses and fungi. They are usually 12 – 100 amino acids longs. The AMPs contain mostly basic (lysin and arginin) and hydrophobic amino acids. They form linear helical structure, β-sheet, loop or their combination. The AMPs bind to bacterial membranes where they form pores (Hancock and Rozek, 2002; Jenssen et al., 2006). The first antimicrobial peptide, cecropin, was isolated from *Hyalophora cecropia* in 1980 (Andreu et al., 1983; BOMAN et al., 1991; Jenssen et al., 2006). To date, hundreds of antimicrobial peptides are known, such as Mersacidin produced by *Bacillus* spp that is active against *Staphylococcus aureus* (Chatterjee et al., 1992), Polymyxin B produced by *Bacillus polymyxa* active against *Pseudomonas aeruginosa,*

Acinetobacter baumanii and *Klebsiella pneumoniae* (Zhang and Gallo, 2016). Cathelicidin functions against *Helicobacter pylori* (Zhang et al., 2016). AMPs are also present on human body (skin, eye, oral mucosa, lung and reproductive tract) and they are produced during infection. Histatins are human antimicrobial peptides in saliva where they play a role in maintaining the healthy state of the oral cavity (Zhang and Gallo, 2016).

2.3.1.2 Peptide nucleic acids

Peptide nucleic acids (PNA) are a new type of antibacterial compounds that form stable complexes with DNA and RNA and could be synthesised for a specific gene. The PNA backbone is composed of N-(2-aminoethyl)-glycine units linked by peptide bonds. The backbone is then "decorated" with purine and pyrimidine bases, reminiscent of nucleic acids. PNAs inhibit translation by binding on mRNA or ribosomes (Lundin et al., 2006). However, there is a problem with their delivery into bacteria (Wojciechowska et al., 2020). The PNA backbone has to be modified to improve PNA solubility and membrane permeability (Sugiyama and Kittaka, 2013) or PNA could conjugate with positively charged amino acids or with molecules able to penetrate bacterial membranes (Wojciechowska et al., 2020; Zanardi et al., 2012). The most effective option how to transport PNA to the bacterial cell is by cell penetrating peptides (CPP). CPP are max. 30 amino acids long cationic or amphipathic peptides. PNA can be bind to CPP through covalent bonds (Barkowsky et al., 2019). In gram-negative bacteria, the lipopolysaccharide layer is the main barrier for PNA. If *E. coli* has a defective lipopolysaccharide layer, this strain is more sensitive to PNA (Good et al., 2000). The First PNA was against the *E. coli acpP* gene (encodes a protein essential in fatty acids biosynthesis, which is conserved among gram-negatives. The *acpP* gene is a frequent target of PNA in human pathogens, *e.g. Brucella suis, Haemophilus influenza, Pseudomona aeruginosa* (Ghosal and Nielsen, 2012; Otsuka et al., 2017; Rajasekaran et al., 2013).

2.3.1.3 Lipophosphonoxins

Recently, a new type of antibacterial agents was developed, called lipophosphonoxins (LPPOs). LPPOs contains four modules: a nucleoside module, an iminosugar module, a

hydrophobic module, and a phosphonate linker module (Figure 20). The mechanism was unknown (Rejman et al., 2011).

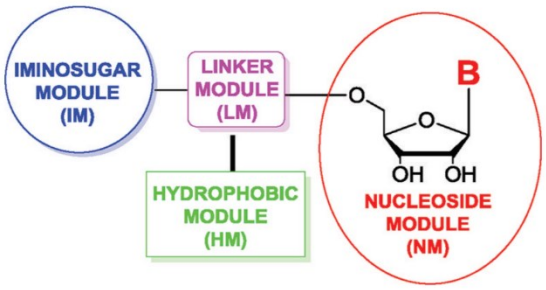

Figure 20. Structure of LPPOs. LPPOs have four parts. Iminosugar module (IM), linker module (LM), hydrophobic module (HM) and nucleoside module (NM) (Rejman et al., 2011).

3. AIMS

I focused on three projects involving bacterial growth. As endogenous factors affecting growth I studied RNase J1 and its cellular role(s) in *Bacillus subtilis* [Project (i)], and a small RNA, called Ms1, and its role(s) in *Mycobacterium smegmatis* [Project (ii)]; as exogenous factors I studied a new type of antibacterial compounds, LPPOs [Project (iii)].

Aims of Project (i):

- Identify effects of RNase J1 on cell growth & morphology.
- Define the RNase J1 regulon.
- Describe the roles and mechanistic functioning of RNase J1 in *Bacillus subtilis*

Aims of Project (ii):

- Confirm *in silico* predicted Ms1 in *Mycobacterium smegmatis in vitro* and *in vivo*
- Determine expression of Ms1 in exponential and stationary phase
- Identify the role(s) of Ms1 in *Mycobacterium smegmatis* – growth, affected genes
- Characterize the stability of Ms1

Aims of Project (iii):

- Characterize the effects of LPPOs on bacterial growth
- Identify binding partners of LPPOs

4. MATERIALS AND METHODS

Cultivation of bacterial cells

Cloning and transformation

DNA and RNA isolation

PCR, qPCR

Reverse transcription

Protein purification

Western and Northern Blots

Scanning electron microscopy and super-resolution microscopy (SIM)

Chromatin immunoprecipitation, ChIPseq, RNAseq

In vitro digestion assay

5. LIST OF PUBLICATIONS

Publication I

Ms1, a novel sRNA interacting with the RNA polymerase core in mycobacteria.

Jarmila Hnilicová, Jitka Jirát Matějčková, Michaela Šiková, Jiří Pospíšil, Petr Halada, Josef Pánek, Libor Krásný.

