

Session II. Regulation of gene expression in Prokaryotes

II.P.1

Search for Rv2998A protein partners using molecular techniques

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Mycobacterium tuberculosis (*M.tb*) is one of the oldest known infectious factor, causing tuberculosis. This is still one of the major causes of mortality. The success of *M. tuberculosis* as a pathogen is connected to its ability to adapt to a wide range of living conditions inside as well as outside the human host. This serious pathogen has spread intensively worldwide and recent years have seen an increase in the number of multi-drug resistant *M. tuberculosis* strains. Comparing to the genetically linked two component signal transduction systems from mycobacteria very little is known about the orphaned elements of the signaling cascade in these bacteria. A typical TCSS composes of membrane histidine sensor kinase and cytosolic response regulator. One of the *Mycobacterium tuberculosis* gene, *rv2998A* is coding for an putative orphan kinase – Rv2998A, whose function was not yet identified.

To better understanding the adaptation of *M. tuberculosis* to environmental changes, Rv2998A was used in functional analysis, which was based on mass spectrometry (MS/MS), pull-down and bacterial two hybrid assays.

Rv2998A tagged to green fluorescence protein (GFP) was expressed in *M.tb* as a bait protein, purified together with its putative partners and identified by MS/MS. The identified partners-interactions were confirmed by using bacterial two hybrid assay consist of reconstitution of active cyclic AMP synthase in *E. coli* host. Further, the selected proteins were expressed in *E. coli* system and purified to be used in pull-down *in vitro* assay to confirm an identified interactions.

Better understanding of the signal transduction pathways could lead to development of specific drugs for efficiently blocking some of the crucial elements of the signaling cascade. This in turn will prevent bacteria from multiplication and would result in improvement in curability of tuberculosis disease.

Key words: *M. tuberculosis*, Rv2998A, TCSS

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II.P.2

Bacterial Mesh-1 enzymes' specificity towards (p)ppGpp analogs

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Bacteria are constantly exposed to changing environmental conditions. To cope with those changes and overcome stress, bacteria had evolved a specific system, called the stringent response, where unusual nucleotides play the major role: guanosine 5'-triphosphate-3'-diphosphate (pppGpp) and guanosine 5'-diphosphate-3'-diphosphate (ppGpp). Stringent response is regulated by the RelA and SpoT proteins. During amino acid starvation, RelA catalyzes synthesis of the signaling nucleotides – (p)ppGpp, that are produced by pyrophosphorylation of GTP using ATP. Conversely, SpoT is able to hydrolyze ppGpp (or pppGpp) into GDP (or GTP) and pyrophosphate. This is very important when environmental conditions improve, because it leads to maintenance of proper levels of (p)ppGpp in the cell. Moreover, during carbon, phosphate and other nutrient limitations, heat or oxidative stress, SpoT can also synthesize (p)ppGpp.

(p)ppGpp is not prokaryote-specific, for instance it also plays a regulatory role in plants. Still, (p)ppGpp synthesis or presence had not been demonstrated in higher eukaryotes. Therefore, recent discovery of a metazoan SpoT homolog, called Mesh-1 in *Drosophila melanogaster* and MESH-1 in *Homo sapiens*, that has been shown to hydrolyze ppGpp was considered a significant breakthrough, although Mesh-1 proteins do not possess (p)ppGpp synthesizing activity. Several bacterial species, for instance *Methylobacterium extorquens* AM1 and *Methylobacterium extorquens* DM4, also contain Mesh-1 homologs. These homologs were here cloned, purified and their ability to hydrolyze (p)ppGpp and its nucleotide analogs were investigated. Experimental results indicate bacterial Mesh-1 homologs to be specific for only one type of 3'-pyrophosphorylated nucleotide.

Key words: (p)ppGpp, Mesh-1, RelA, SpoT

II.P.3

Effect of (p)ppGpp analogs on transcription initiating from the *E. coli* *rrnBP1* promoter

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Precise regulation of gene expression is the key factor which allows bacteria to respond to changing environmental conditions, thus this process shows a high level of complexity. Here, the key enzyme is the RNA polymerase (RNAP) that interacts with multiple protein factors which can act by different modes of action. Primary regulation of transcription occurs via sigma factors, responsible for promoter recognition. Another mechanism of regulation involves DNA binding proteins which recognize canonical sequences on DNA in the proximity of a given promoter. Finally, a third mechanism of transcription regulation is represented by protein factors that interact only with RNAP and not with the DNA template, for example proteins binding to the secondary channel of RNAP, such as GreA, GreB and DksA. RNAP binding competition between these factors has been proposed to be a next level of transcription regulation in bacteria.

