CHARLES UNIVERSITY

Faculty of Nature Sciences

Habilitation Thesis

Transcription: The Pivotal Process of Gene Expression Regulation

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Contents

Summary

Regulation of transcription in bacteria is critically important for the cell's functioning and its ability to respond to changing environment as well as for pathogens to successfully invade and beat the immune systems of their hosts. To understand transcription, the key players must be identified, characterized, and their modes of function established. These result then help both create models of how the bacterial cell functions and aid in designing new antibacterial strategies.

This Thesis covers 27 selected papers that focus on advancing our knowledge of bacterial transcription, dealing mostly with RNA polymerase (RNAP) from model organisms *Escherichia coli*, *Bacillus subtilis*, and *Mycobacterium smegmatis*, representing both gram positives and negatives or being closely related to pathogenic species. The Thesis is arranged into five Chapters. Brief resumes of publications in all Chapters start with the most salient question(s), then follow descriptions of the findings, and they are concluded with answer(s). The five Chapters describe interactions of RNAP with DNA, transcription initiating substrates, small RNAs, and proteins. The last Chapter reports the development and mechanism of action of novel antibacterial compounds, lipophosphonoxins (LPPOs).

The most important results include (i) deciphering the mechanism of incorporation of nicotine amide adenine dinucleotide (NAD) into RNA by RNAP (Nature, 2016), (ii) showing that the transcription start site nucleotide (A or G) dictates the promoter response (increase of decrease in activity) to amino acid starvation (Mol Microbiol, 2008), and (iii) describing rRNA promoter regulation in *B. subtilis*, a novel type of control of ribosome synthesis (EMBO J. 2004).

Chapter 1. DNA

Deoxyribonucleic acid (DNA), which was discovered in 1872, is the prevailing storage medium of the genetic information. Its double stranded structure was revealed almost 70 years ago (Watson and Crick, 1953) and ever since, the pace of its exploration has been accelerating, bringing in novel insights into its organization and function.

The genetic information is organized into units - genes. Genes either encode proteins or have regulatory and/or structural functions by themselves. To realize the genetic information, it is necessary to transcribe it from DNA into RNA. In bacteria, this task is accomplished by a single enzyme, the DNA-dependent RNA polymerase (RNAP). Bacterial RNAP is s multisubunit complex whose core consists of an α dimer that holds together two large subunits, β and β', that form the active site. β' associates with a small subunit ω. This core enzyme is capable of transcription elongation but not initiation. To initiate, it must associate with a σ factor that allows the holoenzyme to recognize and bind specific sequences, promoters (Lewis et al., 2008).

Promoters precede genes and consist of (usually) several conserved elements that interact with RNAP and guide the initiation process. The most common promoters recognized by RNAP with the primary σ factors ($\sigma^{A/70}$) consist of -35 and -10 hexamers, typically spaced ~ 17 bp apart. ~7 bp downstream of -10 is then positioned the transcription start site (TSS) (Feklistov, 2013).

DNA is built from four types of nucleotides, differing in the attached base: adenine, cytosine, guanine, and thymidine. These bases can be modified and various modifications bear important epigenetic information that affects gene expression (Tomkova and Schuster-Bockler, 2018). In this Chapter I describe our research into both natural and artificial types of DNA modification and into the possibility to utilize these modifications for manipulation of gene expression by biorthogonal chemistry. The studies in this Chapter were done in collaboration with Prof. Dr. M. Hocek from the Institute of Organic Chemistry and Biochemistry in Dejvice, Prague.

1. **Raindlová, V., Janoušková, M., Slavíčková, M., Perlíková, P., Boháčová, S., Milisavljevič, N., Šanderová, H., Benda, M., Barvík, I., Krásný, L. and Hocek, M. (2016) Influence of major-groove chemical modifications of DNA on transcription by bacterial RNA polymerases. *Nucleic Acids Res*, 44(7): 3000-12.**

Question: What are the effects of increased bulkiness of base modifications on transcription?

In this study we systematically investigated the effect of base modifications with increasing bulkiness (5-substituted pyrimidines or 7-substituted 7-deazapurines bearing H, methyl, vinyl, ethynyl or phenyl groups, **Fig. 1**) on transcription by RNAPs from two model organisms: gram positive *Bacillus subtilis* and gram negative *Escherichia coli*. The templates were prepared by PCR with corresponding base-modified substrates (dNTPs). The modifications protruded into the major groove of DNA. The modified templates were used in *in vitro* transcription assays with RNAP from *B. subtilis* and *E. coli*. Some modified nucleobases bearing smaller modifications (H, Me in 7-deazapurines) were perfectly tolerated by both enzymes, whereas bulky modifications (Ph at any nucleobase) and, surprisingly, uracil blocked transcription. Some middle-sized modifications (vinyl or ethynyl) were partly tolerated mostly by the *E. coli* enzyme. In all cases where the transcription proceeded, full-length RNA product with correct sequence was obtained indicating that the modifications of the template are not mutagenic and the inhibition is probably at the stage of initiation.

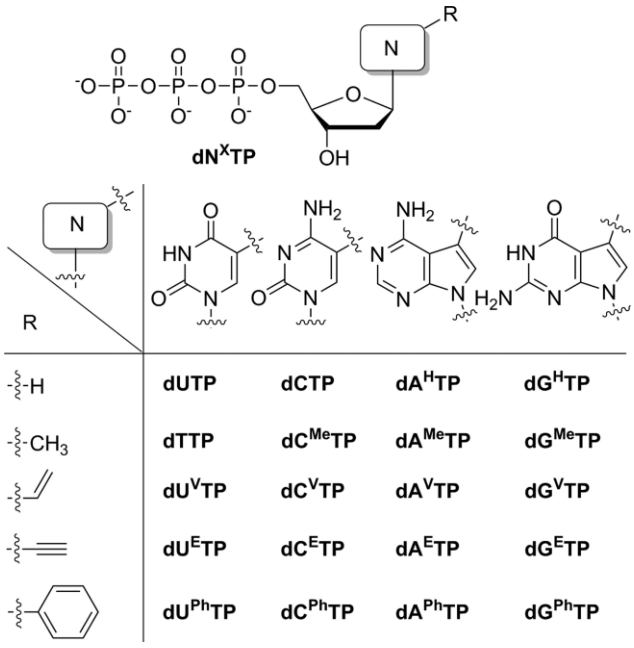


Figure 1. Structures of modified **dN^{X}TP**s used to create modified DNA (N, base; R, substituent).

The results were essential for understanding which modifications are acceptable for RNAP and this paved the way for the subsequent development of bioorthogonal reactions for artificial chemical switching of transcription.

Answer: Modifications with increasing bulkiness progressively inhibit transcription. Uracil was an exception - despite representing a minimal alteration, it displays a strong inhibitory effect.

2. **Janoušková, M., Vaníková, Z., Nici, F., Boháčová, S., Vítovská, D., Šanderová, H., Hocek, M., and Krásný, L. (2017) 5-(Hydroxymethyl)uracil and -cytosine as potential epigenetic marks enhancing or inhibiting transcription with bacterial RNA polymerase. Chem Comm 53(99): 13253-13255.**

Question: What is the effect of two naturally occurring DNA modifications, 5mC and 5mU, on transcription?

Modifications of DNA by epigenetic bases (i.e. 5-methylcytosine, 5mC) play critical roles in regulation of gene expression both in eukaryotes and prokaryotes and their dysregulation may lead to diseases. Recent advances in detection techniques have resulted in the discovery of the new bases 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5caC) (Lu et al., 2015). More recently, also 5-hydroxymethyluracil (5hmU), previously detected in bacteriophages, dinoflagellates and leishmania, has been found in eukaryotic genomes where its level appears to be cell type-specific (Guz et al., 2014; Witmer, 1981). The level of 5-hydroxymethyl-2'-deoxyuridine in blood DNA was investigated as a marker for breast cancer (Djuric et al., 1996). It is being hotly debated whether these modifications function as regulators of gene expression or whether they are just intermediates in active demethylation of DNA or products of oxidative damage. While the role of 5hmC in regulation of transcription has been demonstrated, the role of 5hmU remains unclear (Greco et al., 2016; Olinski et al., 2016). The 5hmU base can be generated by oxidation/hydroxylation of thymine by the Ten-Eleven-Translocation (TET) proteins or result from deamination of 5hmC (Carson et al., 2016).

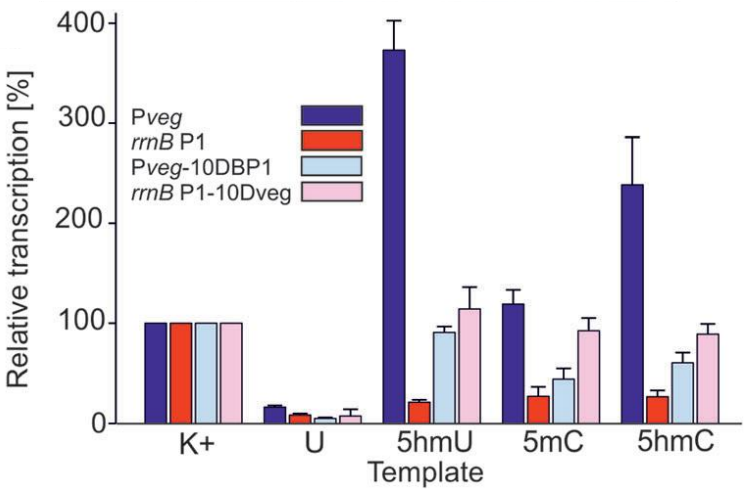


Figure 2. Quantitation of transcriptions from fully-modified templates containing different modifications. K+ is the natural (non-modified) DNA template. Two wt promoters (P*veg, rrnB* P1) and their chimeras were used, demonstrating that the effects of the modifications were promoter-specific.

In this study we used several bacterial promoters and RNAP from *E. coli* to investigate the effect of these modifications on transcription. We showed that both 5hmC and 5hmU affect transcription by bacterial RNAP depending on the promoter sequence (**Fig. 2**). The effects were both inhibitory and, surprisingly, also stimulatory. This study was the first to report strong enhancement of transcription of templates containing 5hmU or 5hmC in an *in vitro* enzymatic assay. We then focused on 5hmU where the effects were more pronounced. In the case of 5hmU, both the enhancement and inhibition were mediated predominantly by interactions of the promoter non-template strand with RNAP. We note that while we used for our studies a well-characterized promoter (P*veg*), other promoters may exist in the genome where random modifications of even single bases may have even more pronounced effects on transcription. Taken together, this illustrates the strong potential of 5hmU to alter gene expression *in vivo*.

Answer: Both 5mC and 5mU inhibit and stimulate transcription (**Fig. 2**). This is probably important for gene expression regulation in the cell.

3. **Slavíčková, M., Janoušková, M., Šimonová, A., Cahová, H., Kambová, M., Šanderová, H., Krásný, L., Hocek, M. (2018) Turning off transcription with bacterial RNA polymerase through CuAAC click reactions of DNA containing 5-ethylenuracil. Chemistry - A European Journal 24(33):8311-8314.**

Question: Can 5-ethyleneuracil incorporated into DNA be utilized in click reactions to turn transcription off?

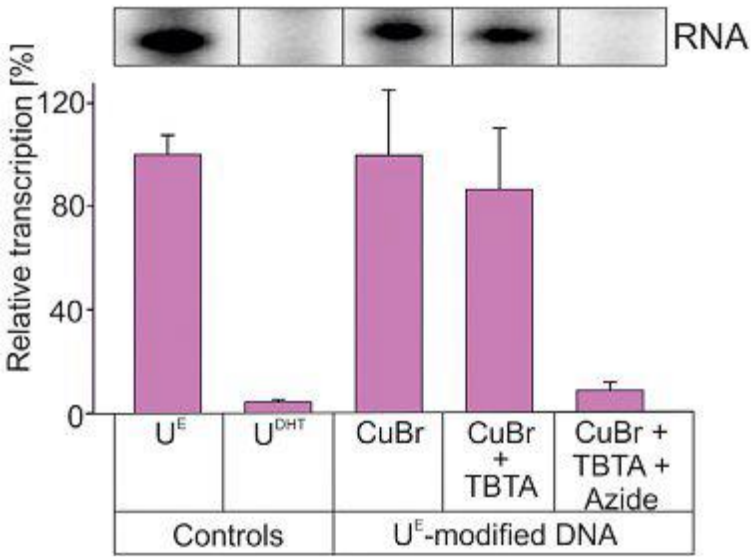


Figure 3. *In vitro* transcriptions from DNA^{E} template. CuBr, the catalyst, does not negatively affect the reaction. TBTA (Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine) facilitated the reaction. 3-azidopropane-1,2-diol (Azide) is the bulky ligand that abolishes transcription upon the click reaction. Primary data (radioactively labeled RNA) are shown above the graph.

In the previous papers (see above) we demonstrated that bulky modifications of bases inhibit transcription. Here, we selected a small modification, 5-ethyleneuracil, that permits transcription with *E. coli* RNAP. Then we tested whether its further modification by click reactions, resulting in dihydroxypropyltriazoles, turns transcription off. The project required massive optimization of reaction conditions. Fortunately, we were able to identify conditions of the click reaction that did not interfere with the integrity of DNA and allowed subsequent transcription experiments. The experiments confirmed the feasibility of the concept, successfully turning transcription off (**Fig. 3**). This, then, will allow for future manipulations of gene expression cells: transforming the cells with modified DNA and then turning the transcription off when required.

Answer: 5-ethyleneuracil in DNA can be modified to dihydroxypropyltriazole by click reactions and stop transcription.

