Sensory and Regulatory RNAs in Prokaryotes

Freiburg, July 18-19, 2019

Dear colleagues,

We welcome you to the meeting “Sensory and Regulatory RNAs in Prokaryotes”, short **sRNA2019**, in Freiburg.

Jens Georg
Wolfgang R. Hess
Annegret Wilde
Viktoria Reimann
Rolf Backofen
Claudia Steglich

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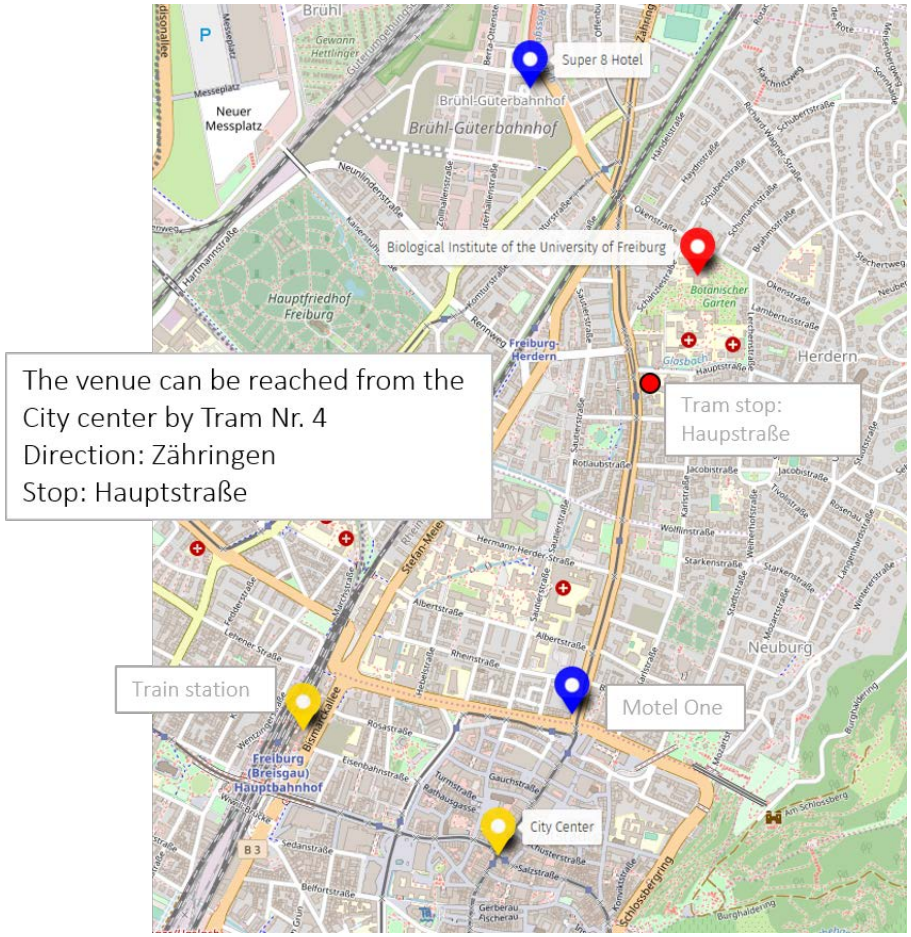

The venue can be reached from the City center by Tram Nr. 4
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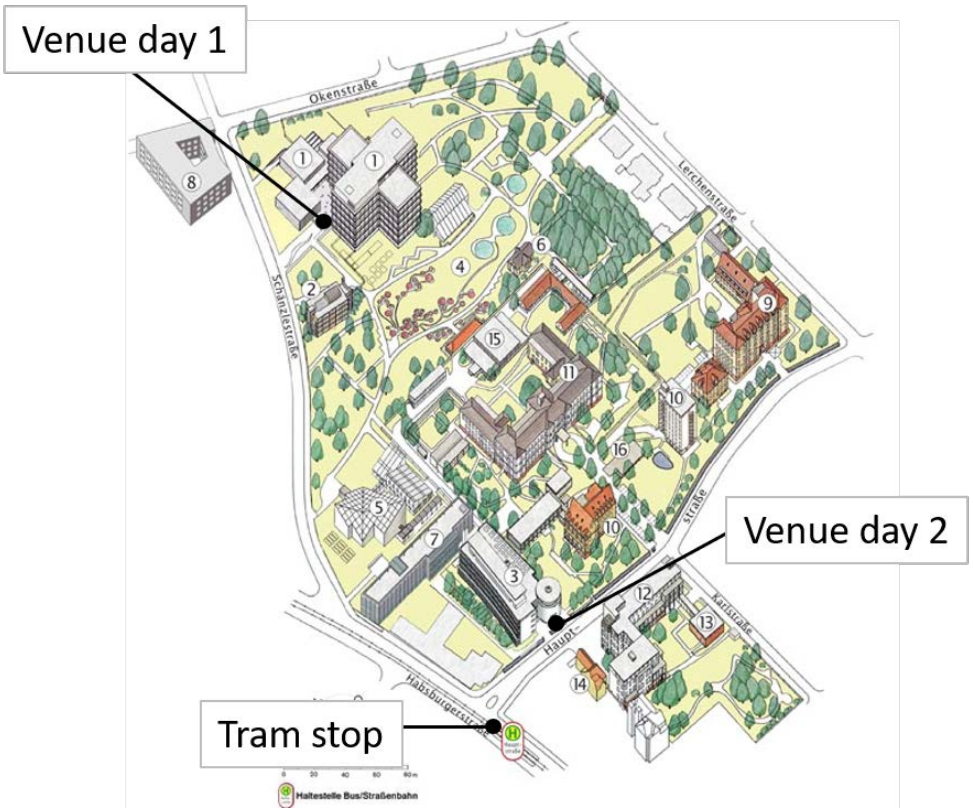
Stop: Hauptstraße