Nucleic Acids Res. 2014 Oct;42(18):11763-76. doi: 10.1093/nar/gku793.

PMID: 25217589

IF 2014: 9.112

The contribution of the author: 10%

I isolated total RNA from *Mycobacterium smegmatis*, analysed Ms1 on polyacrylamide gels and measured the amounts of Ms1 via Northern blots from exponential and stationary phases. I confirmed *in vitro* and *in vivo* that the ~300 nt long band in stationary phase is really Ms1, which had been predicted *in silico*.

Publication II

Insights into the Mechanism of Action of Bactericidal Lipophosphonoxins

Natalya Panova, Eva Zborníková, Ondřej Šimák, Radek Pohl, Milan Kolář, Kateřina Bogdanová, Renata Večeřová, Gabriela Seydlová, Radovan Fišer, Romana Hadravová, Hana Šanderová, Dragana Vítovská, Michaela Šiková, Tomáš Látal, Petra Lovecká, Ivan Barvík, Libor Krásný, Dominik Rejman.

PLoS One. 2015 Dec 30;10(12):e0145918. doi: 10.1371/journal.pone.0145918

PMID: 26716439

IF 2015: 3.360

The contribution of the author: 5%

I measured growth curves with or without lipophosphonoxins and searched for proteins binding to lipophosphonoxins.

Publication III

Ms1 RNA increases the amount of RNA polymerase in *Mycobacterium smegmatis*.

Michaela Šiková*, Martina Janoušková*, Olga Ramaniuk, Petra Páleníková, Jiří Pospíšil, Pavel Bartl, Agnieszka Suder, Petr Pajer, Pavla Kubičková, Ota Pavliš, Miluše Hradilová, Dragana Vítovská, Hana Šanderová, Martin Převorovský, Jarmila Hnilicová, and Libor Krásný

*These authors contributed equally to the paper as first authors.

Mol Microbiol. 2019 Feb;111(2):354-372

PMID: 30427073

IF2019: 3.649

The contribution of the author: 40%

I isolated total RNA from exponential and stationary phases and did RNA-seq of wt and ΔMs1 strains, optimizing the RNAseq method in the process. I analysed and validated RNA-seq data (selected affected genes) by RT-qPCR. I isolated total RNA from exponential and stationary phase and analysed Ms1 degradation in both phases by Northern blotting. I did western blots and analysed amounts of RNAP from stationary phase and during outgrowth in wt and ΔMs1 strain. I did outgrowth of wt, ΔMs1 and CRISPR-Ms1 depleted strains.

Publication IV

The torpedo effect in Bacillus subtilis: RNase J1 resolves stalled transcription complexes.

Michaela Šiková*, Jana Wiedermannová*, Martin Převorovský, Ivan Barvík, Petra Sudzinová, Olga Kofroňová, Oldřich Benada, Hana Šanderová, Ciaran Condon, Libor Krásný

*These authors contributed equally to the paper as first authors.

EMBO Journal, 2020 Feb 3;39(3):e102500. doi: 10.15252/embj.2019102500.

PMID: 31840842

IF 2019: 11.227

The contribution of the author: 60%

I measured growth curves and prepared samples for electron microscopy of wt and Δ*rnjA* strains. I isolated total RNA of wt and Δ*rnjA* strains and prepared libraries for RNAseq. I analysed and verified RNAseq data by RT-qPCR. I performed Chromatin immunoprecipitation of wt and Δ*rnjA* strains, analysed and verified ChIPseq data by qPCR. I did Western blots and

measured amounts of RNAP with antibody against RNAP. I created strains with GFP-RNase J1 and mCherry-RNAP for observations of co-localization of these two proteins and performed the subsequent microscopy. I created the *B. subtilis* strain with His-tagged RNase J1, performed pull down experiment, and subsequent Western blots with antibodies against RNase J1 and RNA polymerase. I isolated RNAP and assembled elongation complexes and analysed the efficiency of RNAP dissociation from elongation complexes by RNase J1, R1 and Xrn1 by Western blotting. I created the *ΔrnjA:Δrho* double deletion strain and performed colony forming units experiments of UV-irradiated cells.

I confirm that this Doctoral Thesis was written by Michaela Šiková and it is based on published data generated during her Ph.D. study between 2014-2020. I hereby declare that her contribution described above is based on truth.

Supervisor: Mgr. Libor Krásný, Ph.D.

6. SUMMARY OF PUBLICATIONS

Publication I

Ms1, a novel sRNA interacting with the RNA polymerase core in mycobacteria.

In this publication, we characterized a small RNA (sRNA), called Ms1, in *M. smegmatis*. This sRNA was discovered as a homologue of 6S RNA *in silico* (Pánek et al., 2011). We found that Ms1 is a ~300 nt long sRNA that is highly abundant in stationary phase. Northern blot analyses have shown that Ms1 is increased ~130-fold in stationary phase compared to exponential phase. By 5'RACE we determined the first transcribed nucleotide of Ms1 – an adenine transcribed from position 6,242,368 in the genome of *M. smegmatis*. Ms1 was identified/predicted also in other microorganisms: *Nocardia* and *Rhodococcus*. Subsequently, we did glycerol-gradient ultracentrifugation and immunoprecipitation (antibodies against RNAP (β). sigma factor ($\sigma^{70/A}$) and IgG as a negative control) to find Ms1 interaction partners. Ms1 binds to RNAP core without sigma factor and to other proteins such as Rho factor and PNPase. The same experiments were done with *E. coli*. Thereafter, we predicted the Ms1 secondary structure – a double stranded RNA with internal single stranded bubble and a long hairpin structure. We synthesised a mutant form of Ms1: Ms1 with deleted internal bubble (Ms1nb) and performed immunoprecipitation experiments. We found that the internal bubble is essential for interaction with the RNAP core. Then, we prepared a strain with overexpressed Ms1 in exponential phase and with overexpressed σ^A in stationary phase. The expression of Ms1 in exponential phase was comparable to endogenous Ms1 in stationary phase. Surprisingly, the overexpressed Ms1 in exponential phase did not change growth rate and interaction with RNAP core was similar as in the case of endogenous Ms1 in stationary phase. The overexpression of Ms1 in exponential phase then did not change the amount of the RNAP holoenzyme, but the overexpression of σ^A in stationary decreased the amount of RNAP:Ms1 complex, suggesting that σ^A is able to displace Ms1 from the RNAP core. We did not find any other sRNA interacting with RNAP core or holoenzyme. This suggest that 6S RNA is not probably present in *M. smegmatis.* Thus, Ms1 is a new type of a sRNA interacting with RNAP. This shows that transcription apparatus in mycobacteria is different from other bacteria, which contains 6S RNA.