Chapter 2. Transcription initiating substrates

Transcription starts from promoters and RNAP typically uses four canonical substrates: nucleoside triphosphates (NTPs: A, C, G, and U). Transcription initiation begins usually with A or G, the remaining two NTPs are used less frequently (Helmann, 1995). The initiating NTPs are termed iNTPs and their concentration plays an important regulatory role in the activity of a number of promoters (Murray et al., 2003). Besides the four canonical substrates, RNAP can utilize alternative metabolites, such as nicotine amide adenine dinucleotide, the essential participant of many redox reactions (Julius et al., 2018; Lin and Guarente, 2003).

To begin transcription, RNAP must first bind promoter DNA and form the so called closed complex. In this complex the two DNA strands are not separated. Next, RNAP isomerizes through at least one kinetic intermediate to form the open complex, where the two DNA strands are unwound and form the transcription bubble. NTPs (or other initiating substrates) can then access the active site, bind with the template strand at +1, and after subsequent binding of the +2 NTP, the first catalytic step can occur (Haugen et al., 2008). All the steps during transcription initiation can be rate limiting. The stability of the open complex is critical for the ability of the promoter to be regulated by the concentration of the iNTP (iNTP binding stabilizes the open complex and stimulates transcription) (Gaal et al., 1997). This type of regulation is especially important for ribosomal RNA (rRNA) promoters that by this mechanism sense the nutritional status of the cell. The open complex is (in gram negative bacteria) also targeted by ppGpp, the alarmone (Barker et al., 2001). This small molecule effector is synthesized by RelA, a protein that associates with ribosomes (Hauryliuk et al., 2015). ppGpp is made in response to amino acid starvation and by destabilizing the open complex at rRNA promoters helps shut down transcription of rRNA operons as new ribosomes are not required in poor nutritional conditions.

This Chapter describes the discovery of how rRNA promoters are regulated in *B. subtilis* by [iNTP] and ppGpp, how this differs from the longer-studied *E. coli*, what promoter sequence elements affect this type of regulation, and how we discovered the mechanism and requirements of NAD incorporation at the 5' end

of RNA. The studies in this Chapter were done at University of Madison, WI, USA while being a postdoc in the lab of Rick Gourse (EMBO J), at Institute of Molecular Genetics, Prague (Mol Microbiol), and the remaining at the Institute of Microbiology, Prague. An important collaborator for several of these studies was Dr. Ivan Barvík from Charles University in Prague.

4. **Krásný, L. and Gourse R. L. (2004) An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. EMBO J. 23:4473-4483.**

Question: What is the regulation of rRNA promoters in *B. subtilis*?

Transcription of rRNA operons (an rRNA operon consists of 16S, 23S, 5S RNA plus tRNAs) represents > 60 % of transcription in exponentially growing cell; to the contrary, in stationary phase cell it is almost nonexistent (Gourse et al., 1996). This is dictated by the need of the cell for new protein synthesis, which is most required when nutrients are abundant and the cell can grow and divide. Therefore, it must be tightly regulated. Historically, rRNA promoters of *E. coli* were the models from which most of our knowledge about ribosome synthesis had been derived (Schneider et al., 2003).

In *E. coli*, the rRNA core promoter (minimal region required to initiate transcription: cca 40 bp including -35 and -10 hexamers) is preceded by the UP element, an AT rich region that interacts with α subunits of RNAP and stimulates transcription (Estrem et al., 1999; Krasny et al., 2000). Further upstream, several FIS binding sites are located, allowing binding of FIS proteins that interact with RNAP and contribute to stimulation of transcription (Bokal et al., 1997). *E. coli* rRNA promoters form highly unstable open complexes and are regulated by iNTP and ppGpp. ppGpp directly binds to *E. coli* RNAP (two binding sites) and reduces the open complex life-time (Ross et al., 2016). iNTPs, on the other hand, increase the stability of the open complex (Gaal et al., 1997). This type of regulation is potentiated by DksA, a protein that binds to *E. coli* RNAP and negatively affects the stability of the open complex (Paul et al., 2004).

E. coli is a gram negative bacterium and at the beginning of this project it was not clear how growth rate regulation of rRNA promoters is achieved in this organism. Even more intriguingly, regulation of rRNA expression in gram positive bacteria was a complete unknown. Hence, I decided to decipher this regulation in *B. subtilis*.

The experiments revealed that the iNTP for rRNA promoters in *B. subtilis* was exclusively G (in *E. coli* it is A, C, or G) and the intracellular GTP concentration regulated their activity (later confirmed by others, e. g. (Kriel et al., 2012; Natori et al., 2009)). ppGpp, on the other hand, did not bind *B. subtilis* RNAP and functioned indirectly by affecting the GTP concentration. This was mediated by (i) direct consumption of GTP (from which (p)ppGpp is made), and (ii) inhibition of the GTP biosynthesis. In addition, I showed that the architectures of *E. coli* and *B. subtilis* rRNA promoters differed. I demonstrated that the AT-rich upstream element (UP element; upstream of the core promoter) that in *E. coli* stimulated rRNA transcription ~50x, stimulated in *B. subtilis* only ~ 3x, suggesting that the *B. subtilis* core promoter is already strong. Finally, no homologue FIS or DksA were found in *B. subtilis*.

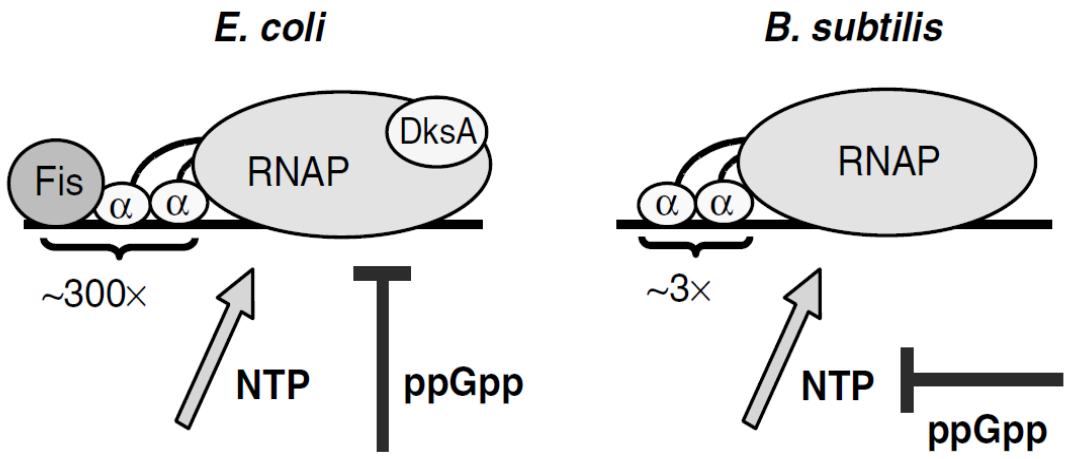


Figure 4. Schematic diagram illustrating mechanisms contributing to *rrn* P1 promoter activity in *E. coli* versus *B. subtilis*. The transcription factor Fis and RNAP αCTD binding to UP element DNA account for the unusually high activity of rrn P1 promoters from *E. coli*, but not *B. subtilis*. Changing NTP and ppGpp concentrations regulate rRNA promoter activities in both bacteria, but in *B. subtilis* ppGpp inhibits rRNA transcription indirectly by reducing GTP levels.

Answer: *B. subtilis* rRNA promoters are directly regulated exclusively by GTP and indirectly by (p)ppGpp. *B. subtilis* and *E. coli* represent two evolutionary mechanisms of regulaton of rRNA expression (**Fig. 4**).

5. **Krásný, L., Tišerová, H., Jonák, J., Rejman, D., and Šanderová, H. (2008) The identity of the transcription +1 position is crucial for changes in gene expression in response to amino acid starvation in *Bacillus subtilis.* Mol Microbiol 69: 42-54.**

Question: Does the identity of the transcription +1 postion (A or G) affect gene expression?

During the previous study we noticed that when the (p)ppGpp level increased in response to amino acid starvation, the GTP level decreased AND, in parallel, the ATP level increased (**Fig. 5A**). This was because when (p)ppGpp inhibited the GTP synthesis, the level of IMP, the last common intermediate for GTP and ATP synthesis increased, and this resulted in an increase in ATP concentration (Kriel et al., 2012; Lopez et al., 1981). This observation led to a hypothesis that the identity of the iNTP might affect the promoter response during amino acid starvation. This hypothesis also correlated with the exclusivity of G as the +1 nucleotide of rRNA promoters, the instability of open complexes of promoters driving transcription of genes encoding proteins for biosynthesis of amino acids

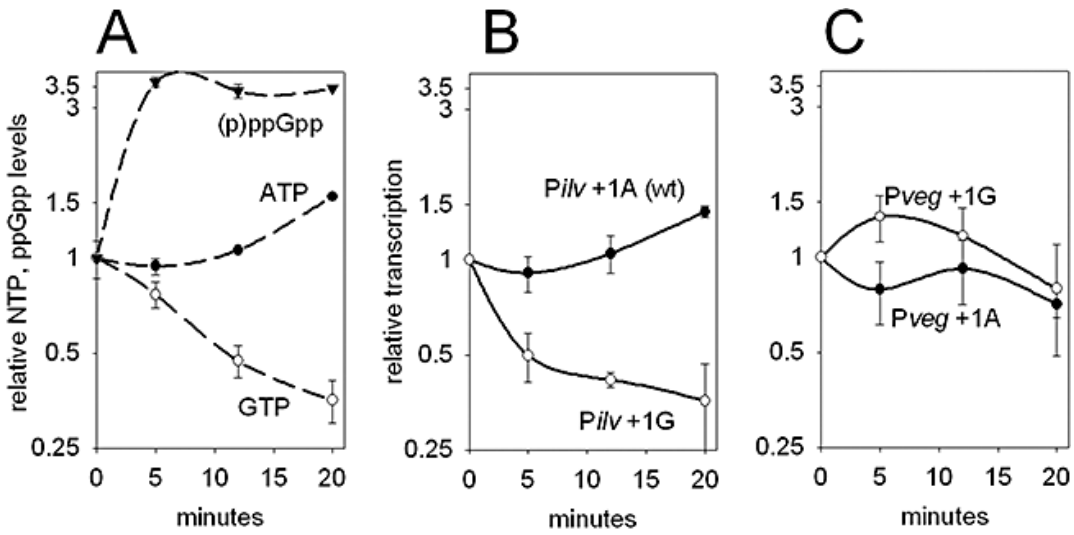


Figure 5. The effects of the identity of +1 on promoter activity of P*ilv* and P*veg* during stringent response in *B. subtilis*. The stringent response was induced at time zero. To enable visual comparison of changes in [NTP] and promoter activity, the values were normalized to 1 at time zero with respect to the induction of the stringent response. wt refers to the identity of +1. **A.** Changes in ATP (closed circles), GTP (open circles) and (p)ppGpp levels (triangles). The GTP levels were 30–40% of the ATP levels at time zero. **B.** Changes in activities of core promoter versions of P*ilv*+1A (closed circles) and P*ilv*+1G (open circles).**C.** Changes in activities of core promoter versions of P*veg*+1A (closed circles) and P*veg*+1G (open circles).

(their activity is stimulated during the stringent response), and the fact that they initiated with ATP.

The experiments revealed that the identity of the transcription +1 position (A or G) plays a pivotal role in the regulation of iNTP-sensitive (i. e. [iNTP]-responding) promoters (*e. g.* P*ilv*). Changing the +1A to +1G and vice versa, reprogrammed the response of these promoters to nutritional stress (**Fig. 5B**). Moreover, we demonstrated that this type of regulation is not restricted to a few promoters but it is a genome-wide phenomenon. P*veg* was a control promoter that was not much affected by iNTP changes (**Fig. 5C**).

Answer: The identity of the transcription +1 position qualitatively affects the change in gene expression (increase or decrease) of iNTP-sensitive promoters initiating with ATP or GTP during amino acid starvation.

6. **Sojka L, Kouba T, Barvík I, Sanderová H, Maderová Z, Jonák J, Krásny L. (2011) Rapid changes in gene expression: DNA determinants of promoter regulation by the concentration of the transcription initiating NTP in *Bacillus subtilis*. Nucleic Acids Res 39(11): 4598-611.**

Question: What promoter sequence elements determine iNTP-regulation in *B. subtilis*?

Not all *B. subtilis* promoters can be regulated by the concentration of the iNTP. This regulation requires relatively unstable open complexes. In this study we used two promoters from *B. subtilis, rrnB* P1, regulated by the iNTP, and P*veg*, insensitive to changes in intracellular iNTP concentrations. We made a set of chimeric promoters, changing P*veg* in a stepwise manner into *rrnB* P1. We tested these promoters for their ability to respond to iNTP concentrations. The experiments revealed that the -10 to +1 region (corresponding to the transcription bubble) contains elements responsible for the regulation (**Fig. 6**). Specifically, -5T, which is almost universally conserved, proved to be critical for the regulation. The identified sequences radically differed from rRNA promoter sequences identified in previous studies with *E. coli* as important for regulation by the [iNTP]

(Gaal et al., 1989). Hence, the two organisms (and by extrapolation gram-positive and gram-negative bacteria) have solved the same problem by different combinations of promoter sequences and structural features of RNAPs.