Program

Thursday July 18-2019	**Day 1**	**GHS Schänzlestr. 1; Institute for Biology II/III**
12:00 - 13:00		Registration
13:00 - 13:10		Welcome address
13:10 - 15:10	**Session 1**	**Chair: Annegret Wilde**
13:10 - 13:50	Pascale Romby	**Keynote lecture:** Regulatory RNAs studies in *Staphylococcus aureus* revealed complex and dynamic networks interconnecting stress responses, metabolism adaptation, decision-making and virulence
13:50 - 14:10	Kathrin Fröhlich	Post-transcriptional regulation by a cell-cycle controlled small RNA in *Caulobacter crescentus*
14:10 - 14:30	Ines Vollmer	Analysis of the RNA-based regulatory mechanisms controlling the type III secretion system in *Yersinia pseudotuberculosis*
14:30 - 14:50	Britta Jordan	A potential type I toxin/antitoxin system in *Methanosarcina mazei*
14:50 - 15:10	Stefano Marzi	*S. aureus* S1 is an RNA chaperone which promotes translation of secreted virulence factors by modulating mRNA structures
15:10 - 15:40	Coffee break	
15:40 - 17:20	**Session 2**	**Chair: Rolf Backofen**
15:40 - 16:20	Sebastian Will	**Keynote lecture:** A brief overview of RNA Bioinformatics
16:20 - 16:40	Martin Raden	The impact of various seed, accessibility and interaction constraints on sRNA target prediction - a systematic assessment
16:40 - 17:00	Jens Georg / Steffen Lott	Using “social networks” for sRNA homolog prediction
17:00 - 17:20	Björn Voss	Data analysis for RNA interactomics
17:20 - 19:30	**Poster session**	**Schänzlestr. 1, Foyer**
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Friday July 19-2019	**Day 2**	**HS1 Hauptstraße 1; Institute for Biology I**
9:00 - 10:40	**Session 3**	**Chair: Kai Papenfort**
9:00 - 9:40	Shoshy Altuvia	**Keynote lecture:** Signal-dependent transcriptional and translational pausing influences the dynamics of RNA folding and formation of alternative conformations
9:40 - 10:00	Matthias Riediger	Gradient profiling by sequencing (Grad-Seq) - on the quest for sRNA-protein interactions in the cyanobacterial model organism *Synechocystis* sp. PCC 6803
10:00 - 10:20	Julian Grützner	DUF1127 protein RSP_6037 is a RNA-binding protein
10:20 - 10:40	Hendrik Melior	Posttranscriptional regulation of ribosomal genes by an antibiotic-dependent complex of the leader peptide peTrpL & the attenuator sRNA rnTrpL
10:40 - 11:20	Coffee break	**Schänzlestr. 1, Foyer**
11:20 - 12:50	**Session 4**	**Chair: Franz Narberhaus**
11:20 - 11:40	Lydia Hadjeras	Global examination of bacterial translatomes using ribosome profiling
11:40 - 12:00	Stephan Pienkoß	Temperature-dependent modulation of the sRNA CyaR
12:00 - 12:20	Florian Sikora	The phosphotransferase protein EIIANtr regulates AcnB aconitase activity in a phosphorylation-independent manner in *Escherichia coli*
12:20 -12:40	Katharina Höfer	Revising RNA Architecture - Characterization of NAD-capped RNA in bacteria
12:40 - 12:50	Closing remarks	