In addition to protein factors that regulate the activity of RNAP, modified nucleotides play important role in transcription regulation, such as (p)ppGpp (guanosine 5'-diphosphate-3'-phosphate and guanosine 5'-triphosphate-3'-diphosphate) that have been found to be the key elements of the stringent response. Specifically, (p)ppGpp was found to inhibit transcription initiating from ribosomal promoters, such as *rrnBP1*. What's more, ppGpp seems to have a stronger effect than pppGpp which would indicate that it is not only 3'-diphosphate that is crucial for these interactions. In addition, DksA was found to enhance (p)ppGpp's action at this promoter, while GreA was found to have a stimulatory effect.

Here, we investigate a possible role of (p)ppGpp analogs, such as ppApp and pppApp, on transcription initiating from *rrnBP1* in the presence and absence of RNAP secondary channel factors, i.e. GreA, GreB and DksA. We hope this study will shed light on how regulation by 3'-diphosphate nucleotides occurs, and specifically if the nucleotide base participates in these interactions.

Key words: transcription, RNAP, *rrnBP1*

II.P.4

A novel stress-inducible system of transcription regulation in *Staphylococcus aureus*

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Possible interactions between the host and the pathogen define a complex living environment. This dynamic complexity requires equally complex regulatory systems which let the pathogen evade the host immune response and survive. Presently, few such systems have been described for *Staphylococcus aureus* and simultaneously proved to be relevant for virulence.

In the study on pemIK_{sa} toxin-antitoxin system [1] a transcript of a hypothetical protein gene was suggested to be resistant to PemK_{sa} toxin endoribonucleolytic activity and classified to a general group of PemK_{sa}-resistant transcripts with putative importance for bacterial cells during exposure to different environmental stresses. The gene was designated as *saoC* [2] and belongs to *saoABC* operon with two other genes. A subsequent *in silico* analysis revealed that SaoC protein potentially possesses two DNA-binding motifs, which suggested it to be a yet uncharacterised transcription factor. In the course of research aimed at functional characterisation of *saoABC* operon further features were revealed. The DNA-binding property of SaoC was confirmed when the protein was expressed in *Escherichia coli* BL21(DE3) and co-purified with DNA. DNase I assays showed that SaoC protects a short fragment of DNA from digestion. Further, the relation between *saoABC* operon and other regulatory systems were investigated and among them a link to the RNA polymerase alternative sigma factor σ^B was uncovered. Another studied aspect was the transcription level of *saoABC* genes during exposure to different stress conditions. Moreover, the phenotype analysis of the null mutants Δ *saoA* and Δ *saoC* was carried out in chicken embryo model of virulence and in intracellular survival model in human fibroblasts and keratinocytes.

The results obtained in the study create a consistent image of *saoABC* operon as a gene cluster coding for a yet uncharacterised transcription regulation system. The data strongly suggests its importance for intracellular survival of the pathogen within the host cells.

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Keywords: sigma factor, staphylococci, *Staphylococcus*, *Staphylococcus aureus*, stress response, transcription factor, transcription regulation

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II.P.5

The functional analysis of PdtaS protein in *Mycobacterium tuberculosis*

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PdtaS protein of *Mycobacterium tuberculosis* is part of a two component signal transduction system (TCSS). It acts as the sensor kinase, with the genomic locus tag Rv3220c. PdtaS is able to self-phosphorylate in the presence of Mg²⁺/ATP and subsequently transfer the phosphoryl group to a response regulator PdtaR (Rv1626). This TCSS acts at the level of transcriptional antitermination. Effective action of TCSS in *Mycobacterium tuberculosis* is essential for the effective and rapid response to changing environmental conditions. This ability of mycobacteria to adapt to the environment seems to be crucial for the pathogenicity of tubercle bacilli considered as the most dangerous human bacterial pathogen.