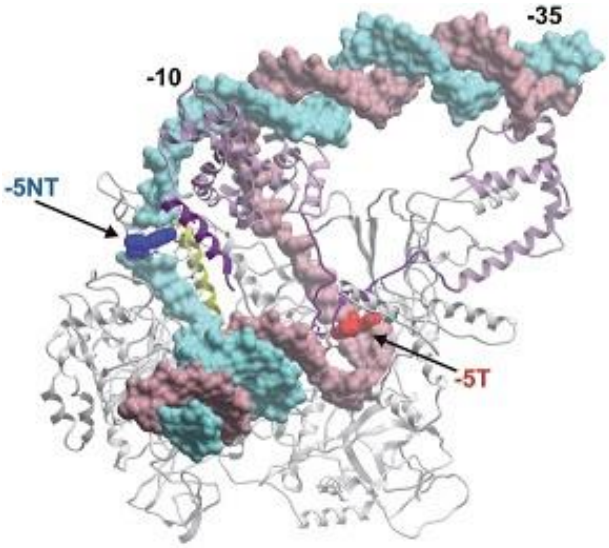


Figure 6. Model of the promoter -5 position nucleotides and their possible interactions with *B. subtilis* RNAP. The β' subunit was removed to view the areas of interest. Light gray, β; light magenta, σ^A; light pink, DNA template strand; light blue, DNA non-template strand. The regions of β and σ^A that contain amino acids that may interact with the -5 position bases are in yellow or magenta, respectively. The DNA non-template -5 position is in blue and indicated with '-5NT'; the DNA template -5 position is in red and indicated with '-5T'. -10 and -35 hexamers are indicated.

Answer: The transcription start site-proximal regions of rRNA promoters corresponding to the transcription bubble contain specific sequences, namely -5T, that make suboptimal interactions with RNAP. This consequently sets up the physiologically relevant stability of the complex, allowing for regulation of the promoter activity by the concentration of the iNTP both *in vitro* and *in vivo*.

7. **Bird, J. G., Zhang, Y, Tian, Y, Panova, N, Barvík, I, Greene, L, Liu, M, Buckley, B, Krásný, L, Lee, J. K, Kaplan, C.D., Ebright, R.E., Nickels, B. E. (2016) The mechanism of RNA 5' capping with NAD+, NADH, and desphospho-CoA. Nature, 535(7612):444-447.**

Question: Can RNAP utilize coenzymes as non-canonical substrates of transcription initiation?

Chen and coworkers published surprising findings (Chen et al., 2009), identifying NAD and dephospho-CoA as part of RNA in bacteria. Subsequently, Hana Cahova developed a chemical method that allowed to pull out and sequence the NAD-modified RNAs in *E. coli* (Cahova et al., 2015). During a discussion with her

I asked by what mechanism NAD was attached to RNA. The reply was that nobody knew at the time. People had tried to use RNAP and failed (Jaschke et al., 2016). Nevertheless, considering the structure of NAD – the adenine nucleotide part is perfectly capable of base-pairing with a T in the template strand, and the 3' OH end of the ribose permits attachment of another nucleotide – I proposed that RNAP was still the likeliest enzyme responsible for this RNA modification. We used a different approach, however, than the earlier unsuccessful attempts. We used a radio-labeled NAD in a defined cell-free *in vitro*

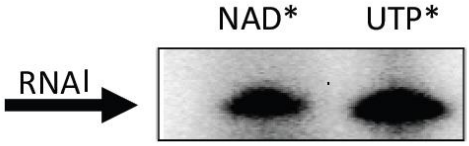


Figure 7. *In vitro* transcription from the RNA I promoter with *E. coli* RNAP. The identity of the labeled compound used for transcription is indicated above the primary data.

transcription system. The very first experiment then yielded a positive result: NAD utilization as the transcription initiating NTP was proven! (**Fig. 7**)

Subsequently, we performed a panel of additional experiments, showing that the incorporation indeed depended on the +1 position being an A, and that different promoters displayed different propensities for NAD incorporation into RNA. Additionally, we created an *in silico* model of RNAP initiating with NAD (**Fig. 8**). Then, at the Phage Meeting in Madison, WI, USA, I met Jeremy Bird from the lab of

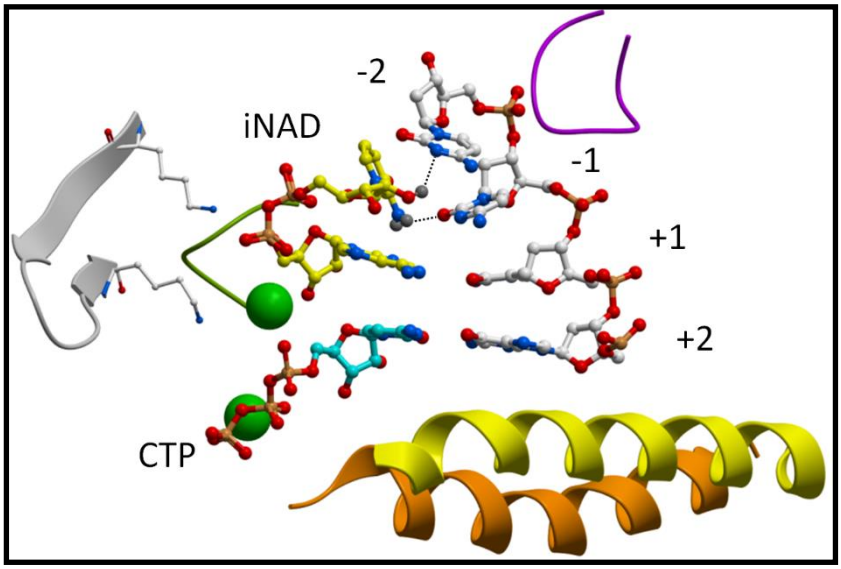


Figure 8. *De novo* transcription initiation complex with initiating NAD (replacing the first iNTP) and CTP in the active site of RNAP. Nucleic acids and amino acid side chains of Lys-β838, Lys-β846 are shown as stick models, and the bridge helix (yellow), trigger loop (orange), DFDGD motif (green), and sigma region 3.2 (magenta) are shown as cartoon models. Initiating NAD (iNAD) binds at the active site of RNAP through multiple interactions, including base pairing with the +1 DNA base, hydrogen bonding of the carboxamide group with the -1 DNA base (dashed line), ribose-base interactions with the -2 DNA base (dashed line), and salt bridges with conserved lysine side chains. CTP is stabilized by aspartate residues of the DFDGD motif of the β' subunit coordinating the Mg2+ (green spheres).

Bryce Nickels who gave a talk describing essentially the same results. After that, we got into contact with Bryce and put our results together. Importantly, the Nickels lab also had a 3D crystallographic model of the RNAP complex with NAD that corroborated the whole idea and confirmed also our *in silico* modeling. *In vivo*, then, the presence of the cap (NAD) stabilized RNA against degradation, prolonging its biological half-life.

Answer: NAD (and also NADH and dephospho-CoA) can be utilized by RNAP as transcription initiating substrates thereby introducing a novel type of an RNA cap into RNA.

8. **Vvedenskaya, I. O., Bird, J. G., Zhang, Y, Zhang, Y, Jiao, X., Barvík, I., Krásný, L., Kiledjian, M., Taylor, D. M., Ebright, R. H., Nickels, B. E. (2018) "CapZyme-Seq" comprehensively defines promoter-sequence determinants for RNA 5' capping with NAD+. Mol Cell 3;70(3):553-564.**

Question: What are the promoter sequence determinants of NAD utilization by bacterial RNAP?

In the previous study, we had shown an example how mutating the promoter sequence affected the efficiency with which NAD was utilized as the transcription initiating substrate. Here, in collaboration with the Nickels lab, we identified sequences around the +1 position that either stimulated or inhibited NAD incorporation. The study was performed both *in vitro* and *in vivo*, employing a newly developed approach for identification of NAD-capped RNAs. The identified promoter consensus that enhances NAD-capping has a potential to be utilized in bio-engineering/biotechnological applications, increasing the biological half-life of selected mRNAs, and thereby increasing expression of selected genes.

Answer: This study identified promoter sequences flanking the transcription start site (-4 to +4) as crucial for NAD incorporation.

9. **Barvík, I., Rejman, D., Panova, N., Šanderová, H., Krásný, L. (2017) Non-canonical transcription initiation: The expanding universe of transcription initiating substrates. FEMS Microbiol Rev 41(2): 131-8.**

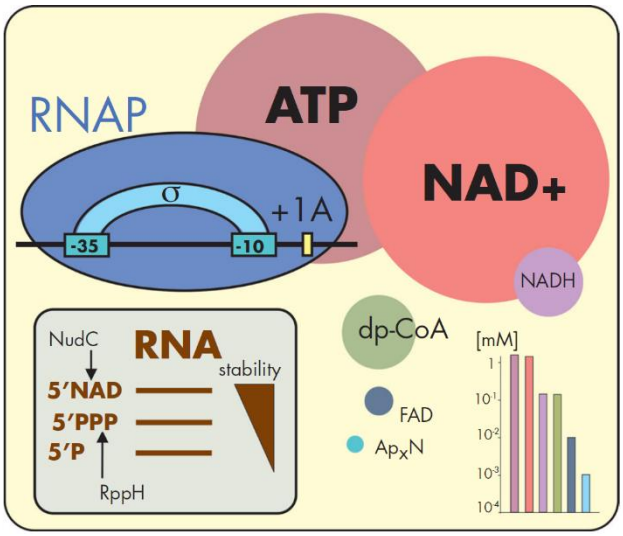


Figure 9. Known (ATP, NAD⁺, CoA) and hypothetical substrates (NADH, FAD, Ap_xN) of RNAP in vivo at promoters where +1 encodes adenine. RNAP is schematically depicted in complex with σ bound to promoter DNA; -35 and -10 consensus hexamers are indicated. The size of the circles approximately represents the relative pools of the inscribed small molecules in the cell. The graph in the bottom right hand corner shows the amounts of the depicted molecules based on the literature as discussed in the text. The color coding of the bars is the same as for the circles. In the inset, three types of 5' RNA ends are depicted in brown; the triangle on the right hand side shows the effect of the 5' end status on RNA stability. The arrows show where hydrolysis occurs and the respective enzymes responsible.

In this review we summarized the current knowledge about transcription initiation with a specific focus on various initiating substrates, including iNTPs, nanoRNAs (oligoribonucleotides), and coenzymes, such as NAD (**Fig. 9**). At this stage, it became apparent the NAD capping might be a universal phenomenon as reports had appeared about its incorporation into yeast and human RNA (Jiao et al., 2017; Walters et al., 2017). Interestingly, almost 50 % of all transcripts contained some percentage of NAD caps in human RNA (Jiao et al., 2017). Then, very recently, mitochondrial RNAs from yeast and humans were reported to contain 50 % and 10 % of NAD caps (e. i. 1/2 and 1/10 of total RNA is modified!), respectively (https://www.biorxiv.org/content/early/2018/07/31/381160). These studies, together with our findings, then refuted the previous speculations about other molecular machineries responsible for (as proposed) posttranscriptional modification of RNA (Luciano and Belasco, 2015). Moreover, NAD-capping by RNAP has been thus firmly established as trait likely present in all branches of life.

Chapter 3. sRNAs interacting with RNAP

sRNAs are non-coding RNAs, typically of <300 nt in length. They may have structural and/or regulatory roles. The very first sRNA ever discovered was 6S RNA from *E. coli*, which is visible as a distinct band in gels containing total RNA from stationary phase cells (Storz et al., 2011). Its amount in exponential phase is much lower. Although 6S RNA was discovered in 1960-ies, it was only in 2000 when Karen Wassarman determined that 6S RNA bound to RNAP, specifically to its holoenzyme form in complex with the main sigma factor (Wassarman and Storz, 2000). Later on, it was established that the secondary structure of 6S RNA reminded of a promoter with an open transcription bubble. To this bubble, RNAP binds, mimicking thus the transcription initiation complex (Steuten et al., 2013). When the RNAP holoenzyme is sequestered in complex with 6S RNA, it is transcriptionally inactive. By this inactivation, this fraction of RNAP is "conserved", still present in the cell, yet not engaged in active transcription. This mechanism helps the cell switch gears from the rapid growth in exponential phase to the more subdued state in stationary phase. When the conditions change, RNAP, which is by definition a DNA-dependent RNA polymerase, can initiate transcription from the 6S RNA template, transcribe a short 14-19 nt transcript, and dissociate from it. 6S RNA is then rapidly degraded and RNAP resumes transcription (Wassarman and Saecker, 2006). At the start of our studies, 6S RNA had been identified in many but not in all species. Most notably, it seemed to be absent from mycobacteria, and we wished to determine whether it was so.

10. **Pánek, J., Krásný, L., Bobek, J., Ježková, E., Korelusová, J., Vohradský, J. (2011) The suboptimal structures find the optimal RNAs: homology search for bacterial non-coding RNAs using suboptimal RNA structures. Nucleic Acids Res. 39(8):3418-26.**

Question: Can we find 6S RNA genes in bacterial genomes based on *in silico* approaches?

This study was initiated by and done in collaboration with Dr. Josef (Pepa) Pánek from the Vohradsky lab. Pepa developed an *in silico* approach to search for and

identify genes of structured non-coding RNAs (ncRNAs) in DNA databases. These RNAs often display non-conserved nucleotide sequences but possess conserved secondary structures. As a case study, we selected 6S RNA with its extensive base-paired regions and the essential central bubble. The developed *in silico* approach was based on the assumption that unlike optimal and consensus structures, suboptimal structures are capable of capturing RNA homology even in divergent bacterial species. A computational procedure for the identification of homologous ncRNAs using suboptimal structures was created. The suggested procedure was applied to many strongly divergent bacterial species and was capable of identifying homologous 6S RNAs. We subsequently showed that a 6S RNA homolog (termed Ms1) identified in mycobacteria was indeed a *bona fide* gene, highly expressed in stationary phase, reminiscent of 6S RNA. Nevertheless, immunoprecipitation of the RNAPσ^A holoenzyme from *M. smegmatis* revealed no interaction with Ms1. What a disappointment! (A control experiment with *B. subtilis* RNAPσ^A yielded a positive result – interaction with *B. subtilis* 6S RNA.) (**Fig. 10**).