Keynote lectures

Regulatory RNAs studies in *Staphylococcus aureus* revealed complex and dynamic networks interconnecting stress responses, metabolism adaptation, decision-making and virulence

Emma Desgranges, Laura Antoine, David Lalaouna, Stefano Marzi, Isabelle Caldelari and Pascale Romby

Architecture et Réactivité de l'ARN, Université de Strasbourg, CNRS, IBMC, 67084 Strasbourg, France

It is now well recognized that RNAs are key intracellular effectors in fast adaptive processes. In many cases, they co-regulate the expression of functionally related genes at the post-transcriptional level. Today, more than 200 potentially regulatory RNAs have been identified so far in *S. aureus*. In order to define their direct targets and the regulatory networks in which they belong, we have recently applied the MS2-affinity purification approach coupled with RNA sequencing to several non-coding RNAs in *S. aureus* (1,2). Using a combination of *in vivo* and *in vitro* approaches, numerous of the co-purified and enriched mRNAs have been validated as direct targets. Several non-coding RNAs will be used to illustrate the intricate interactions between RNAs, two-component systems and transcriptional regulatory proteins and their implication in stress responses (including oxidative and NO), sugar metabolism and virulence.

(1) Bronesky et al. (2019) *A multifaceted small RNA modulates gene expression upon glucose limitation in Staphylococcus aureus. EMBO J*. Mar 15; 38(6).

(2) Tomasini et al. (2017) *The RNA targetome of Staphylococcus aureus non-coding RNA RsaA: impact on cell surface properties and defense mechanisms.* Nucleic Acids Res. 45(11):6746-6760.

A brief overview of RNA Bioinformatics

Sebastian Will

Theoretical Biochemistry Group, Institute for theoretical Chemistry, Universität Wien

A central theme of RNA Bionformatics is the prediction of secondary structure. But why? Ultimately, RNA Bioinformatics ---like RNA Biology--- wants to learn about the function of RNAs and their interplay. It's the approach, where Bioinformatics differs from classical Biology: gaining insights by computation from biological data. This requires appropriate models and methods to compose analysis work-flows.

RNA Bioinformatics faces one crucial challenge: typically, RNA function is hard to explain from sequences alone, but depends on their emergent structures. Here, RNA Bioinformatics responds by leveraging secondary structure as a proxy of RNA function. This approach is promising since secondary structures provides valuable information (beyond sequences alone) *and* can be predicted computationally. Effectively utilizing this approach requires to clearly understand its capabilities and limitations.

Starting from elementary secondary structure prediction, bioinformaticians developed a large tool set. More advanced prediction methods, available in the ViennaRNA package, provide insights into the structure equilibrium of RNAs. Two major ideas substantially improve the reliability of structure prediction further. Firstly, integrating prior knowledge in the form of experimental data from structure probing. Secondly, harvesting information from comparing homologous or analogous RNAs. The latter is supported by fast and accurate computational tools like LocARNA. Another closely related and highly relevant branch of RNA Bioinformatics is the prediction of RNA-RNA interactions as performed by IntaRNA.

Signal-dependent transcriptional and translational pausing influences the dynamics of RNA folding and formation of alternative conformations

Shoshy Altuvia

Hebrew University of Jerusalem

One class of regulatory RNAs comprises mRNA leaders that affect expression in *cis* by adopting different conformations in response to cellular and/or environmental signals, including stalled ribosomes, uncharged tRNAs, elevated temperatures, or small molecule ligands. I this talk I will discuss the mechanism of regulation of two unique riboregulators: (I) The pH responsive riboregulator in which the dynamics of mRNA folding is driven by alkaline conditions via an effect on RNA polymerase pausing during transcription elongation; and (II) the ornithine decarboxylase riboregulator in which the rate of ribosome progression during translation of the upstream ORF, in response to amino acid availability results in formation of an mRNA structure that is susceptible to degradation by ribonuclease. It is interesting to note that the mRNA structure formed due to RNA polymerase pausing during transcription is subjected to translational control, whereas the mRNA structure formed due to an effect on the rate translation elongation is susceptible to the degradation machinery.

Oral Presentations

Post-transcriptional regulation by a cell-cycle controlled small RNA in *Caulobacter crescentus*

Dr. Kathrin Fröhlich

LMU Munich

Small RNAs (sRNAs) are a prominent class of regulators of bacterial gene expression. Most sRNAs characterized today act at the post-transcriptional level, and engage in direct base-pairing interactions to modulate translation and/or stability of target mRNAs. In many cases, the ubiquitous RNA chaperone, Hfq, contributes to the establishment of functional interactions.