Publication II

Insights into the Mechanism of Action of Bactericidal Lipophosphonoxins

This article describes the mechanism of action of the first generation of new antibacterial compound called Lipophosphonoxins, and their effect on bacterial growth. We studied the effect of LPPOs on RNA, DNA, protein, lipids and cell wall synthesis. LPPOs did not have any effect on these processes. Subsequently, we looked at whether LPPOs have effects on bacterial cells by transmission electron microscopy. We showed that LPPOs cause huge cell envelope damage. Then, we did HPLC experiments that revealed that LPPOs localise to the cytoplasmic membrane. Thus, LPPOs are membrane-targeting compounds that create pores on the membrane. We proved that LPPOs are not toxic to human cells. Thereafter, we did pull-out experiment with biotinylated LPPOs to find whether LPPOs are perhaps assisted by proteins in their action. We did not find any proteins bound to LPPOs. We also characterized the stability of LPPOs. Using LC-MS we show that LPPOs are stable at low pH. Finally, we determined that LPPOs have bactericidal effect on gram-positive bacteria including pathogenic bacteria and antibiotic resistant bacteria.

Publication III

Ms1 RNA increases the amount of RNA polymerase in Mycobacterium smegmatis.

This publication continues with the story about Ms1 in *M. smegmatis*. Based on RNAseq data, we found that Ms1 is the most expressed non-rRNA in stationary phase. Its level is ~115-fold higher in stationary phase compared to exponential phase. We prepared a strain with deletion of the Ms1 gene (ΔMs1 strain) and studied phenotypes as well as performed RNAseq. Surprisingly, Ms1 did not display large changes in the transcriptome compared to wt strain. We found, that Ms1 has effect on the amount of β and β' subunits of RNAP. The lower amount of β and β' subunits of RNAP was observed also at the protein level. We prepared a complemented Ms1 strain to confirm that the affected genes in the ΔMs1 strain are indeed affected because of the absence of Ms1. We found, that the ΔMs1 strain had a problem to adapt to new condition (during outgrowth). As a control of whether Ms1 is truly beneficial for adaptation, we prepared a knock-down Ms1 strain by the CRISPR method. Further, we studied synthesis and degradation of Ms1. We characterized a putative promoter of Ms1. The

upstream region is important for Ms1 synthesis. We found that Ms1 is very stable in stationary phase (half-life ~8h) compared to exponential phase (~8 minutes). From RNAseq data, we found three most expressed RNases in exponential phase, which can play role in Ms1 degradation. We knocked-down these three RNases (PNPase, RNase E and RNase J) by CRISPR. By Northern blotting we demonstrated that Ms1 is more accumulated upon depletion of PNPase. Further, we synthesised an *in vitro* Ms1 and its mutant forms (Ms1 without internal bubble and Ms1 without tails) and prepared His-PNPase to find which structure is important for degradation. We found that tails are important part of Ms1 to degradation. Ms1 is important molecule for regulation of RNAP pool in stationary phase and it is beneficial for cell during outgrowth and under the stress.

Publication IV

The torpedo effect in *Bacillus subtilis*: RNase J1 resolves stalled transcription complexes.

In this article, we characterized RNase J1 in *B. subtilis*. RNase J1 was the first discovered 5'→3' exoribonuclease in bacteria. We showed that RNase J1 has an effect on cell growth, morphology and half of the *B. subtilis* genome. By ChIPseq experiments, we identified RNAP to be accumulated on ~one third of all genes. These genes were mostly less transcribed. Comparisons of RNAseq and ChIPseq data suggested that RNAP is stalled on most of these genes. Subsequently, we found an interaction between RNAP and RNase J1 that is mediated via RNA. We demonstrated that RNase J1 resolves stalled transcription elongation complexes *in vitro* and *in vivo* and showed that RNase J1 terminates transcription (of stalled complexes) by a 'torpedo' mechanism. In this mechanism, RNase J1 in the 5'→3' direction cleaves RNA protruding from the stalled RNAP, and after contacting RNAP, torpedoes the complex, leading to dissociation of RNAP from nucleic acids. This is a new concept in bacterial cells.

7. DISCUSSION

Both endogenous and exogenous factors affect bacterial growth. Bacteria have the ability to quickly adapt to their environment and thus they can survive in harsh conditions. My Thesis describes effects of two endogenous factors and one exogenous factor on bacterial growth and their cellular roles in bacteria. The goal of the first project was to characterize the cellular role(s) of RNase J1 in *Bacillus subtilis* (Šiková et al., 2020). The goal of the second project was to study a small RNA, called Ms1, in *Mycobacterium smegmatis* and its function in the cell (Hnilicová et al., 2014; Šiková et al., 2019). The last project of this Thesis was to determine the effect(s) of antibacterial compounds, lipophosphonoxins on the cell (Panova et al., 2015). In the following text I focus primarily on results and interpretation/discussion of my own experiments. Wherever necessary, however, I include also relevant information obtained from experiments of my colleagues and duly acknowledge the author.