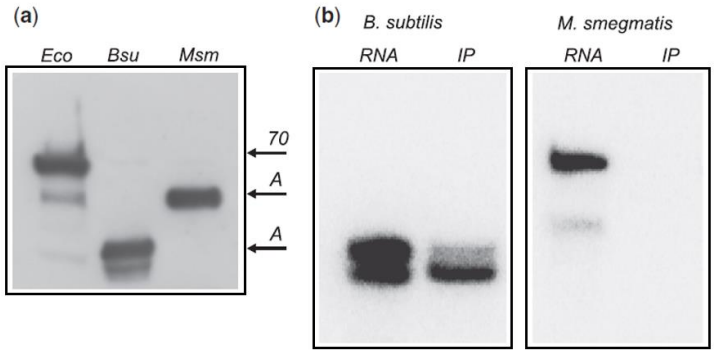


Figure 10. Ms1 does not bind to RNAP holoenzyme. Imunoprecipitation of 6S RNA. **A.** Western blot of total protein from *E. coli, B. subtilis* and *M. smegmatis* probed with monoclonal antibody against σ^{70} from *E. coli*. The antibody reacts with the main housekeeping sigma factors from all three organisms. **B.** Northern blot of total RNA (RNA) and the products of immunoprecipitation experiments (IP). The *B. subtilis* blot (positive control) was probed with an oligonucleotide against 6Sb RNA demonstrating the presence of this ncRNA on *B. subtilis* RNAP containing σ^A. The *M. smegmatis* blot was probed with an oligonucleotide against Ms1 RNA, demonstrating that it not a *bona fide* 6S RNA.

Answer: A novel *in silico* approach based on suboptimal structures was developed and demonstrated to be able to identify 6S RNA genes in many divergent bacteria where this gene had not been identified previously.

Nevertheless, the RNA encoded by the predicted 6S RNA gene in mycobacteria (Ms1), despite its 6S-like predicted secondary structure, did not bind to RNAPσ^A and thus was not a 6S RNA.

11. **Hnilicová, J., Jirát Matějčková, J., Šiková, M., Pospíšil, J., Halada, P., Pánek, J., Krásný, L. (2014) Ms1, a novel sRNA interacting with the RNA polymerase core in mycobacteria. Nucleic Acids Res 42(18):11763-76.**

Question: What is the binding partner of Ms1 in the cell?

The previous study prompted us to start from scratch with respect to identification of the Ms1 binding partner(s). We employed a combination of several approaches, including glycerol gradient ultracentrifugation and pull-down with biotinylated Ms1 as the bait. After the glycerol gradient ultracentrifugation we identified fractions (separated by the density of the gradient) with Ms1. Then, by mass spectrometry, we analyzed the protein content of these fractions. With the biotinylated Ms1, we pulled out proteins that interacted with this bait in cell lysates. This was followed by identification of the proteins by mass spectrometry. Both approaches yielded several proteins. Most notably, and by both approaches, we identified subunits of the RNAP core but

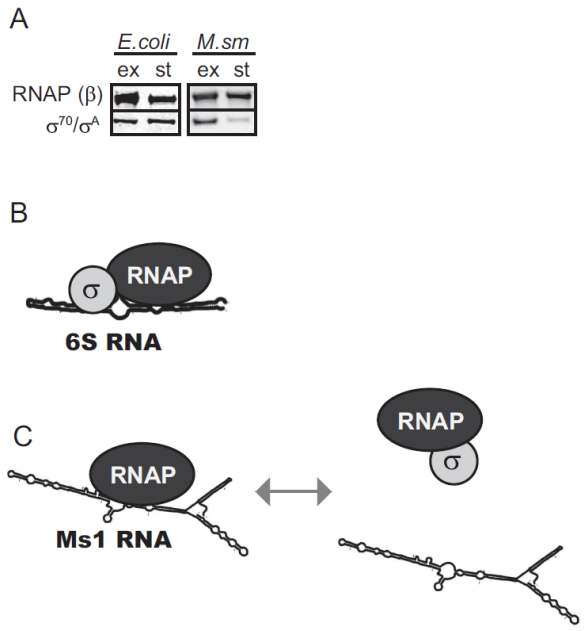


Figure 11. Modes of interaction of sRNAs with bacterial RNAP. **A.** The level of σ^A relative to β dropped in *Mycobacterium smegmatis* cells harvested 12 h after entry into stationary phase. In *Escherichia coli,* the relative protein level of σ^{70} to β remained unchanged even after 16 h in stationary phase. The experiment was repeated 3× with identical results. **B.** 6S RNA (e. g. *E. coli, Bacillus subtilis*) binds to RNAP containing the main *σ* factor. **C.** Ms1 (mycobacteria) binds to the RNAP core in the absence of *σ* factors and the presence of σ^A decreases this interaction.

not the main sigma factor or any alternative sigma factor among these proteins. The difference in the interaction of the two types of sRNAs (Ms1 or 6S RNA) with RNAP possibly reflects the difference in the composition of the transcriptional machinery between mycobacteria and other species. Contrary to *E. coli*, stationary phase *M. smegmatis* cells contain relatively few RNAP molecules in complex with σ^A (**Fig. 11**).

Answer: Ms1 binds to the RNAP core, representing a novel type of a sRNA interacting with RNAP.

12. **Šiková, M., Janoušková, M., Ramaniuk, O., Páleníková, P., Pospíšil, J., Bartl, P., Suder, A., Pajer, P., Kubičková, P., Pavliš, O., Hradilová, M., Vítovská, D., Šanderová, H., Převorovský, M., Hnilicová, J. and Krásný, L. (2019) Ms1 RNA increases the amount of RNA polymerase in *Mycobacterium smegmatis*. *Mol Microbiol* (in press).**

Questions: How is Ms1 accumulation regulated? What is the effect of Ms1 on the transcriptome?

In this follow-up study we identified the Ms1 promoter in both *M. smegmatis* and *M. tuberculosis*. In addition, we showed that the core promoter was relatively weak and was stimulated by cca. two orders of magnitude by upstream DNA sequences that bind yet unknown transcription factors. The activity of the promoter region, surprisingly, was about the same in both exponential and stationary phases. Therefore, the Ms1 accumulation must stem from its differential degradation. Consistent with this model, we identified PNPase, an RNase that degrades Ms1 both *in vitro* and *in vivo*, as a factor that contributes to the differential accumulation of Ms1.

Next, we created an ΔMs1 strain and performed RNAseq experiments. Surprisingly, the absence of Ms1 (a sRNA binding RNAP) did not have huge effects on gene expression during steady state. In stationary phase, only handful of genes were affected. Importantly, however, genes for β and β' subunits of RNAP were among those genes, and this was also reflected at the protein level. As a consequence, when stationary phase cells were diluted into fresh medium,

the mutant strain displayed slower outgrowth, suggesting that the pool of RNAP sequestered with Ms1 in wt, is utilized early on in this process, and its lack in the mutant leads to slower outgrowth. The mechanism of how Ms1 mediates this regulation is unknown and will be the subject of further studies.

Answers: Accumulation of Ms1 is achieved by its differential degradation and increased stability in stationary phase. Ms1 affects the level of transcripts encoding the two largest subunits of RNAP. This is important for the ability of the cell to start rapidly growing when stationary cells are diluted into fresh medium (**Fig. 12**).

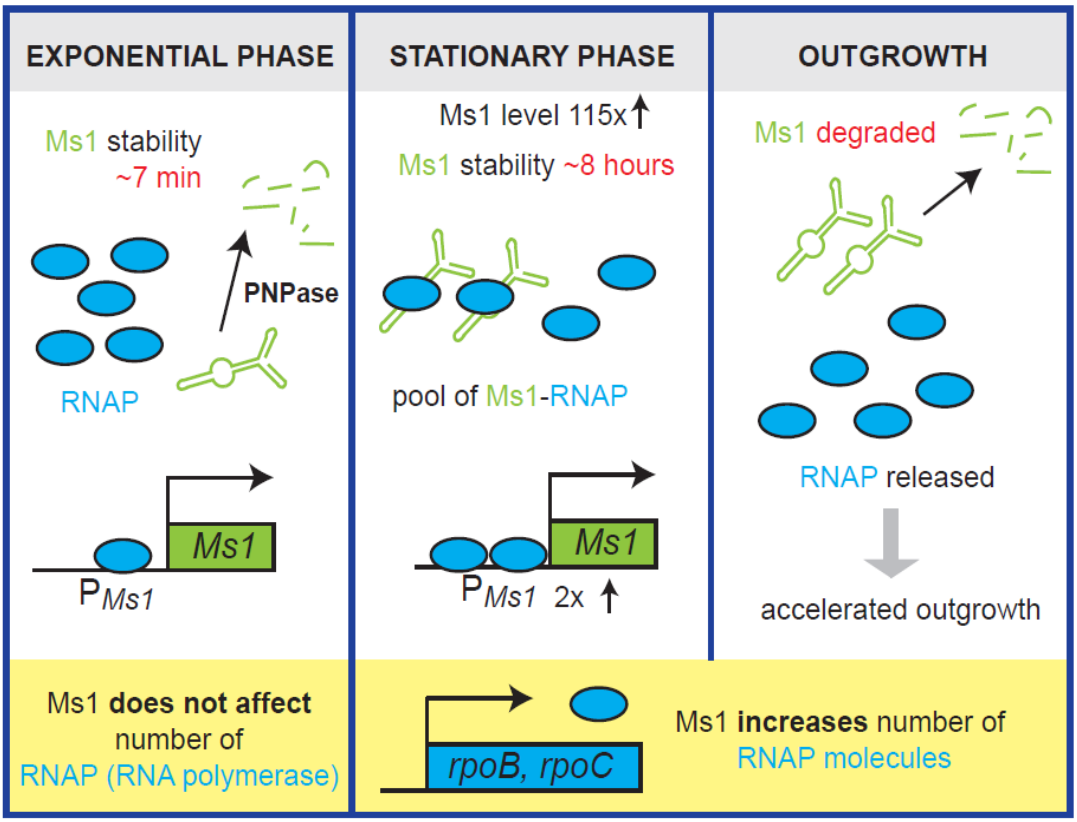


Figure 12. (LEFT) In exponential phase in mycobacteria, Ms1, a sRNA, is unstable and rapidly degraded. PNPase contributes to the degradation. The level of RNAP is about the same in both the Δ*Ms1* and *wt* strains. (MIDDLE) In stationary phase, the PNPase level is decreased, contributing to accumulation of Ms1. Ms1 interacts with, and sequesters a fraction of the RNAP core in a complex. In Δ*Ms1*, this fraction of the cellular RNAPs is absent. Nevertheless, the pool of RNAPs directly involved in transcription remains similar both in *wt* and Δ*Ms1* strains. (RIGHT) The absence of this extra pool of RNAP is phenotypically manifested during outgrowth from stationary phase - the difference between RNAP levels (Δ*Ms1* versus *wt*) remains similar for ~30 min and the growth Δ*Ms1* is slowed down for ~3 hours.

Chapter 4. RNAP and its protein interacting partners

Transcription is massively regulated by protein factors (Helmann, 2009). These factors interact with the DNA and/or RNA, and/or RNAP. Examples of DNA interacting factors are repressors that by various mechanisms inhibit transcription e. g. (Brinsmade, 2017). Rho factor is an example of a protein interacting with RNA and subsequently with RNAP. It is a transcription termination factor that was proposed to function by the "spider strategy: trap first, kill later" (Epshtein et al., 2010). According to this model, Rho first interacts with nascent RNA (the recognition sequences are poorly defined; generally, they are C-rich) and upon catching up with RNAP, alters the conformation of RNAP and rapidly inactivates the complex. This is followed by a subsequent, relatively slow dissociation step. Yet some transcription regulators interact with RNAP only. Examples of these factors are proteins interacting with the secondary channel of RNAP, by which NTPs enter the active site (Paul et al., 2004; Stepanova et al., 2009). GreA,B are such factors, acting on RNAPs during elongation when transcription gets stalled. Under such situations, RNAP tends to backslide and the 3' OH end of the most recently added nucleotide is not in the active site anymore but protrudes in the forward direction (Toulme et al., 2000). Therefore, RNAP cannot continue synthesizing further RNA. To resolve the situation, GreA binds to the secondary channel and by its coiled-coil domain reaches into the channel, reaching the vicinity of the active site. There, it alters the conformation of the active site, reversing the catalytic activity of RNAP. Instead of synthesis, it hydrolyzes the phosphodiester bond in the active site, cleaving off the few protruding nucleotides, and creates a new 3' OH end. This subsequently allows RNAP to resume transcription. Another example is DksA from *E. coli* (Paul et al., 2004). This protein is important for transcription initiation. Structurally, DksA and GreA are homologs. DksA increases the requirements of RNAP for the concentration of the iNTP for maximal transcription, and is essential in the cell for the physiologically relevant regulation of rRNA promoters by this mechanism. Moreover, it is required for RNAP regulation by ppGpp in gram-negative bacteria (Ross et al., 2016). In gram-positive bacteria, no DksA-like protein has been identified to date.

This Chapter focuses of several protein interacting partners of RNAP that either had been poorly characterized at the beginning of our studies or were discovered by us as new transcription factors. The first of these proteins is the small δ subunit of *B. subtilis* RNAP, an enigmatic protein (Weiss and Shaw, 2015), for which we solved the structure and defined its cellular roles. Next comes ε, another small subunit of *B. subtilis* RNAP (the name "ε" originated in our laboratory as well as the gene name, *rpoY*). We characterized its structure, binding to RNAP and attempted to decipher its cellular role. The next protein is HelD, a helicase-like protein, which we and others discovered to be a binding partner of *B. subtilis* RNAP (Delumeau et al., 2011). We characterized its effects on transcription. Next, Spx from *B. subtilis* is a protein that is important for the cell's response to various types of stress, most notably oxidative stress (Zuber, 2009). We characterized the genome-wide effect of this protein and unraveled its mechanistic functioning on selected promoters. Finally, and very recently, we were able to solve the 3D structure of two forms of mycobacterial RNAP, creating thus a basis for further studies of this enzyme complexes with selected factors.