The water-dwelling alpha-proteobacterium C. crescentus is an important model organism for the bacterial cell cycle, however its fundamental principles of RNA-mediated regulation have not been studied in detail. Here we report on the identification of sRNAs associated with Hfq in C. crescentus, and for the first time the characterization of Hfq-dependent post-transcriptional regulation in this organism. Using transcriptomics we have uncovered a large set of targets controlled by one Hfq-bound sRNA which is differentially expressed over the C. crescentus cell cycle, and have validated its activity both in vivo and in vitro.

Analysis of the RNA-based regulatory mechanisms controlling the type III secretion system in *Yersinia pseudotuberculosis*

Ines Vollmer[1], Maria Kusmeriek[2], Ann Kathrin Heroven[2], Petra Dersch[1]

[1]ZMBE, Muenster
[2]HZI, Braunschweig

The human pathogen Yersinia pseudotuberculosis is able to adhere to the epithelial cell layer and evade the host immune response after passage through the M-cells of the small intestine, allowing the pathogen to replicate in deeper tissues and cause disease.

Virulence regulation in Yersinia is strictly controlled and influenced by numerous environmental factors such as an increase in temperature from 25°C to 37°C or contact to host cells. This ensures an organised expression of virulence factors necessary for infection establishment. Among the virulence proteins are those involved in the initial infection phase, such as adhesins and flagella, but also proteins of the on-going infection including the type III secretion system and its secreted Yop effector proteins encoded on the virulence plasmid pYV. Many of these virulence factors are under the control of the plasmid-encoded transcriptional regulator LcrF, whose 5'-UTR undergoes a temperature-dependent opening of a thermo-loop, leading to efficient translation of the lcrF mRNA.

RNases play an important role in a variety of cellular processes. Among them are the endo- and exoribonucleases RNaseE and PNPase, which are part of a multi-enzyme complex called the degradosome, and RNaseIII. Recent data from our lab suggests that these RNases are involved in the regulation of different virulence traits such as type III and invasion. These roles are currently under further investigation, for example by RNAseq. This will give us an important insight into the versatile role of RNases in the infection process of this enteric pathogen.

A potential type I toxin/antitoxin system in *Methanosarcina mazei*

Britta Jordan[1], Daniela Prasse[1], Katrin Weidenbach[1], Ruth Schmitz-Streit[1]

[1]Christian-Albrechts-Universität Kiel

Methanosarcina mazei is a methanogenic archaeon and represents an archaeal model organism. Genome-wide differential RNA-seq and term-seq analysis discovered more than 70 small transcripts containing potential sORFs ranging from 12 to 61 amino acids [1].

One transcript identified is the small protein encoding (sp)RNA24. A putative antisense (as)RNA94 was detected, which directed in the opposite orientation overlaps with the spRNA24 around 60 %. spRNA24 encodes for three potential sORFs, one of which is a predicted hydrophobic protein with one transmembrane helix. Northern blot analysis showed an increase of spRNA24 during virus infection. RNAseq analysis and growth experiments with overexpression mutants argue for a potential type I toxin/antitoxin system. The effect(s) of the small protein is currently analyzed in the native background M. mazei and in E. coli (in collaboration with AG Berghoff).

Following experiments will characterize the potential toxin and its antitoxin. Furthermore, the localization of the toxin in the cell and its association to the membrane of M. mazei will be studied.

[1] Jäger, D.; Sharma, C. M.; Thomsen, J.; Ehlers, C.; Vogel, J.; Schmitz, R. A. (2009): Deep sequencing analysis of the Methanosarcina mazei Gö1 transcriptome in response to nitrogen availability. In: Proceedings of the National Academy of Sciences of the United States of America 106 (51), S. 21878–21882. DOI: 10.1073/pnas.0909051106.

S. aureus S1 is an RNA chaperone which promotes translation of secreted virulence factors by modulating mRNA structures

Alessandra Marenna[1], Javier Rol[1], Karen Moreau[2], Lucas Herrgott[1], Anne-Catherine Helfer[1], Alejandro Toledo-Arana[3], Francois Vandenesh[2], Pascale Romby[1], Isabelle Caldelary[1], Stefano Marzi[1]

[1]CNRS, Strasbourg, France
[2]CIRI, Lyon, France
[3]University of Navarra, Spain