The aim of project is to investigate the involvement of the PdtaS histidine kinases of *M. tuberculosis* in the transduction of signal onto cellular regulatory elements. Project objectives are realized by studies of phenotypical changes associated with altered levels of investigated histidine kinases. For this purpose, by using protocol for homologous recombination, it has been prepared *M. tuberculosis* mutant defective in synthesis of PdtaS (Δ pdtaS) as well as merodiploid strain overproducing this sensory kinase under inducible promoter. The named strains are being exposed to various environment conditions such as reactive oxygen intermediates (menadione), hypoxia, nitric oxide donor -DATA/NO and tested for growth kinetics *in vitro*, survival by determining the colonies forming unit (CFU) and morphological changes (microscopic analyzes). In the subsequent stages of research, we will search for cellular partners of PdtaS. Moreover, recombinant PdtaS protein was expressed and purified in order to produce antibodies and to monitor its kinase activity.

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Key words: Two component signal transduction system, Rv3220c, PdtaS, antitermination

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II.P.6

The effect of GraL overexpression on its putative gene targets

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Small noncoding RNAs (sRNAs) are involved in regulation of many different processes in bacteria, including responses to various stresses and control of virulence, and provide rapid and cost-effective response for the cell. They are often found in intergenic regions and can act *in cis* or *in trans*. By pairing with their mRNA targets, they can either repress or activate gene expression by affecting mRNA stability or influencing the initiation of translation.

GraL is a recently discovered *Escherichia coli* sRNA encoded in the *greA* leader region. What is unique, is that it appears to be collection of products differing in length, rather than one definitive transcript. There are two sets of GraL products coming from two overlapping promoters, and the transcripts additionally diverge by 1–10 nucleotides due to imprecise termination. Another oddity is that GraL seems to act both *in cis* and *in trans*, which seems to be the first such case reported to date.

Overall, 13 most probable gene targets for GraL have been identified by using the sTarPicker software. Translational *lacZ* fusions with promoter and leader regions of the genes of interests, containing putative GraL binding regions, were constructed. β -galactosidase assays were then performed to investigate the effect of GraL overproduction on the expression level of each fusion. Scrambled version of GraL was used as control to assess a possible effect of elevating the level of any sRNA in the cell. The results obtained will be applied for analysis of GraL-mRNA interactions in subsequent *in vitro* assays.

Key words: GraL, sRNA, β -galactosidase

II.P.7

The role of DksA and ppGpp in regulation of transcription from tRNA promoters in *Escherichia coli*

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The adaptation to specific environmental conditions is crucial for all living organisms, because it enables them to grow in variety of ecosystems and allows to thrive in new niches. In order to survive, *Escherichia coli* developed various mechanisms to adapt to different environmental conditions. Amongst them, one of the most far-reaching is the stringent response, mediated by guanosine tetraphosphate (ppGpp) – a specific nucleotide, synthesized by RelA and SpoT, which binds to RNA polymerase (RNAP). Stringent response down-regulates the energy-consuming processes such as ribosomal promoters activity or up-regulates the life-protecting processes like amino acid biosynthesis, virulence and many more. Another transcription regulation factor – DksA protein, plays important role in the stringent response by which stabilizes ppGpp interaction with RNA polymerase and affects stability of open complexes. During stringent response several tRNA promoters like pArgX, pLeuV, pLeuX and pMetT also are under negative control of ppGpp.

Nevertheless, the mechanism of transcription regulation from tRNA promoters by ppGpp and DksA still remains unknown. To answer this question we have isolated RNA from *Escherichia coli* MG1655: wild type, $\Delta relA \Delta spoT$; $\Delta dksA$; $\Delta relA \Delta spoT \Delta dksA$ strains at two time points, first for exponential growth and second for stationary phase. The analysis of the regulation of tRNA promoters has been studied by RT-PCR method.

Here we described analysis for 18 tRNA promoters. Our results indicated that the activity of tRNA promoters *in vivo* in stationary phase for the wild strain is at the different level. This data supported some previous information about negative control of tRNA promoters by ppGpp but also showed that this effect is specific for each of them.