Studies presented in this Chapter were possible also thanks to our collaborators, namely Dr. Lukáš Žídek from Masaryk University in Brno (NMR studies). The number of joint publications vastly exceeds those presented in this Habilitation Thesis. Next comes Dr. Míša Wimmerova (crystallographer, Masaryk University in Brno), then Dr. Peter Lewis (structural biologist) from Newcastle University in Australia, Dr. Jan Dohnálek (crystallographer, Biocev, Vestec), Dr. Tomáš Kouba (Cambridge, UK), and Dr. Olivier Delumeau and Dr. Philipe Noirot (genomic studies) from INRA Micalis, France.

4.1. Delta

13. Motáčková V., Šanderová H., Zídek L., Nováček J., Padrta P., Švenková A., Korelusová J., Jonák J., Krásný L., Sklenář V. (2010) Solution structure of the N-terminal domain of *Bacillus subtilis* delta subunit of RNA polymerase and its classification based on structural homologs. Proteins, 78(7):1807-10.

Question: What is the structure of the N-terminal domain of δ from *B. subtilis*?

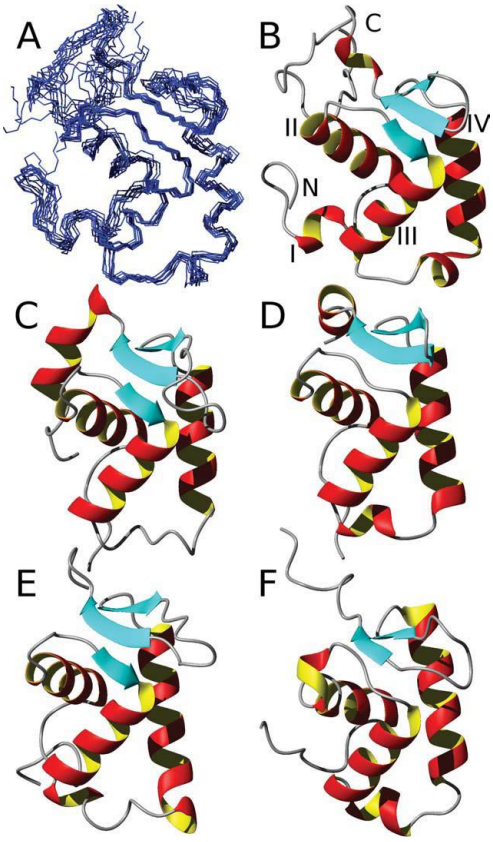


Figure 13. The N-terminal domain of δ and its structural homologs. **A.** Superimposed backbone traces of 10 Nδ structures with the lowest energy **B.** The representative (lowest energy) structure of Nδ compared to its closest structure homologs **C.** Forkhead Box Protein K2,2C6Y.PDB; **D.** Forkhead Box Protein P2, 2A07.PDB; **E.** Forkhead Box Protein 01, 3CO6.PDB; **F.** and Yeast linker Histone Hho1p, 1YQA.PDB. The N and C termini and the helices I to IV are labeled in Panel B.

While the $\alpha_2\beta\beta'\omega$ composition is conserved across the bacterial kingdom, Gram-positive Firmicutes contain an additional subunit, δ, which is encoded by the *rpoE* gene in the model bacterium *B. subtilis*. The δ subunit was first reported as an endogenous protein present in RNAP from phage SP01-infected *B. subtilis* cells, which was required for its accurate middle gene transcription (Pero et al., 1975). The *rpoE* gene specifies a protein of 173 amino acids (aa) with a molecular mass of 20.5 kDa. The protein is highly acidic (pI, 3.6) (Lampe et al., 1988). As determined by circular dichroism (CD) spectroscopy, it consists of two domains: (i) the N-terminal domain (NTD), which is structured; and (ii) the C-terminal domain, which is unstructured and whose amino acid composition - stretches of glutamic and aspartic acid residues - makes it virtually a polyanion (Lopez de Saro et al., 1995).

This study focused on solving the structure of the NTD. We prepared the appropriate construct and purified the protein with ^{13}C and

^{15}N for NMR studies. The structure of NTD was well-defined, consisting of four α-helices (helices I, II, III, and IV, formed by residues Q8-K12, L16-H27, F33-L44, G52-N63, respectively) and an antiparallel β-sheet composed of three short β-strands (residues V31-P32, F68-A70, and T75-L78) at the top of a ‘‘twisted tripod’’ formed by helices II, III, and IV (**Fig. 13**). The C-terminal part of NTD starting from residue R79, and containing the artificial His-tag region L93–H100, was unstructured. With the structure in hand, we searched for its structural homologs (no sequence homologs were identified by BLAST searches). Several successful hits of the were retrieved, including three proteins (PDB codes 2C6Y,32 2A07,33 and 3CO634) from the forkhead domain PFAM family (PF00250), and the GII domain of the yeast linker histone Hho1p (1YQA) (Fig. 13). All these proteins, including NTD, contained DNA/RNA fold. However, in the case of NTD, no DNA/RNA binding has ever been detected, indicating that this fold has a different function in δ.

Answer: The 3D structure of NTD of *B. subtilis* δ has been determined by NMR.

14. **Papoušková, V., Kadeřávek, P., Otrusinová, O., Rabatinová., A., Šanderová, H., Nováček, J., Krásný, L., Sklenář, V., Žídek, L. (2013) Structural study of the partially disordered full length delta subunit of RNA polymerase from *Bacillus subtilis*. Chembiochem 14(14): 1772-9.**

Question: What is the structure of the full-length δ?

In this study we structurally characterized the full-length δ from *B. subtilis*. This was especially challenging due to the high flexibility of the C-terminal domain (CTD). Various ^{15}N relaxation experiments were employed to describe the flexibility of both domains. The structure of NTD was essentially the same as that of the isolated NTD reported in the previous study. The relaxation data revealed that the C-terminal domain is more flexible, but its flexibility was not uniform. By using paramagnetic labels, transient contacts of the C-terminal tail with the N-terminal domain and with itself were identified. A propensity of the C-terminal domain to form β-type structures was detected by chemical shift analysis (the negatively charged CTD interacted with the K-tract, a positively charged stretch

at the junction of the two domains. Thus, CTD is an example of an intrinsically disordered protein (IDP) that, nevertheless, is not totally chaotic. Its sequence molds its spatio-temporal architecture, and underlies its physiologically relevant functioning. IDPs appear to be the rule rather than exceptions among proteins, with many functions in the cell, both in health and disease (Sigalov, 2016). Understanding the determinants of their transient conformations is an urgent future challenge.

Answer: The NTD in full-length δ possesses the same structure as isolated NTD, validating our previous results. Further, the CTD is highly flexible; nevertheless it displays a propensity towards interactions with the K-tract, creating thus an array of spatiotemporal conformations that are likely essential for functioning of the protein.

15. **Demo, G., Papoušková, V., Komárek, J., Kadeřávek, P., Otrusinová, O., Srb, P., Rabatinová, A., Krásný, L., Žídek, L., Sklenář, V., and Wimmerová, M. (2014) X-ray vs. NMR structure of N-terminal domain of delta subunit of RNA polymerase. J Struct Biol 187(2): 174-86.**

Question: Is there a difference in 3D structures of *B. subtilis* δ NTD obtained by crystallography and NMR?

This study was a bonus. Obtaining protein crystals is always a challenge. Hence, we were pleasantly surprised when we learned that Misa Wimmerova, a colleague of Lukas Zidek from Masaryk University, as a 'side project' managed to crystalize the *B. subtilis* δ NTD. Then came another surprise, the two structures, one from crystallography and the other from NMR, differed! We systematically investigated the cause of the discrepancies between the NMR and X-ray structures of δ NTD, addressing the pH dependence, presence of metal ions, and crystal packing forces. We showed that the crystal packing forces, together with the presence of Ni^{2+} ions, were the main reasons for the difference. In summary, the study illustrated that the two structural approaches might give unequal results, which need to be interpreted with care to obtain reliable structural information in terms of biological relevance.

Answer: The 3D structures of *B. subtilis* δ NTD obtained by crystallography and differed significantly. Further studies then determined that the NMR structure was the one which was physiologically relevant.

16. **Rabatinová, A., Šanderová, H., Jirát Matějčková, Korelusová, J., Sojka, L., Barvík, I., Papoušková, V., Sklenář, V., Žídek, L., and Krásný, L. (2013) The delta subunit of RNA polymerase is required for rapid changes in gene expression and competitive fitness of the cell. J Bacteriol 195(11): 2603-11.**

Question: What is the cellular role of δ in *B. subtilis*?

Despite extensive previous research from other labs (Weiss and Shaw, 2015) the full physiological role of δ was unknown. As δ binds RNAP, we systematically investigated the effect of δ on transcription. We discovered that δ decreased the stability of the open complex, changing thereby requirements of RNAP for the concentration of the iNTP. In the absence of δ, RNAP required relatively low concentration of the iNTP for maximal transcription (e. i. increased affinity for iNTP that was not physiologically relevant). The absence of δ then altered regulation of iNTP-responsive promoters *in vivo*, and severely compromised the competitive fitness of the cell. Further, we showed that GreA and YdeB, two proteins structurally/sequentially reminiscent of DksA from *E. coli*, did not affect RNAP regulation by the [iNTP].

Answer: *B. subtilis* δ affects the affinity of RNAP for the iNTP. This is essential for regulation of iNTP-responsive promoters, and, consequently, the competitive fitness of the cell.

4.2. Sigma factors

17. **Holátko, J., Šilar, R., Rabatinová, A., Šanderová, H., Halada, P., Nešvera, J., Krásný, L., Pátek, M. (2012) Construction of *in vitro* transcription system for *Corynebacterium glutamicum* and its use in the recognition of promoters of different classes. Appl Microbiol Biotechnol 96(2): 521-9.**

Question: Can we engineer a cell free *in vitro* transcription system from *Corynebacterium glutamicum*?

Our previous experience with defined *in vitro* transcription systems of *B. subtilis* and *E. coli* encouraged us to engineer a similar system for *C. glutamicum*. This organism is a rod-shaped gram-positive bacterium, which is extensively used in the industry as an amino acid producer (Nesvera and Patek, 2011). Here, by putting a His-tag on the β' subunit of RNAP in *C. glutamicum* we managed to create a strain for purification of the RNAP core. In parallel, we cloned and purified *C. glutamicum* σ^A and σ^H factors. We reconstituted RNAP holoenzymes *in vitro* and obtained transcription from σ^A and σ^H–dependent promoters.

Answer: We prepared a new tool for studies of transcription regulation in *Corynebacterium glutamicum*: an *in vitro* transcription system.

18. **Zachrdla M, Padrta P, Rabatinová A, Šanderová H, Barvík I, Krásný L, Žídek L (2017) Solution Structure of Domain 1.1 of the σA Factor from *Bacillus subtilis* is Preformed for Binding to the RNA Polymerase Core. J Biol Chem 292(28): 11640-17.**

Question: What is the structure of Domain 1.1 of the σ^A factor from *Bacillus subtilis*?

σ^A is the main, vegetative sigma factor in *B. subtilis*, homologous to other primary sigma factors in other organisms (e. g. σ^{70} in *E. coli*). These factors consist of several domains, 1 to 4. These domains are further divided into subdomains. The

subdomains of primary sigma factors and their interactions with promoter DNA have been extensively studied. Domains 4.2 and 2.4 interact with promoter consensus hexamers, -35 and -10, respectively (Davis et al., 2017). The structure of domain 1.1, then, has been elusive for many years as the available crystal structures lacked electron density for this part of the protein, suggesting a significant degree of movement. This had changed, and at the start of our study two 3D structures of 1.1 were available: from *E. coli* in complex with RNAP and from *T. maritima* solved free in solution (Bae et al., 2013; Schwartz et al., 2008). However, these two structures significantly differed, and it was unclear whether this difference was due to an altered conformation upon RNAP binding or to differences in intrinsic properties between the proteins from these two distantly related species. Therefore, we solved the solution structure of 1.1 from the Gram-positive bacterium *Bacillus subtilis* by NMR (**Fig. 14**). We found that *B. subtilis* 1.1 is highly compact because of additional stabilization not present in1.1 from the other two species and that it is more similar to *E. coli* 1.1. Moreover, modeling studies suggested that *B. subtilis* 1.1 requires minimal conformational changes for accommodating RNAP in the DNA channel, whereas *T. maritima* 1.1 must be rearranged to fit therein. Thus, the mesophilic species *B. subtilis* and *E. coli* share the same 1.1 fold, whereas the fold of 1.1 from

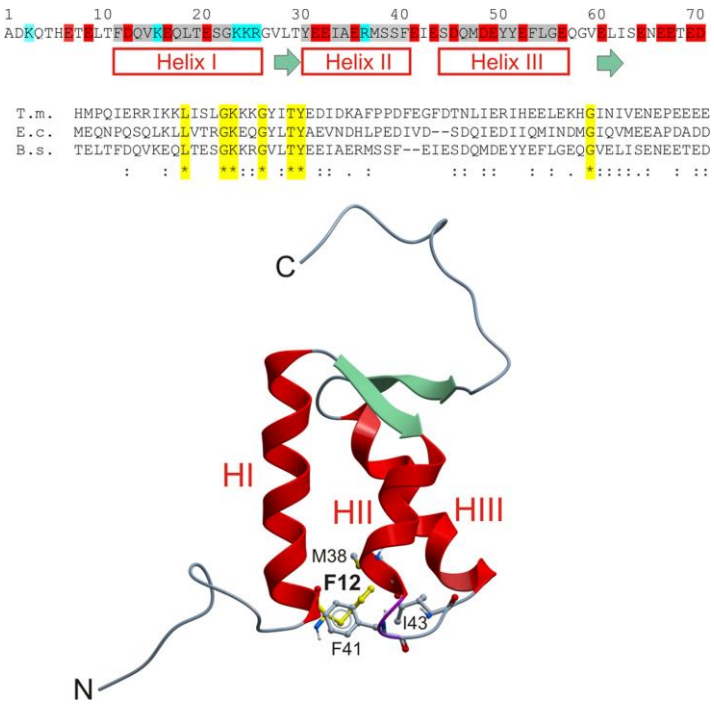


Figure 14. σ1.1 of *B. subtilis*. *Top panel*, amino acid sequence of *B. subtilis* σ1.1 with helices indicated below the sequence. Negatively charged residues are shown in *red*, and positively charged residues are in blue. An alignment of amino acid sequences from *T. maritima*, *E. coli*, and *B. subtilis* is shown below. Asterisks indicate fully conserved amino acid residues, colons indicate conservation of amino acid residues with strongly similar properties, and *dots* indicate conservation of amino acid residues with weakly similar properties. *Bottom panel*, solution structure of σ1.1 of *B. subtilis*. Helices HI–HIII, N/C termini and selected amino acid residues are indicated.

the thermophile *T. maritima* is distinctly different.