7.1 Cellular role(s) of RNase J1 in *Bacillus subtilis*

mRNA degradation is one of the important processes in gene regulation in all organisms. RNases are necessary for RNA turnover and contribute to maintaining the correct level of mRNA for subsequent protein synthesis and also play a role in maturation of non-coding RNAs: tRNA, rRNA and sRNA. Previously, it was believed that the 5'→ 3' exonuclease activity is characteristic only for eukaryotes. In 2005, RNase J1 was discovered in *B. subtilis* as a functional homologue of RNase E in *E. coli* with an endonuclease activity (Even et al., 2005). However, two years later Mathy et al. (2007) discovered that RNase J1 possesses a 5' → 3' exonuclease activity, the first such ribonuclease in bacteria. Based on the known information about RNase J1, the following questions were asked: How the absence of RNase J1 affects cell growth and cellular processes? Which genes are affected? What are the mechanistic details of functioning of RNase J1?

Phenotype of the Δ*rnjA* strain

To characterize the effect(s) of RNase J1 in *B. subtilis*, we first prepared a strain with a deleted RNase J1 (Δ*rnjA*). We showed that Δ*rnjA* strain has a significant effect on the cell growth rate.

The doubling time is ~2-times longer in the Δ*rnjA* strain. Likewise, bacterial growth slows down also in the absence of other RNases, such as RNase Y, the main endoribonuclease in *B subtilis* (Figaro et al., 2013), or a double mutant of RNases HII and HIII, important enzymes for restart of paused replication (Lang et al., 2017; Yao et al., 2013) in *B. subtilis*. It suggests that RNase J1 plays also a similarly important role in the cell. Moreover, the *ΔrnjA* strain has significantly different morphology from the wt strain. These results are consistent with previously published data (Figaro et al., 2013) but we describe these changes at a more detailed resolution.

The absence of RNase J1 affects the transcriptome and RNAP occupancy

Due to the effect of RNase J1 deletion on growth and also to other phenotypic parameters we decided to investigate in detail expression of which genes is affected in the mutant strain. Previously, it had been assumed that RNase J1 was an essential gene (Even et al., 2005; Hunt et al., 2006). So, historically, the first experiments addressing the regulon of RNase J1 were done with a depleted strain where it had an effect on 1,261 genes (at least two-fold change) (Durand et al., 2012). We performed RNAseq with a Δ*rnjA* strain. RNase J1 had an effect on ~40% of genes (1,740 genes, at least two-fold change). Compared with the depleted strain, there was an overlap in most genes. However, we characterized ~1,000 new genes affected in Δ*rnjA* compared to the RNase J1-depleted strain.

Subsequently, we performed chromatin immunoprecipitation and ChIPseq and compared ChIPseq (RNAP occupancy on DNA) and RNAseq data. We observed four situations. First two situations were expected - the more RNAP, the more RNA transcripts and *vice versa*. Then, we observed other situation – an increased RNA level in Δ*rnjA* but unchanged or possibly decreased RNAP occupancy. This suggest that these RNAs are more stable in the mutant strain, and, therefore, direct targets of RNase J1. The last observed situation was – a decreased RNA level whereas the RNAP occupancy on respective genes was increased or unchanged. We observed this effect mainly for genes with less abundant transcripts. Generally, RNAPs are stalled mainly in weakly transcribed genes because there are not another RNAPs for transcription initiation of specific gene. In strong transcribed genes, stalled

RNAP is translocated by trailing RNAPs and so stalled RNAP can overcome obstacles and continue with transcription (Epshtein et al., 2003).

Role of RNase J1 in *B. subtilis*

We found 1654 genes where RNAP is stalled more often in the Δ*rnjA* strain (the fourth situation). In *B. subtilis*, GreA helps resolve stalled RNAPs within in promoter-proximal regions but this effect was shown only for a few genes (35 genes; (Kusuya et al., 2011)). However, transcribing RNAP can be paused also within genes, not only in the vicinity of promoters. Consequently, other factors must be required to resolve stalled RNAP on DNA within genes. One of them is Mfd, which is involved in nucleotide excision repair and transcription-coupled repair. Mfd recognises stalled RNAP and resolves stalled RNAP by its ATPase and translocase activity (Ragheb and Merrikh, 2019). Another factor is RNase J1, functioning by a torpedo model like mechanism, similarly to 5'→3' exonucleases in eukaryotes (Kim et al., 2004; West et al., 2004).

In eukaryotes, 5'→3' exonucleases (Xrn1, Xrn2, Rat1) have been well studied (Connelly and Manley, 1988; Kim et al., 2004; West et al., 2004). It was shown that these RNases are important for transcription termination. When transcribing RNAPII recognizes the polyadenylation (polyA) site (usually AAUAAA hexamer), an endonuclease cleaves the nascent pre-mRNA. However, RNAPII continue transcribing of 'junk' RNA (RNA which is useless). This junk RNA serves as a substrate for a 5'→3' exoribonuclease. The 5'→3' exoribonuclease cleaves the junk RNA up to RNAPII and then causes dissociation of RNAPII from DNA. This mechanism of dissociation of RNAPII and transcription termination is called the 'Torpedo model' – the exoribonuclease hits RNAPII and pushes RNAPII off DNA (Cortazar et al., 2019; Kim et al., 2004; Miki et al., 2017; West et al., 2004).