Key words: stringent response, guanosine tetraphosphate, tRNA promoters, DksA protein

II.P.8

Transcriptional slippage is common but its efficiency is RNA polymerase-dependent

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By using NGS approach we have analyzed the polymorphism of a T7 RNA polymerase-generated mRNA of *mbolIM2* gene. We have found that RNA polymerase exhibits relatively high level of template-dependent transcriptional infidelity (RNA editing). The multiple insertions in A- and T-rich tracts of homopolymers in mRNA cause the epigenetic changes resulting in site-dependent rescue of a single or even double InDel frame shift mutants. It increases the repertoire of phenotypic variants. Moreover, the production of the mixture proteins consisting of functional and non-functional variants creates a heterogeneous pool of proteins of almost the same molecular mass, indistinguishable to each other upon ordinary analysis. We show that bypass of InDel mutations through a transcriptional slippage is common, but its efficiency is RNA polymerase-dependent (e.g. *E. coli* host RNA polymerase is several fold less efficient than T7 RNAP).

Key words: RNA editing, transcriptional slippage, insertion/deletion mutations, epigenetics

II.P.9

Analysis of biochemical and biophysical properties of the *Escherichia coli* Hfq protein: formation of helical structures inside the cell

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The Hfq protein is a highly conserved and abundant RNA chaperone found in different bacteria. Its best-characterized function is to affect the regulation of post-transcriptional events, including mRNA translation and degradation. It is possible mostly by mediation of interactions between small non-coding RNAs and other RNA molecules. In conjunction with its low specificity of binding nucleic acids, Hfq can be involved in different cellular processes, like regulation of gene expression and plasmid DNA replication. Previous studies have demonstrated that Hfq forms helical structures under the inner membrane of the bacterial cell. These structures appear to be similar to those formed by different proteins involved in RNA metabolism, particularly included in the degradosome. Hfq is able to form long fibers which is dependent on the function of its C-terminal domain. However, it is not known how Hfq is attached to the membrane. The aim of this work was to determine the role of the N-terminal domain of Hfq in the formation of fibers, and to characterize chemical properties allowing this protein to attach to the membrane. Electron microscopic studies were performed to investigate how the N-terminal domain assembles. To test if Hfq is a subject for post-transcriptional modification, this protein was purified under various conditions, using different chromatography techniques, like IMAC and Hydrophobic Interaction Chromatography followed by MALDI-TOF mass spectroscopy. Our analysis demonstrated that the N-terminal domain of Hfq cannot form long fibers. We suggest that it is not required in the assembly of the Hfq into the helical structure. Natural affinity of Hfq to nickel cations is useful to purify the non-modified protein, however, using the methods mentioned above, we were not able to find any post-transcriptional modifications.

II.P.10

Protein acetylation and aggregation in stationary *E. coli* cultures

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Protein lysine acetylation is a reversible post-translational modification (PTM) more prevalent than phosphorylation in bacteria. In the model bacterium *Escherichia coli*, the ε-amino group of a lysine residue can be acetylated either catalytically by acetyl-coenzyme A and lysine acetyltransferases or non-enzymatically with acetyl phosphate. On the other hand, only one lysine deacetylase (the sirtuin CobB) has been identified in *E. coli*. It is known that the acetylation level of proteins increases when bacteria enter the stationary phase. One of the changes observed in stationary aging *E. coli* cultures is accumulation of damaged and aggregated proteins. We found that protein aggregation in stationary cells was affected by acetylation/deacetylation. The lack of deacetylase activity in the *cobB* mutant resulted in increased protein aggregation. However, enhanced protein aggregation did not affect the viability of *cobB* cells during the stationary phase and after transfer of the bacteria into fresh medium. In the *ackA/pta* strain which is defective in non-enzymatic protein acetylation, the protein aggregates were not acetylated but their formation and removal during recovery from stationary phase were only slightly affected. The aggregates in *ackA/pta* cells contained an increased level of the heat shock IbpA/B proteins which are known to bind aggregation-prone denatured polypeptides and facilitate their subsequent refolding by ATP-dependent molecular chaperones. Since depletion of acetyl phosphate results in reduced rate of proteolysis, it is plausible that under recovery from stationary phase aggregated proteins in *ackA/pta* cells are refolded rather than degraded.