Answer: The 1.1 domain of σ^A from *B. subtilis* is similar to its counterpart from *E. coli* and these two structures differ from the same protein region from *T. maritima*. Moreover, the *B. subtilis* domain 1.1 is even more compact than that one of *E. coli*, and appears preformed for binding to the DNA channel of RNAP.

19. Ramaniuk O, Černý M, Krásný L, Vohradský J (2017) Kinetic modelling and meta-analysis of the *B. subtilis* SigA regulatory network during spore germination and outgrowth. Biochimica et Biophysica Acta (BBA) - Gene Regulatory Mechanisms 1860(8):894-904.

Question: Which genes are regulated by RNAP complexed with σ^A during germination and outgrowth from spores in *B. subtilis*?

σ^A (SigA) is the main, vegetative sigma factor in *B. subtilis* with hundreds of genes identified in its regulon (Nicolas et al., 2012). The goal of this study was to define this regulon during spore germination and outgrowth. The approach was a combination of bioinformatics (data mining) and wet-lab experiments. We performed a meta-analysis and kinetic modelling of gene expression control by sigma factor SigA based on microarray data from 14 time points (Keijser et al., 2007). The analysis computationally modeled the direct interaction among SigA, SigA-controlled sigma factor genes (sigM, sigH, sigD, sigX), and their target genes. Of the> 800 known genes in the SigA regulon, as extracted from databases (Zhu and Stulke, 2018), 311 genes were analysed, and 190 were confirmed by the kinetic model as being controlled by SigA. For the remaining genes, alternative regulators satisfying kinetic constraints were suggested. The kinetic analysis suggested another 214 genes as potential SigA targets. The modelling was able to (i) create a particular SigA controlled gene expression network that is active under the conditions for which the expression time series was obtained, and where SigA is the dominant regulator, (ii) suggest new potential SigA target genes, and (iii) find other possible regulators of a given gene or suggest a new mechanism of its control by identifying a matching profile of

unknown regulator(s). We tested selected predicted regulatory interactions experimentally (**Fig. 15**). Out of the ten tested putative SigA dependent genes, five proved to be regulated by SigA without the requirement for additional factors.

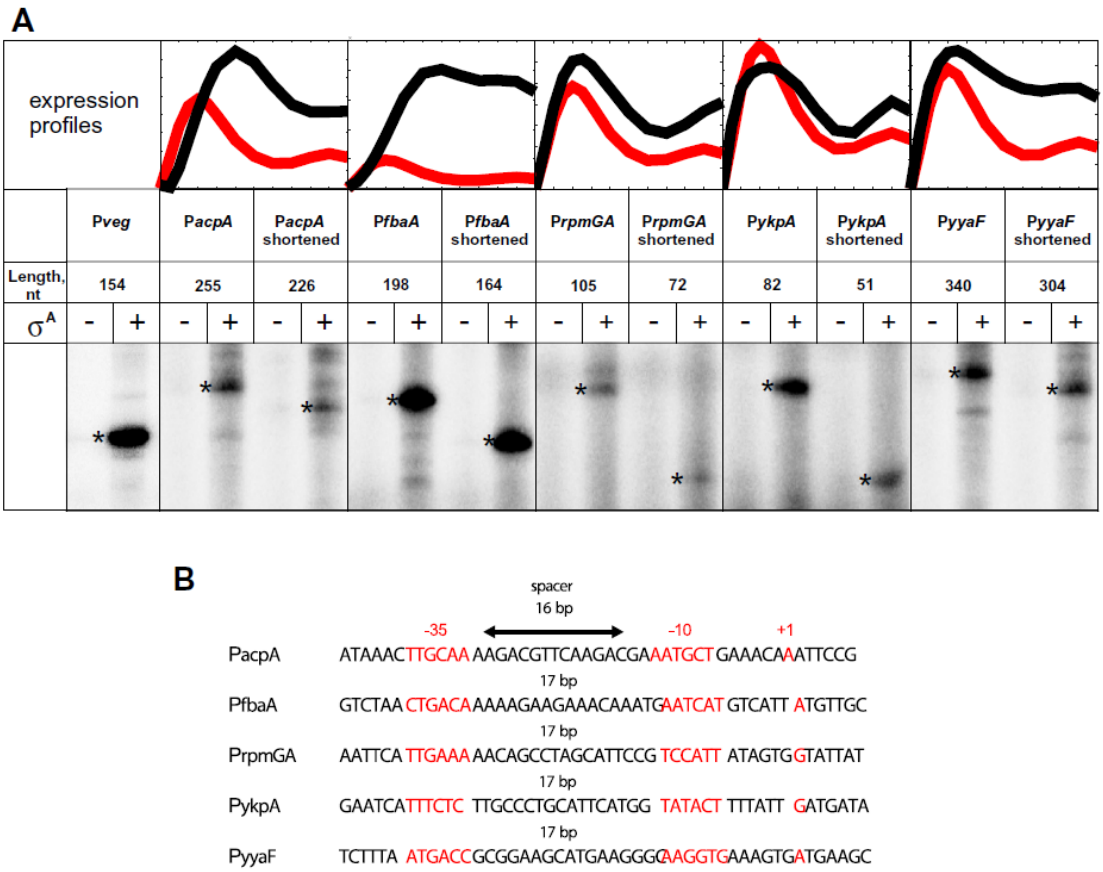


Figure 15. *In vitro* multiple round transcriptions of DNA fragments derived from upstream regions of genes predicted to be SigA-dependent. **A.** Representative primary data. Each reaction was performed with the RNAP core and SigA-containing holoenzyme to demonstrate that the core had not been contaminated with sigma factors prior to its reconstitution with SigA. Radioactively labelled samples were loaded onto polyacrylamide gels. P*veg* was used as a positive control. Transcript length was calculated with an RNA ladder (data not shown). The differences in length between the long and shortened fragment variants were in the 29–36 bp range. The distance between the long and shortened transcript variants in the gel differed for different promoters because each transcript had a unique length. Asterisks indicate the specific transcripts. The upper part of the figure shows respective kinetic modelling results. Red, SigA mRNA; black, specific gene. The scaling between boxes varies to accommodate the graphs as the levels of specific transcripts differed over a wide range. **B.** Alignment of putative promoter sequences identified by in vitro transcription assays. The −35 and −10 hexamers and the transcription start sites (+1) are indicated in red. Spacer regions between −35 and −10 hexamers are indicated.

Answer: In addition to known SigA-dependent genes, we identified >200 genes potentially regulated by this sigma factor. We validated the prediction and 50 %

of the tested genes proved to be *bona fide* targets of SigA regulation, thus extending our knowledge about the SigA regulon and its kinetics during spore germination and outgrowth.

20. Ramaniuk O, Převorovský M, Pospíšil J, Vítovská D, Kofroňová O, Benada O, Schwarz M, Šanderová H, Hnilicová J, Krásný L. (2018) σ^I from *Bacillus subtilis*: Impact on gene expression and characterization of σ^I-dependent transcription that requires new types of promoters with extended -35 and -10 elements. J. Bacteriol 200(17): e00251-18.

Question: What is the regulon of SigI in *B. subtilis*?

SigI was the least explored/defined sigma factor regulon in *B. subtilis* at the beginning of our studies (Liu et al., 2017). Considering the industrial importance of this model organism (Harwood et al., 2018), we decided to define its regulon and cellular roles. SigI had been previously implicated in adaptation of the cell to elevated temperature (Zuber et al., 2001). In this study, we provided a comprehensive characterization of this transcriptional regulator. By transcriptome

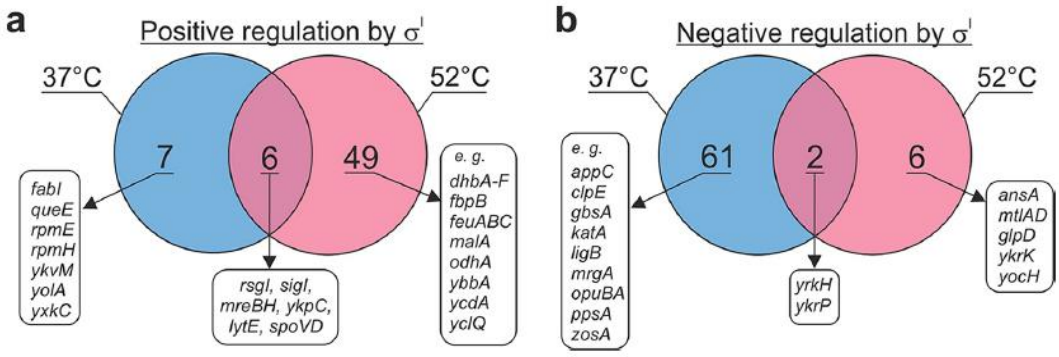


Figure 16. Genes in *B. subtilis* affected by σ^I. *B. subtilis* Δ*sigI-rsgI* and wt strains were grown at 37°C and 52°C in LB broth to an OD_{600} of ~0.45. RNA was extracted and libraries were prepared for transcriptome sequencing (RNA-seq). **A.** Genes positively regulated by σ^I. These genes were downregulated in the Δ*sigI-rsgI* strain compared to in the wt strain. **B.** Genes negatively regulated by σ^I. These genes were upregulated in Δ*sigI-rsgI* compared to wt.

sequencing (RNA-seq) of wild-type (wt) and SigI-null strains at 37°C and 52°C, we identified cca 130 genes affected by the absence of SigI (**Fig. 16**). Further

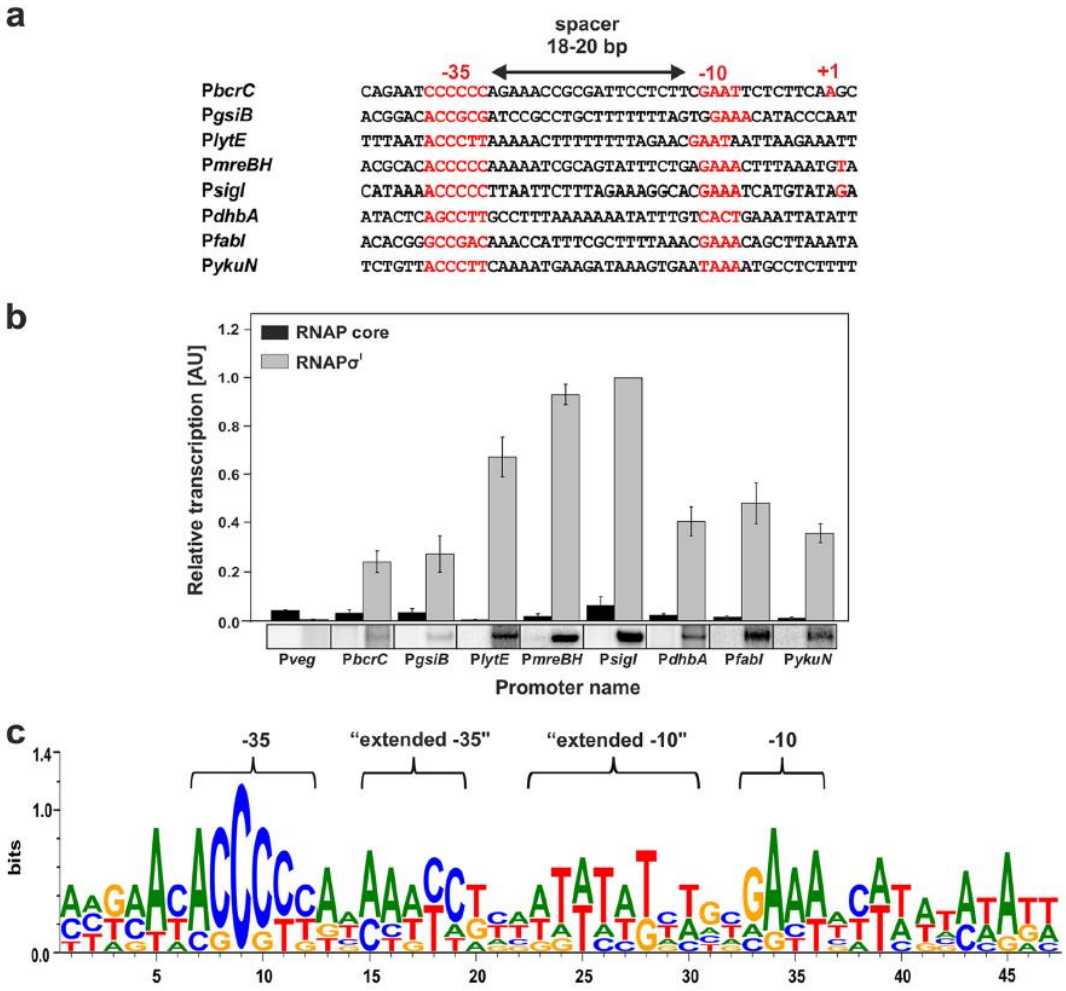


Figure 17. Multiple-round *in vitro* transcription assays with promoter regions of σ^I-regulated genes and RNAP σ^I. **A.** Alignment of σ^I-dependent promoters. The -10 and -35 elements and +1 position for P*sigI*, P*mreBH*, and P*bcrC* are in red. **B.** Transcription was performed with the RNAPσ^I holoenzyme and the RNAP core. Transcription with the RNAP core was used to assess potential contamination of the RNAP core with σ factors. Promoter P*sigI* was used as a control and its transcription was set as 1. P*veg* (σ^A dependent) was used as a negative control for RNAPσ^I. Primary data (radioactively labeled transcripts resolved on polyacrylamide [PAA] gels) are shown below the graph. The error bars show averages from three independent experiments ±SD. **C.** The σ^I consensus logo was created from the 8 promoter sequences shown in panel A. Conserved promoter elements are indicated above the logo.

analysis revealed that the majority of these genes were affected indirectly by SigI. The SigI regulon, i.e., the genes directly regulated by SigI, consisted of 16 genes, of which eight (the *dhb* and *yku* operons) are involved in iron metabolism. The involvement of SigI in iron metabolism was confirmed phenotypically. Next, we set up an *in vitro* transcription system and defined and experimentally validated the promoter sequence logo that, in addition to -35 and -10 regions, also contains

extended -35 and -10 motifs (**Fig. 17**). Thus, SigI-dependent promoters are relatively information-rich in comparison with most other promoters. In summary, this study supplied information about the least-explored SigI factor from the industrially important model organism *B. subtilis*.