If RNase J1 is involved in dissociation of stalled RNAP from DNA, these two enzymes have to interact. Previously, it was published that RNase J1 is localized on cell poles (Cascante-Estepa et al., 2016). We performed super-resolution microscopy with a strain containing GFP-RNase J1 and mCherry-RNAP and we found an overlap between these two enzymes, especially on the periphery of the nucleoid. Moreover, pull-down of RNAP or RNase J1 via His-tag confirmed the interaction between these two enzymes via RNA.

Subsequently, we observed that RNase J1 is able to dissociate RNAPs from transcription elongation complexes. Approximately 50% of RNAPs were released from TECs after addition of RNase J1. It was confirmed by *in vitro* experiment (performed by Jana Wiedermannová). RNase J1 degraded RNA up to RNAP, leaving 17-18nt long RNA. This 17-18nt long RNA is protected by RNAP. Park et al. (2015) study *in vitro* whether eukaryotes Rat1 is able to degrade RNA up to *E. coli* RNAP but the termination of transcription (*e.i.* the torpedo effect) did not occur. We performed similar experiment with a eukaryotic 5'→3' exoribonuclease Xrn1 (Sun et al., 2013). Xrn1 dissociated fewer *B. subtilis* RNAPs from TECs (~30%). Another *in vitro* experiment (performed by Jana Wiedermannová) also showed that Xrn1 did not dissociate RNAP complexes as efficiently as RNase J1. Moreover, Xrn1 generated more diverse stubs (RNA degradation products ending at the outer edge of the RNA exit channel of RNAP) than RNase J1. It suggests that the behaviour of RNase J1 and Xrn1 is different relative to RNAP.

Relatively recently, an RNase with a 5'→3' exonuclease activity was discovered in *E. coli*, named RNase AM (Ghodge and Raushel, 2015; Jain, 2020). RNase AM was shown to be involved in maturation of all three rRNAs (Jain, 2020). However, it has not been studied yet whether RNase AM terminates transcription by torpedo model (if it has such the function at all, *E. coli* contains other factors for resolution of stalled RNAP –e.g. Gre factors (Toulmé et al., 2000), UvrD (Epshtein et al., 2014) and whether it has also other cellular targets. This is a question for further work.

Transcriptome and phenotype correlation

Then, we looked at how the RNAseq data correlate with Δ*rnjA* phenotypes including its slower growth. The slower growth of the Δ*rnjA* strain can be affected by many factors. The Δ*rnjA* strain has lower amounts of the main subunits of RNAP (α, β, β'). Previously it was shown that slowly growing *E. coli* or *B. subtilis* have ~8-fold fewer molecules of RNAP per cell then fast growing cells (Bremer and Dennis, 1996; Ishihama et al., 2008; Klumpp and Hwa, 2008; Maass et al., 2011; Shepherd et al., 2001). Moreover, the slow growth rate of the Δ*rnjA* strain correlates with expression of translation elongation factors. These factors were also downregulated.

Slower growth of the Δ*rnjA* strain can be also caused by slower rate/increased pausing of replication. RNase HII is responsible for removal of rNMPs incorporated into DNA during replication. Its absence increases the likelihood of mutations and may lead to replication pausing (Yao et al., 2013) and this is a problem for growing cells. When replication is paused, transcription could collide with the replication machinery. RNase HII was downregulated in the Δ*rnjA* strain.

Further, replication-transcription collisions generate R-loops that could cause genome instability and replication stalling (Lin and Pasero, 2012). RNase HIII, which resolves R-loops (Lang et al., 2017), was upregulated (~1.6-fold) in the Δ*rnjA* strain. Moreover, DNA repair genes for restart of replication after replication-transcription collision (AddA and AddB) (Krajewski et al., 2014; Shepanek et al., 1989) were the most upregulated repair genes. Other repair genes were also upregulated, such as mismatch repair genes [MutS, MutL (LeBlanc et al., 2018; Liu et al., 2016)], base excision repair [MutM, MutT, Ung (Lenhart et al., 2012)]. It suggests that the Δ*rnjA* strain contains many mutations and transcription collides with replication more often. The cells are trying to repair the damage, but the extent of the damage is large and, consequently, RNAP might be more frequently stalled and accumulated on genes.

In the Δ*rnjA* strain, RNase J2 was the most upregulated RNase. RNase J2 has a weak 5'→3' exoribonuclease activity than RNase J1 (Mathy et al., 2010). Probably, the cells try to compensate for the loss of RNase J1 and try to overexpress RNase J2 to replace its degradation function or/and its function in resolution of stalled RNAP. However, the 5'→3' exoribonuclease activity of RNase J2 is too weak and even its increased level is not sufficient to compensate for the absence of RNase J1. Stalled RNAP are a problem for cells and this also affects growth.

Effect of Rho factor

As I mentioned in the Literary review, Rho is involved in transcription termination and release of RNAP from DNA. The mechanism of RNAP dissociation is probably different from RNase J1. The mRNA level of the *rho* gene was decreased in the Δ*rnjA* strain. We performed UV sensitivity assay with single (Δ*rho* or Δ*rnjA*) and double mutant (Δ*rho* and Δ*rnjA*) strains. UV irradiation increases stalling of RNAP due to the formation of pyrimidines dimers or 6,4

pyrimidine-pyrimidones (Goodsell, 2001) and this creates obstacles for transcribing RNAP. The *Δrho* strain did not display any significant changes (relative to wt) after UV irradiation. The Δ*rnjA* strain displayed a decreased cell viability after UV irradiation compared to wt cell. The double mutant strain was more sensitive to UV irradiation than the Δ*rnjA* strain. Therefore, these two proteins function in analogous manner but in different pathways.

To sum up this part of the Thesis, we found a new mechanism of bacterial transcription termination – the torpedo model. RNase J1 liberates RNAP from stalled transcription complexes. RNase J1 degrades incomplete RNA and makes stalled RNAP available for other transcription.