Key words: lysine acetylation, protein aggregation, IbpA/B

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II.P.11

Natural C-independent expression of restriction endonuclease in a C protein-associated restriction-modification system

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Restriction–modification (R-M) systems are highly prevalent among bacteria and archaea, and appear to play crucial roles in modulating horizontal gene transfer and protection against phage. There is much yet to learn about these diverse and useful enzyme systems, including their regulation. Type II R-M systems specify two independent enzymes: a restriction endonuclease (REase) and protective DNA methyltransferase (MTase). Their activities represent a toxin and antitoxin, and need to be finely balanced. Some R-M systems rely on specialized transcription factors, called C (controller) proteins. These proteins play a vital role in the temporal regulation of R-M gene expression and thus, indirectly, modulate the horizontal transfer of their genes. Typically, C protein-mediated timing delays expression of the toxic REase activity while DNA modification occurs in a new host cell. This mechanism involves co-transcription of the C and REase genes, with C protein both activating and repressing their shared promoter. We report novel regulation of a C-responsive R-M system that involves a C protein of a poorly-studied structural class – C.Csp231I. Here, the C and REase genes do not share a single transcript; REase and C transcription originate from separate promoters. High REase activity was observed, regardless of C gene presence, unlike other C-linked R-M systems. However, despite the structural distinction of C.Csp231I and its C-box, some features of transcriptional auto-control seen in other C-regulated R-M systems are conserved. These include activation associated with a more upstream operator and repression with a more downstream one. The results reveal unexpected regulatory variation among R-M systems.

Key words: restriction modification system, Csp231I, C-protein

II.P.12

Inhibition of growth and gene expression in Gram-negative bacteria by antisense peptide nucleic acids

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Pseudomonas aeruginosa and *Enterobacteriaceae* causes common and severe infections in hospitals in particular with immune compromised patients. Especially ESBL strains (extended-spectrum β -lactamases) highlight the necessity to develop new therapeutic strategies. One promising strategy could be the use of antisense oligonucleotides (AS-ODN) that target gene transcripts in a sequence-specific manner and modify their function. In this study, we will introduce antisense PNA (peptide nucleic acid) conjugated with cell penetrating peptide (KFF)3K as antibacterial agent against Gram-negative bacteria. We previously described new potential targets – mRNA transcripts encoding proteins essential for bacterial growth: 1) *dnaG* which encodes bacterial DNA primase and 2) *dnaA* which encodes chromosomal replication initiator protein. Moreover, to evaluate the potential for antisense effects we will first target the *rfp* (red fluorescein protein) reporter gene. We constructed a broad-host-range reporter vector expressing *rfp* – plasmid pBBR(*rfp*) and transferred it, using triparental mating and transformation, to all tested strains to observe the level of *rfp* expression. The results will verify two new potential targets for antisense technology in the fight against Gram-negative bacteria, and indicate if PNA conjugated with the peptide (KFF)3K are active against Gram-negative strains *in vitro*.

Key words: peptide nucleic acid, *rfp*, Gram-negative, antisense oligonucleotides

II.P.13

A method for rapid scanning of cDNA libraries for factors activating the *rrnBP1* promoter

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Due to high energy cost of protein synthesis, many regulatory networks modulate the activity of rRNA (ribosomal RNA) promoters that direct the synthesis of RNA which forms ribosomes and is essential for translation of mRNA to proteins. One of such bacterial promoters is the well-studied *rrnBP1*, known to be negatively regulated by ppGpp (the stringent response) and positively regulated by GreA. Here, we reasoned that preparing cDNA or gDNA library from selected prokaryotic or metazoan species and simultaneous transformation of a bacterial strain carrying the *rrnBP1*-gene reporter fusion could result in discovery of proteins, chemical compounds or transcription factors with potential to regulate this promoter. Using this approach may lead to discovery of new regulatory circuits.

In order to measure the *rrnBP1* promoter activity we utilized a strain carrying *rrnBP1-lacZ* chromosomal transcriptional fusion. The first step was to establish proper growth conditions such that the basic level of *rrnBP1* and other *rrn* promoters activity would be high enough to allow for cellular growth and yet low enough to allow for scoring an increase in this promoter's activity. This included varying concentrations of the carbon source, X-gal (for monitoring the fusion's activity) and IPTG (for induction of overexpression of genes present in the cDNA or gDNA libraries). A plasmid overproducing GreA served as a positive control at this stage.

Having established these basic conditions, we are able to perform scans of the libraries for constructs that lead to up regulation of *rrnBP1*. After plasmid isolation and retransformation to confirm their activating properties, the clones are sequenced and are then further exploited to elucidate their biological properties.

Key words: *rrnBP1*, cDNA library, *in vivo* screening