Answer: The SigI regulon is small, consisting of 16 genes. Nevertheless, it is important for cell shape maintenance, heat-resistance, and iron metabolism. Moreover, the SigI-dependent promoters are unusually information rich, with four identified conserved motifs.

4.3. Epsilon

21. Keller, A., Yang, X., Wiedermannová, J., Delumeau, O., Krásný, L., Lewis, P. J. (2014) Epsilon: A new subunit of RNA polymerase found in Gram positive bacteria. J Bacteriol 196(20): 3622-32.

Question: What is the structure/position on RNAP/function of the ω_1 (ε) subunit of RNAP from *B. subtilis*?

RNAP from *B. subtilis* contains additional subunits/binding factors compared to RNAP from *E. coli* (Weiss and Shaw, 2015). In the model Gram-positive organism *B. subtilis*, core RNAP has the subunit composition $\alpha_2\beta\beta'\delta\omega_1\omega_2$. The occurrence of two ω subunits in *B. subtilis* at approximately 11 kDa (ω_1) and 9 kDa (ω_2) has been known for some time (Helmann, 2003). The ω_2 subunit is encoded by *rpoZ* (synonym, *yloH*) and is similar to the ω subunit present throughout eubacteria. The ω_1 subunit has been referred to as the second analogous ω subunit simply because it has a size similar to that of the true ω subunit. This annotation has been accepted despite there being no evidence to suggest that these two proteins are related in function. Using mTRAQ (mass differential tags for relative and absolute quantification) mass spectrometry, it has been shown that both of the ω subunits are approximately equimolar with the β subunit (Doherty et al., 2010). The *ykzG* gene is present in an operon with the physiologically important RNase RNaseJ1 (RnjA), and the two proteins are transcriptionally and translationally linked (Nicolas et al., 2012). So far, no functional link between these two proteins

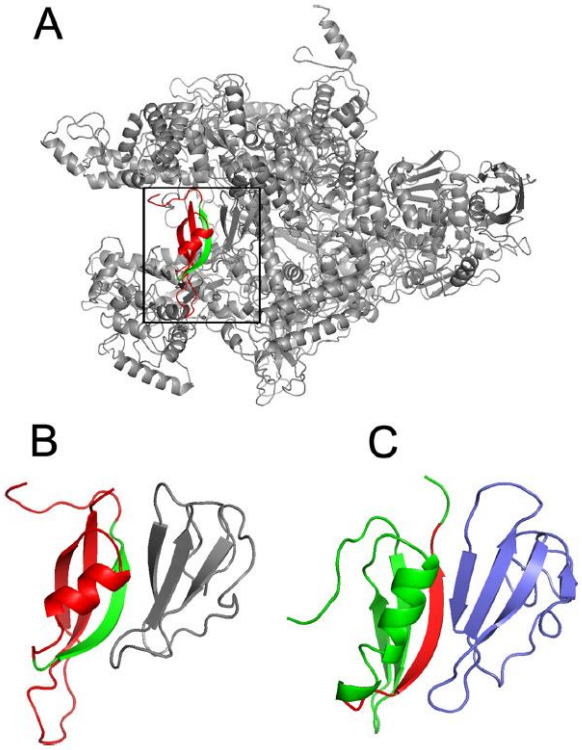


Figure 18. Model of ε bound to RNAP. **A.** ε docked against the jaw region of an homology model of *B. subtilis* RNAP using the Gp2-jaw structure (PDB 2LMC) as a guide. (B and C) ε-jaw and Gp2-jaw complexes, respectively. In panel **B**, ε is shown in red, with the β3 strand thought to be involved in interaction with *B. subtilis* RNAP shown in green. In panel **C**, Gp2 is shown in green with the β3 strand known to be involved in interaction with the jaw of *E. coli* RNAP (pale blue), shown in red.

has been demonstrated. In this study we demonstrated that ω_1 is a *bona fide* subunit of RNAP and (to avoid confusion) we renamed it as ε and the corresponding gene as *rpoY*. We determined the structure of ε, examined the phenotype generated on its deletion, and determined its location on RNAP (**Fig. 18**). The structural analysis indicated that ε is similar to phage T7 Gp2 which inhibits host cell transcription through interaction with RNAP (Bae et al., 2013). Mutagenesis studies indicated that both ε and Gp2 interact with RNAP via a shared structural motif. Due to the lack of phenotype on its loss, location within an RNAP complex, and similarity to phage T7 Gp2, we proposed that ε may help protect against phage infection from Gp2-like proteins by occupying their binding sites.

Answer: The structure of ε is almost identical with that of the phage T7 Gp2 protein and so is the binding site on RNAP. Remarkably, considering it is an RNAP subunit, a deletion mutant for the ε-encoding gene does not show changes in gene expression. Hence, we propose that ε may serve as an immunity protein, protecting *B. subtilis* RNAP against phages employing analogous strategy to T7 via Gp2.

4.4. HelD

22. **Wiedermannová, J., Sudzinová, P., Kovaľ, T., Rabatinová, A., Šanderová, H., Ramaniuk, O., Rittich, Š., Dohnálek, J., Zhihui, F., Halada, P., Lewis, P., Krásný, L. (2014) Characterization of HelD, an interacting partner of RNA polymerase from *Bacillus subtilis*. Nucleic Acids Res 42(8): 5151-63.**

Question: What is the function of HelD, a newly identified binding partner of RNAP in *B. subtilis*?

Jana Wiedermannová from my lab discovered a new binding partner of RNAP from *B. subtilis* (it copurified with RNAP and mass spectrometry revealed its identity). In parallel, the same protein was described as interacting partner of RNAP by others (Delumeau et al., 2011). In this study we set out to characterize HelD. We first verified its binding to RNAP and, by the far-Western approach identified its approximate binding region on RNAP. HelD binds the RNAP core between the secondary channel of RNAP and the α subunits. Next, we created a HelD-null strain and determined that the absence of the protein results in prolonged lag phase. We created recombinant His-tagged HelD and performed experiments *in vitro*. These experiments revealed that HelD stimulateed transcription in an ATP-dependent manner by enhancing transcriptional cycling and elongation. Interestingly, we observed that the stimulatory effect of HelD could be amplified by the small subunit of RNAP, δ. Thus, there seems to be a synergism between these two proteins.

Answer: HelD binds RNAP between the secondary channel of RNAP and the alpha subunits and promotes transcriptional elongation and cycling. Future studies will be required to define its overall effect on gene expression and the biology of the cell.

4.5. Spx

23. **Rochat T, Nicolas P, Delumeau O, Rabatinová A, Korelusová J, Leduc A, Bessières P, Dervyn E, Krásný L, and Noirot P. (2012) Genome-wide identification of genes directly regulated by the pleiotropic transcription factor Spx in *Bacillus subtilis*. Nucleic Acids Res 40 (19): 9571-9573.**

Question: Which genes are regulated by Spx in *B. subtilis*?

Spx is a transcription factor in *B. subtilis* with a key role in affecting gene expression with respect to maintenance of redox homeostasis (Zuber, 2009). Spx binds to α subunits of RNAP and allosterically affects its binding to DNA, based on its redox state. It becomes active when it is oxidized (Zuber, 2009). In this study, we catalogued the regulon of Spx by chromatin immunoprecipitation followed by microarray hybridization. This part of the project was performed by our French collaborators. We, then, validated the results by detailed *in vitro* transcription studies with selected genes (their regulatory sequences) and identified new types of regulation of RNAP by Spx. Overall, 283 discrete chromosomal sites potentially bound by the Spx–RNA polymerase (Spx–RNAP) complex were identified. Three quarters of these sites were located near SigA-dependent promoters, and upon diamide treatment (*e. i.* oxidative stress), the fraction of the Spx–RNAP complex increased in parallel with the number and occupancy of DNA sites. Correlation of Spx–RNAP-binding sites with gene differential expression in wild-type and Δspx strains exposed or not to diamide revealed that 144 transcription units comprising 275 genes were potentially under direct Spx regulation. Spx-controlled promoters exhibited an extended -35 box in which nucleotide composition at the -43/-44 positions strongly correlated with observed activation. *In vitro* transcription confirmed activation by oxidized Spx of seven newly identified promoters, of which one was also activated by reduced Spx. This study thus globally characterized the Spx regulatory network, revealing its role in the basal expression of some genes and its complex interplay with other stress responses.

Answer: Over 200 sites binding the complex of RNAP-Spx were genome-wide identified in *B. subtilis*, underscoring the importance of this transcription factor

with respect to the number of regulated genes. Interestingly, subsequent detailed analysis revealed that Spx, depending on the gene, can act both in oxidized and reduced forms.

4.6. RNAP

24. Tomáš Kouba, Jiří Pospíšil, Jarmila Hnilicová, Hana Šanderová, Ivan Barvík, and Libor Krásný (2019) The Core and Holoenzyme forms of RNA Polymerase from *Mycobacterium smegmatis*. J. Bacteriol (in press).

Question: What is the structure of the core and holoenzyme forms of mycobacterial RNAP?

In this study we described 3D structures of core and holoenzyme forms of *M. smegmatis* RNA polymerase (RNAP) solved by cryo-EM (**Fig. 19**). These structures fill the so far empty spots in the gallery of the pivotal forms of mycobacterial RNAP and illuminate the extent of conformational dynamics of this

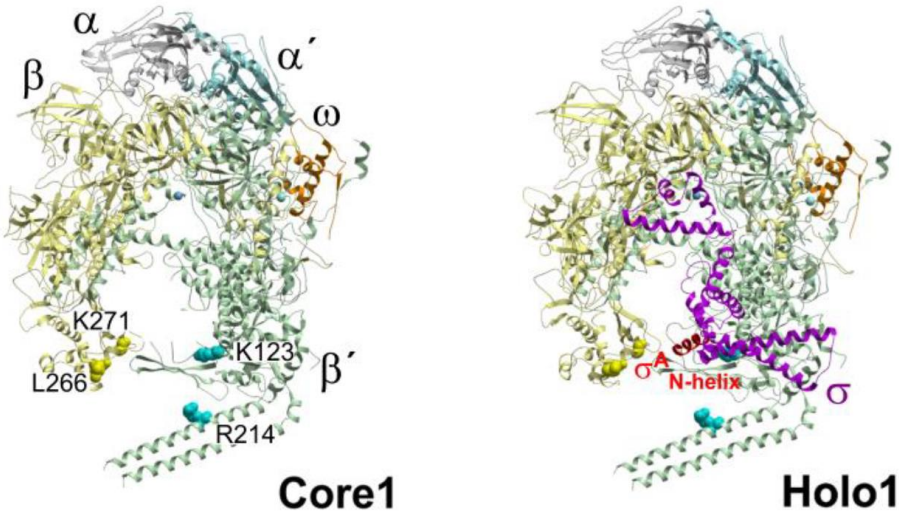


Figure 19. Secondary structures of two forms of RNAP from *Mycobacterium smegmatis* as observed in the experimental cryo-EM densities: core (**Left**) and holoenzyme (**Right**, RNAP in complex with primary sigma factor, σ^A), subunits are colored as follows: β – yellow, β'– green, α – grey, α' – cyan, ω – orange, σ^A – magenta. Amino acid residues used to quantitate the range of motions of the primary channel pincers are shown in the left structure. In the right structure, the $\sigma^A{}_{N\text{-}helix}$ (of domain 1.1) is shown in red. The mycobacteria-specific β'i1 structural element is the prominent helix-coil-helix containing R214.

enzyme, showing that the mycobacterial enzyme is highly flexible, and providing topological details for region 1.1 of SigA and the mycobacteria-specific β'i1 structural element. The presented findings may facilitate future designs of antimycobacterial drugs targeting RNAP, and provide a basis for structural studies of mycobacterial RNAP in complex with auxiliary factors.