7.2 Ms1, a new type of small RNA in *Mycobacterium smegmatis*.

Ms1 was originally found as a potential homologue of 6S RNA by an *in silico* approach (Pánek et al., 2011). In the first study (Publication I), the questions were: Does the ~300 nt long major RNA identified in total *M. smegmatis* RNA contain the predicted Ms1? How is it expressed during growth? Is Ms1 a homologue of 6S RNA?

We isolated total RNA and we observed ~300 nt long RNA in stationary phase. This correlated with our previous article, where the predicted Ms1 had been detected by Northern blot mainly in stationary phase (Pánek et al., 2011). By RNase H digestion experiments we confirmed that the ~300 nt long RNA was really the predicted Ms1 in *M. smegmatis*.

Similarly to the Ms1 homologue (MTS2823) in *M. tuberculosis* (Arnvig et al., 2011) and 6S RNA in *E.coli* (Wassarman and Storz, 2000), Ms1 in *M. smegmatis* accumulates in stationary phase. We quantified the relative amounts of Ms1 in exponential and stationary phase of growth by Northern blotting and calculated the amounts of Ms1. Ms1 increased ~130-fold in stationary phase compared to exponential phase and we estimated ~600 Ms1 molecules per cell. In contrast to 6S RNA in *E. coli*, Ms1 has ~16-fold lower expression in stationary phase (Wassarman and Storz, 2000). Subsequently, we found that Ms1 interacts with the RNAP core without any sigma factor. This is in contrast with 6S RNA that binds to RNAP holoenzyme (RNAP with σ factor) (Wassarman and Storz, 2000). Therefore, we classified Ms1 as a new type of sRNA interacting with RNAP. Approximately 30% of Ms1 binds to RNAP (~200

molecules of RNAP is in complex with Ms1). The differences between expression of Ms1 in *M. smegmatis* and 6S RNA in *E. coli* can be explained by the different number of RNAP molecules in respective cells. *M. smegmatis* grows more slowly compared to *E. coli* and a slower growth rate may require fewer RNAPs per cell. It was calculated that slowly growing *E. coli* or *B. subtilis* have ~1,400 molecules RNAP per cell (Bremer and Dennis, 1996; Ishihama et al., 2008; Maass et al., 2011) whereas fast growing *E. coli* has ~11,400 molecules of RNAP per cell and ~17 % of RNAP is free in cytoplasm (Shepherd et al., 2001). If we assume that stationary phase *M. smegmatis* has a similar total amount of RNAP and free RNAP per cell as slowly growing *E. coli*, almost all free RNAPs are in complex with Ms1. Based on our study, Ms1 is not a homologue of 6S RNA, although it has a similar structure. It follows that bioinformatically predicted sRNAs must be confirmed experimentally. Ms1 is a new type of a small RNA interacting with RNAP.

7.3 Ms1 RNA increases the amount of RNAP in *Mycobacterium smegmatis*.

This publication is a follow-up of the previous study where we found that Ms1 is a new type of sRNA, interacting with the RNAP core. . Ms1 was also predicted in other bacteria, e.g. *Rhodococcus*, *Nocardia* (Hnilicová et al., 2014) and Ms1 homolog was also found in pathogenic *M. tuberculosis* (Arnvig et al., 2011). Our questions were: What genes are affected/regulated by Ms1? What is the role of Ms1?

Expression of Ms1

To characterize Ms1 in *M. smegmatis*, we prepared a ΔMs1 strain and performed RNAseq. Ms1 was found as the most expressed gene in stationary phase. In exponential phase, Ms1 is still within the top twenty most expressed genes. Based on RNAseq data, Ms1 is ~175-fold more expressed in stationary phase. Then, we quantified an amount of Ms1 by quantitative PCR (qPCR) from both phases and we observed ~115-fold increase in stationary phase. In the previous study, where we measure amount of Ms1 in both phases by Northern blots, Ms1

increased expression ~130-fold in stationary phase (Hnilicová et al., 2014). The expression of Ms1 thus nicely correlates between the three independent methods.

Ms1 affects the amount of RNAP

Surprisingly, the absence of Ms1 from cells had only a minor effect on gene expression, despite its high accumulation in stationary phase. Ms1 affected fourteen genes in stationary phase. In *E. coli*, the absence of 6S RNA (Ec6S RNA) had a bigger effect on gene expression. In stationary phase, Ec6S RNA affected 133 genes ([~3% of all genes were affected] (Lal et al., 2018) but this was still not a large change considering its own massive expression. The strain with deleted 6S RNA had then a phenotypic effect only on survival during late stationary phase (Trotochaud and Wassarman, 2004).

Interestingly, we observed that Ms1 had an effect on the levels of β and β' subunits (mRNA) of RNAP in stationary phase. They decreased ~2-fold in the ΔMs1 strain. The RNAP level was affected also on the protein level (measured by Western blot). In the ΔMs1 strain, the level of the β subunit was decreased by ~30 %. The decreased level of RNAP was surprising. We expected that the ΔMs1 strain will have a higher level of free RNAPs (those RNAPs that in wt are otherwise in complex with Ms1) and this would influence gene expression. However, RNAP decreased in ΔMs1 and thus lacks a reservoir of RNAPs, which are in the complex with Ms1. In the previous study (Hnilicová et al., 2014), we observed that almost 40% of RNAP binds to Ms1. In addition, when we overexpressed Ms1 in exponential phase, we pulled-down ~20 % of RNAP with Ms1, but the amount of the RNAP holoenzyme was comparable to the wt strain. It could mean that RNAPs bound to Ms1 could be inactive. Thus, the amount of RNAP available for transcription could be comparable to the wt strain. It explains the same growth rate (doubling time in exponential phase) of wt and the ΔMs1 strain. How are the levels of β and β' subunits of RNAP regulated is not known yet. This is the subject of another study, currently in progress.

Ms1 is important during outgrowth

The growth rate of the wt strain and ΔMs1 strain was similar. We asked whether the higher level of RNAP in the wt strain is advantageous during outgrowth, in other words, whether the wt strain adapts to new conditions faster compared to the ΔMs1 strain. We inoculated both strains to the same optical density and cultivated into stationary phase (24 h cultivation). Then we reinoculated cells into fresh medium to the same optical density. Before inoculation to the fresh medium, we measured colony forming unit (CFU). The viability and cell numbers of both strains were similar. During outgrowth, we observed differences in the first ~3 h. Wt cells grew faster immediately after reinoculation to the fresh medium compared to the ΔMs1 strain. Moreover, we prepared a depleted Ms1 strain by the CRISPR method (Rock et al., 2017) to confirm that the presence of Ms1 plays a role in outgrowth. The knockdown Ms1 strain grew slower for 4 h. Based on these results, we concluded that Ms1 (and a higher RNAP level in stationary phase) is important for better and faster adaption during outgrowth.

A homolog of Ms1 in *M. tuberculosis*, MTS2823, is present at a high concentration during chronic lungs infection and could help to survive in the hostile environment (Arnvig and Young, 2012; Arnvig et al., 2011). If we targeted to the homolog of Ms1 in *M. tuberculosis* (or possible other pathogens containing Ms1 or its homolog) by antibacterial compounds, we could reduce their survival in host cells. The PNA can be one of the options for targeting MTS2823 RNA and reduce its amount in *M. tuberculosis* during infection. In *E. coli*, deletion of 6S RNA led also to reduction of the RNAP level (both on mRNA and protein levels) in stationary phase (Lal et al., 2018) and it caused a decrease survival in stationary phase.

Degradation of Ms1

We found that Ms1 is unstable during exponential growth (half-life ~8 min vs ~8h in stationary phase). Hence, Ms1 is likely rapidly turned over by RNases during exponential phase. It suggests that Ms1 bound to RNAP is protected against degradation during stationary phase.

Based on RNAseq data we found three RNases that were upregulated in exponential phase – PNPase, RNase J and RNase E. We showed that PNPase is partially responsible for the degradation of Ms1 in exponential phase (this result was done with the collaboration with

Martina Janoušková). The Ms1 level increased by ~30% in the knock-down PNPase strain. It is consistent with our previous study about Ms1 (Hnilicová et al., 2014), where we found PNPase as an interaction partner of Ms1. The 30% increase, however, was moderate; we had expected a more pronounced effect on the Ms1 level. This suggests that PNPase is only one of the degradation factors of Ms1 and other factor(s) must exist. In *E. coli*, RNase BN is the direct RNase involved in degradation of 6S RNA. In exponential phase, expression of RNase BN is high and keeps the 6S RNA level low. In stationary phase, RNase BN level dramatically decreases and 6S RNA is more stable (Chen et al., 2016). RNase BN is a member of the RNase Z super family (De La Sierra-Gallay et al., 2005) and it was also predicted in mycobacteria (Taverniti et al., 2011). It is possible, that this RNase could also play a role in degradation of Ms1. This is question for future studies.

Dissociation of Ms1 from RNAP

How is Ms1 released from the Ms1-RNAP complex and the role of Ms1 protection in the complex is still unknown. Generally, 6S RNA binds to RNAP-holoenzyme and also serves as a template for pRNAs synthesis during outgrowth. Synthesis of pRNA destabilizes 6S RNA-RNAP holoenzyme complex, RNAP-holoenzyme dissociates from 6S RNA:pRNA duplex and 6S RNA is degraded by RNases (Beckmann et al., 2012; Cavanagh et al., 2012; Wassarman and Saecker, 2006). Rifampicin stabilizes 6S RNA-RNAP holoenzyme complexes and thus 6S RNAs are protected from degradation during outgrowth (Beckmann et al., 2012; Wurm et al., 2010). We observed the opposite result with Ms1. Rifampicin did not protect Ms1 against degradation during outgrowth. This suggests that RNAP is released from Ms1 in a different way compared to 6S RNA. Ms1 could be dissociated from RNAP during outgrowth by either σ^A or Rho protein. σ^A could bind to RNAP and change its conformation, leading to dissociation of Ms1. Rho factor was found as an interaction partner of Ms1 (Hnilicová et al., 2014). RNAP might transcribe an RNA from the Ms1 template and the Rho factor may bind the emerging RNA and after catching up with RNAP, the Rho factor may RNAP from Ms1. Another option could be that RNAP transcribes RNA, leading to changes in Ms1 structure in the central bubble region and this would lead to RNAP release similarly to 6S RNA:RNAP complex dissociation (Beckmann et al., 2012; Panchapakesan and Unrau, 2012; Steuten et al., 2014). The study of dissociation of RNAP from Ms1 will be addressed by future studies.

In conclusion, Ms1 is the most expressed non-rRNA in *M. smegmatis* that regulates the amount of RNAP in stationary phase. A higher level of RNAP in stationary phase (in wt) is beneficial during outgrowth or under stress condition. Ms1 thus appears to be important for better adaptation to new conditions.

7.4 Lipophosphonoxins, new antibacterial compounds

During the last years, bacteria are becoming more resistant against antibiotics and, consequently, development of new antibacterial compounds is becoming more important. In recent years, ~700,000 people die per year due to resistance of bacterial strains against known antibiotics. This number is likely to increase if we do not find new antibacterial compounds (Willyard, 2017). Recently, antibacterial compounds called lipophosphonoxins (LPPOs) have been developed (Rejman et al., 2011). It was shown that LPPOs are membrane binding antibacterial compounds that cause bacterial cell damage.