Answer: We have solved the 3D structures of *M. smegmatis* core and holoenzyme forms of RNAP. The two forms complement the known structures and provide a complete movie of the main kinetic intermediates of this enzyme, showing that *M. smegmatis* RNAP is highly flexible compared to other species.

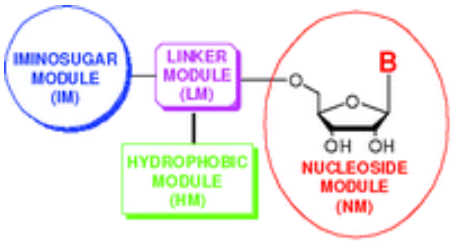
Chapter 5. Antibacterial compounds

Currently, antibiotics in the clinical practice are often found ineffective, due to the rise of resistant bacterial strains (Jiang et al., 2018; Schwartz and Morris, 2018). The situation is dire and some physicians even say that "We have already entered the post-antibiotic era" (Kostakioti et al., 2013). What, then, needs to be done? Clearly, new antibiotics are required, preferably such that would elicit zero to low level resistance in pathoges. What is the ideal target of such antibiotics? Undoubtedly, it is a proces/bacterial cell structure that is difficult to be altered/mutated due to its essentiality for the cell. What is such a structure? An ideal target is the plasmatic membrane as the phospholipid composition is difficult to change; however, efflux pumps scavenging antibiotics from the membrane may in some species provide a means for resistance to develop (Alav et al., 2018). Importantly, there are salient differences between pro- and eu-karyotic plasma membranes, with the former being more negatively charged on the surface, whereas the latter contain anionic lipids sequestered to the monolayer facing the inside of the cell. Antibiotic compounds targeting the bacterial plasma membrane have been described in the past (Bastos et al., 2018). These antibacterial peptides may alter the physico-chemical properties of the membrane and thereby adversely affect the cell. A team from the Institute of Organic Chemistry and Biochemistry in Prague, led by Dr. Dominik Rejman, has synthesized novel compounds targeting the bacterial membrane and we closely collaborated (also with others) on their development and characterization. The results of these efforts are contained in the following three papers.

25. **Rejman, D., Rabatinová, A., Pombinho, A., .Kovačková, S. ,Pohl, R, Zborníková, E., Kolář, M., Bogdanová, K., Nyč, O., Šanderová, H., Látal, T., Bartůněk, P., Krásný, L. (2011) Lipophosphonoxins – new modular molecular structures with significant antibacterial properties. J Med Chem 54(22): 7884-7898.**

Question: Can we develop novel antibacterial compounds?

At the onset of this study was the idea to screen for novel inhibitors of bacterial RNAP. To do this, we teamed up with Dr. Dominik Rejman from the Institute of Organic Chemistry and Biochemistry in Prague, clinical microbiologists from Motol and Olomouc (Drs. O. Nyč and M. Kolář), the private company Trios Ltd (Dr. T. Látal) and also several others. We performed an extensive *in vitro* screen of the nucleotide-, nucleoside-like compounds from the library of Dr. Rejman and came up with a number of hits. However, while some of these compounds were active against purified RNAP, it was difficult to transport them into cells due to their negative charge. Nevertheless, in parallel we tested the effect of all the library compounds on bacterial strains regardless of their potential mechanism of action. By this approach we identified a set of compounds, collectively termed lipophosphonoxins (LPPOs), that displayed significant antibacterial activities against gram positive (**Fig. 20**) but not against gram negative bacteria. Importantly, they showed low cytotoxicity against a panel of human cell lines.



	MIC µg/ml			
	E. faecalis CCM 4224	*S.aureus* CCM 4223	*B. subtilis*	*S.agalactiae*
12a	12.50	-	6.25	3.13
12b	6.25	25.00	3.13	3.13
12h	12.50	25.00-12.50	6.25	6.25
12i	6.25	12.50- 6.25	3.13	1.56

Figure 20. LPPOs are active against gram-positive bacteria. The modular structure of LPPO sis shown on the left. Minimal inhibitory concentrations (MIC) are listed for the most active compounds and respective species on the right.

Answer: We identified novel compounds, termed lipophosphonoxins (LPPOs), with significant activities against gram-positive bacteria and low cytotoxicity.

26. Panova, N., Zborníková,E., Šimák, O., Pohl, R., Kolář, M., Bogdanová, K., Večeřová, R., Seydlová,G., Fišer, R., Hadravová, R., Šanderová, H., Vítovská, D., Šiková, M., Látal, T., Lovecká, P., Barvík, I., Krásný, L., Rejman, R. (2015) Insights into the mechanism of action of bactericidal lipophosphonoxins. PLOS ONE, 10(12)e0145918.

Question: What is the mechanism of action of LPPOs?

In this study we systematically searched for the target(s) of LPPOs. We tested their effect on DNA, RNA, protein, and cell wall synthesis and showed that none of these processes was affected by LPPOs. Next, we investigated the functioning of LPPOs by microscopy and noticed a dramatic effect of LPPOs – they appeared to create pores in the cell envelope (**Fig. 21**). This was further studied by a number of approaches that confirmed the pore-forming activity of LPPOs. Dr. G. Seydlová and Dr. R. Fišer from Charles University in Prague were indispensable for these experiments. Importantly, the pore-forming activity was restricted to bacterial membranes and eukaryotic membranes were much less affected.

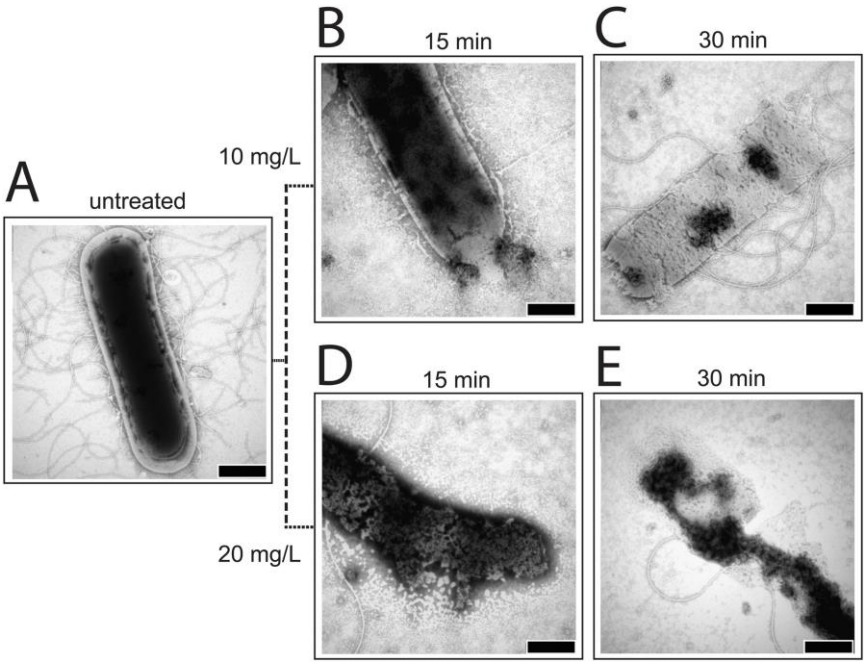


Figure 21. LPPOs destroy the cell envelope. TEM pictures of *B. subtilis* cells. 0.25% phosphotungstic acid at pH 7.3 was used for staining. **A**. Untreated. **B.** Treated with 10 mg/L of DR5026 for 15 min. **C.** Treated with 10 mg/L of DR5026 for 30 min. **D.** Treated with 20 mg/L of DR5026 for 15 min. **E.** Treated with 20 mg/L of DR5026 for 30 min. The scale bars in the right-hand corners of the pictures represent 500 nm.

Answer: LPPOs function by creating pores in the bacterial membrane.

27. **Seydlová G, Pohl R, Zborníková E, Ehn M, Simak O, Panova N, Kolar M, Bogdanova K, Vecerova R, Fiser R, Šanderová H, Vítovská D, Sudzinová P, Pospíšil J, Benada O, Křížek T, Sedlak D, Bartunek P, Krasny L, Rejman D. (2017) Lipophosphonoxins II: Design, Synthesis and Properties of Novel Broad Spectrum Antibacterial Agents. J Med Chem. 60(14):6098-6118.**

Question: Can LPPOs be improved with respect to efficiency and range of bacteria against which they are active?

As LPPOs from the first generation (LPPOs I) were not active against gram negative bacteria, Dr. D. Rejman designed new LPPOs (second generation, LPPOs II) with a higher positive charge compared to LPPOs I. These compounds were more efficient and, importantly, active also against gram negative bacteria including a number of pathogens and resistant strains. Parallel mechanistic studies revealed that LPPOs II functioned in the same manner as LPPOs I, and retained their low cytotoxicity (**Fig. 22**).

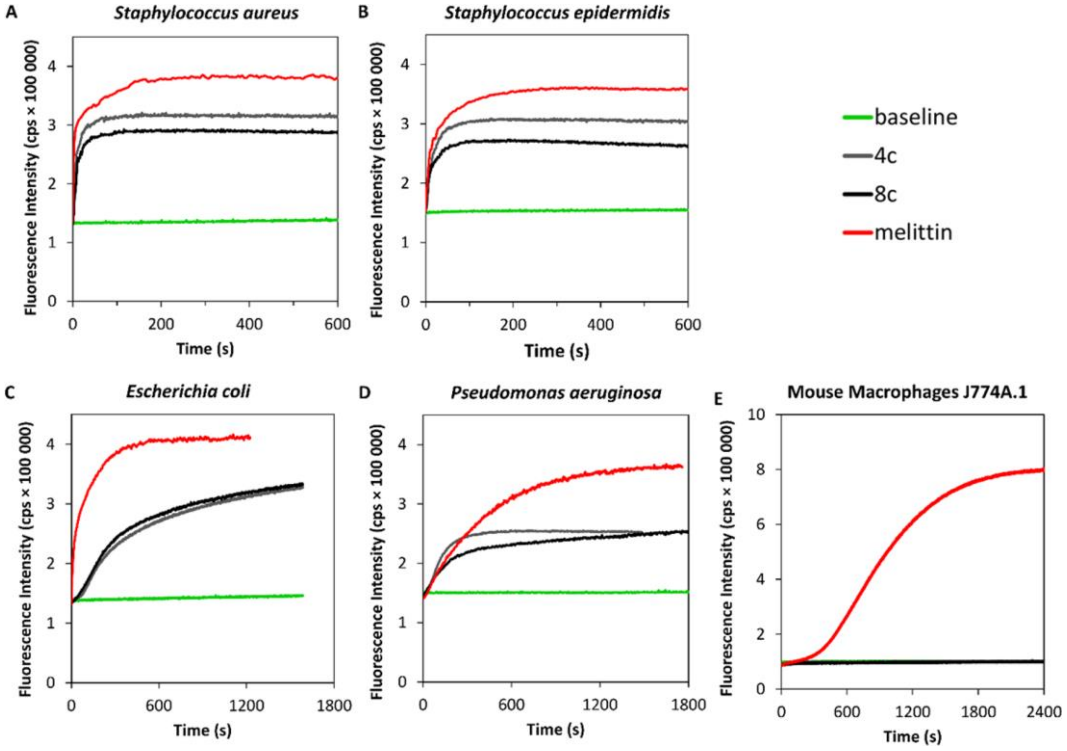


Figure 22. LPPOs II are specific for bacterial cell membranes. Permeabilization of the cytoplasmic membrane of live cells by LPPOs 4c and 8c, and melitin (positive control). Permeabilization of the cytoplasmic membrane of Gram-positive (**A, B**) and Gram-negative bacteria (**C, D**) and mouse macrophages (**E**) induced by LPPO II measured as the increase in fluorescence intensity of propidium iodide. LPPO II and melittin concentrations were 5 mg/L and 5 μM, respectively.

Answer: The second generation LPPOs was more efficient than the first generation and active against both gram-positive and –negative bacteria. They are now being tested in practical applications.

Future Challenges

The way an organism functions/looks like/communicates depends on gene expression. Studies of gene expression regulation are gaining momentum as large scale approaches allow to amass and tackle increasing amounts of data. Subsequently, this must be followed up by detailed verifications to build reliable models of the regulatory processes. We are at the beginning of this era, and the quest for the ultimate understanding of the cell has just begun in earnest. Completely new concepts and breakthroughs are expected. The new knowledge will be utilized in development of new antibacterial compounds and/or biotechnologies.

In transcription, future challenges lie in detailed descriptions/explorations and cataloguing of the key players interacting with RNAP, and in gaining insights into the mechanistic details of their functioning. This task will be multidimensional, as the architecture of the transcription apparatus changes with changing environmental conditions. A dynamic view of the process, as complete as possible, is the goal.

In cell-to-cell communication, a relatively new phenomenon, the so called nanotubes, is making the waves (Baidya et al., 2018). Their existence seems to be firmly established. However, their formation and function are still poorly defined. As they in principle transform bacteria into a multicellular organism (and change thus the paradigm of how we think about them), their research is an urgent need. The challenges include defining the genetic apparatus and its regulation as well as defining the types of metabolites/molecules that can be trafficked via nanotubes.

Finally, the demand for antibacterial compounds is on the increase. Novel/improved antibiotics (increased specificity, lower possibility for resistant strains to occur) have to be developed. Importantly, their implementation into clinical practice will need to take place (testing of LPPOs II as additives to surgical bone cements is already in progress), and a more intense interaction with pharmaceutical companies is desirable.

I am looking forward to being part of the quest.

Index - List of Publications (in alphabetical order based on first author)

Kouba, T, Pospíšil, J., Hnilicová, J., Šanderová, H., Barvík, I., and Krásný, L. **(2019)** The Core and Holoenzyme forms of RNA Polymerase from *Mycobacterium smegmatis*. ***J. Bacteriol*** (in press).

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