Their antibacterial activity was shown on a wide range of gram-positive bacteria (*Enterococcus faecalis*, *Streptococcus agalactiae* and *Bacillus subtilis*). The best antibacterial activity was for LPPOs which had (i) uridine as nucleoside module (NM), hexadecyl ester as hydrophobic module (HM) and (3R,4R)-3,4-dihydroxypyrrolidine as iminosugar module (IM), (ii) uridine as NM, hexadecyloxypropyl as HM, and (3R,4R)-3,4-dihydroxypyrrolidine as IM and (iii) cytidine as NM, hexadecyloxypropyl as HM, and (3R,4R)-3,4-dihydroxypyrrolidin (Rejman et al., 2011).

LPPOs do not need any other proteins for their activity as I showed in experiments with biotinylated LPPOs and cell lysates. LPPOs bind exclusively to the bacterial membrane where they penetrate its phospholipid bilayer and form pores, leading to membrane disintegration. In this respect, they are similar to antimicrobial peptides are also pore-forming antibacterial compounds and do not use any other binding proteins for their activity (Hancock and Rozek, 2002; Jenssen et al., 2006).

We tested LPPOs ((i) and (iii)) on bacterial growth in *M. smegmatis* as a model organism for *M. tuberculosis* (agent of tuberculosis). Tuberculosis is a serious problem for human health and it is one of the top 10 causes of death in the world and drug resistant *M.*

tuberculosis strains are becoming more frequent. In 2018, ~500,000 cases of tuberculosis were caused by rifampicin resistant strains (WHO, 2019). However, none of both tested LPPOs had any effect on mycobacterial growth. A possible reason is that mycobacteria have a different cell wall composition compared to other bacteria. Mycobacteria are classified as gram-positive bacteria but their cell wall shares some similarities also with gram-negative bacteria (Alderwick et al., 2015). This is in accord with the fact that LPPOs have an effect only on gram-positive bacteria but not on gram-negative bacteria.

Two years after the completion of the just described project, the second generation LPPOs, called LPPOs II, were synthesized. In contrast to the first generation, LPPOs II have an antibacterial effect against both gram-positive, including *M. smegmatis*, and gram-negative bacteria (Seydlová et al., 2017).

8. CONCLUSIONS

My doctoral Thesis centred around the ability of bacteria to grow/divide and I studied the influence of selected factors on this process. I focused on two endogenous factors acting at the transcriptional level (RNase J1 in *B. subtilis* and Ms1 RNA in *M. smegmatis*) and one exogenous factor (LPPOs), affecting the cell membrane.

In the first project, I studied a recently discovered new type of RNase in *B. subtilis*, RNase J1. I showed that RNase J1 has a large effect on the cell – it affects growth, morphology, and gene expression. By several methods I discovered a new mechanism of transcription termination in *B. subtilis* – the Torpedo model. This model was previously identified in eukaryotes but never described in prokaryotes. Importantly, the bacterial torpedo resolves stalled transcription complexes whereas its eukaryotic counterpart terminates transcription. However, it is possible that in eukaryotes 5'→3' RNases may function also by removing stalled transcription complexes. This needs to be addressed by future studies.

The second project was focused on a recently discovered small RNA (Ms1) in *M. smegmatis* that interacts with RNAP. We showed that Ms1 is not a homologue of 6S RNA in *E. coli*. It is a new type of small RNA interacting with the RNAP core. Notably, Ms1 is the most expressed non-rRNA in stationary phase but does not have large effects on cell morphology and gene expression. However, we observed that Ms1 has an effect on the RNAP level and it is beneficial in adaptation to new conditions. Ms1 is important for faster adaptation during outgrowth. How is RNAP regulated by Ms1 and how Ms1 is released from RNAP is now under investigation.

The last project describes effects of recently discovered antibacterial compounds, called Lipophosphonoxins (LPPOs). We showed that LPPOs are effective against gram-positive bacteria. They form pores in plasma membranes and thus kill bacteria, abruptly ending their growth. For their function, they do not need any other proteins. Currently, new generations of LPPOs are being developed and tested for potential applications.

9. LIST OF ABREVIATIONS

5'UTR	5' untranslated region
AMP	ampicilin
AMPs	antimicrobial peptides
B. subtilis	*Bacillus subtilis*
bp	base pair
CFU	colony forming units
ChIP	chromatine immunoprecipitation
CPP	cell penetrating peptides
CRISPR	clustered regularly interspaced short palindromic repeats
CTD	C-terminal domain
E. coli	*Escherichia coli*
HPLC	high performance liquid chromatography
iNTP	initiation nucleotidtriphosphate
LC-MS	liquid chromatography–mass spectrometry
Log phase	logarithmic phase
LPPOs	lipophosphonoxins
M. smegmatis	*Mycobacterium smegmatis*
Mbp	Mega base pair
mRNA	messenger RNA
NAD	nicotinamide adenine dinucleotide
Np4N	dinucleoside tetraphosphates
nt	nucleotide
NTD	N-terminal domain
NTP	nucleotidtriphosphate
PBP	phosphate binding pocket
PfkA	phosphofructokinase
PNA	peptide nucleic acids

PnpA	polynucleotide phosphorylase
pRNA	product RNA
RNAP	RNA polymerase
RNase	ribonuclese
rNMP	ribonucleotidmonophosphate
RPc	closed promoter complex
RPo	open promoter complex
RppH	pyrophosphohydrolase
rRNA	ribosomal RNA
scRNA	small cytoplasmic RNA
sRNA	small RNA
TET	tetracycline
tmRNA	transfer messenger RNA
tRNA	transfer RNA
wt	wild type

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