Staphylococcus aureus is a formidable human pathogen that uses secreted cytolytic factors to injure immune cells and promote infection of its host. Among these proteins, the PSM family of pore-forming toxins plays critical roles in S. aureus pathogenesis. The regulatory mechanisms governing the expression of these toxins are incompletely defined. Differential transcriptomics and proteomics of extracellular proteins, and in vivo and in vitro translation analyses, revealed that ribosomal protein S1 (SauS1), which in not associated with the ribosome, but directly binds several mRNAs and sRNAs, influences the expression and production of exotoxins like PSMs, α-haemolysin, δ-haemolysin and γ-haemolysins and exoenzymes (proteases and lipases). We demonstrated that SauS1 is required for cellular cytotoxicity (leukocytes, erythrocytes and monocytes) and for dissemination of the infection in a mouse model. By recognizing a specific structure/sequence motif present in the vicinity of the Ribosome Binding Sites (RBS) of the target mRNAs, SauS1 promotes local opening of the inhibitory structures present on these mRNAs and their translation initiation. SauS1 mediated activation of translation of the different PSM peptides, from the αpsm 1-4 operon, from hld locus encoded into the long regulatory RNAIII gene and from the βpsm operon, is contributing to the strictly regulated temporal control in the production of these toxins. Thus, SauS1 belongs to a new class of RNA chaperones that play key roles in the regulation of translation in S. aureus. Its role in virulence and in sRNAs regulations will be also discussed.

The impact of various seed, accessibility and interaction constraints on sRNA target prediction - a systematic assessment

Martin Raden[1], Teresa Müller[1], Stefan Mautner[1], Rick Gelhausen[1], Rolf Backofen[1]

[1]Bioinformatics, University of Freiburg

Seed and accessibility constraints are core features to enable highly accurate sRNA target screens based on RNA-RNA interaction prediction. IntaRNA provides various ways to define such constraints such as seed length, its accessibility, minimal unpaired probabilities, or energy thresholds. Here, we present a systematic assessment of their impact on sRNA target prediction. The evaluation is done both on a qualitative as well as computational level and thus provides on the one hand new biological hypothesis to be tested and on the other hand recommendations for upcoming in silico methods and approaches to predict and filter sRNA targets.

Using “social networks” for sRNA homolog prediction

Steffen Lott[1], Dominik Rabsch[1], Wolfgang R. Hess[1,2] and Jens Georg[1]

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[2]Freiburg Institute for Advanced Studies, University of Freiburg, Albertstr. 19, D-79104 Freiburg, Germany.

The investigations of the evolutionary conservation of a (newly detected) sRNA is an important initial step in sRNAs analysis. The existance of homologs enables the usage of comparative genomics for various downstream analyses such as secondary structure prediction, promoter analysis, investigation of the peptide coding potential and last but not least comparative sRNA target prediction. Unfortunately, homologous sRNAs often feature only a poor overall sequence conservation. For that reason, the most scuccesfull sRNA homolog prediction methods mostly rely on a combination of primary sequence -and structure conservation.

Here we present a fundamentally different approach. Analog to social networks, which allow to predict the behavior or the group membership for individual persons, we construct a network from the sRNA gene neighbourhood/synteny. The nodes of the network are scored by Googles PageRank algorithm to identify the most important neighbouring genes. The final network is used to score potential homolog candidates with high specificity and sensitivity.

Data analysis for RNA interactomics

Björn Voß[1]

[1]University of Stuttgart

Recent advances in methods for the direct detection of interacting RNA strands so-called Direct Duplex Detection (DDD) methods, also pose new challenges for data analysis. I will present our solutions to some of these problems and their integration into our analysis software RNAnue. It combines quality control, mapping of chimeric reads, annotation matching, and statistical as well as thermodynamic assessment. Benchmarks on existing data sets and a comparison to other tools prove the performance of RNAnue. Furthermore, the application of RNAnue to the different kinds of DDD data sets, provide insight into the peculiarities of the respective library preparation protocols.

Gradient profiling by sequencing (Grad-Seq) - on the quest for sRNA-protein interactions in the cyanobacterial model organism *Synechocystis* sp. PCC 6803

Matthias Riediger[1], Wolfgang R. Hess[1,2]

[1]University of Freiburg, Faculty of Biology, Schänzlestr. 1, D-79104 Freiburg, Germany;
[2]Freiburg Institute for Advanced Studies, University of Freiburg, Albertstr. 19, D-79104 Freiburg, Germany.

During the last twenty years, sRNA-mediated posttranscriptional regulation has evolved as a major research topic within the field of gene regulation. Numerous studies could show its importance by either adjusting specific transcript levels prior to translation or altering the Shine-Dalgarno-Sequences' accessibility for the small ribosomal subunit. These regulatory processes are ubiquitous and cover diverse functions and many well-characterized examples exist.

While the initial emphasis was mainly put on the characterization of sRNAs and their physiological relevance, research on RNA-chaperones facilitating sRNA-mRNA interactions is a rather poorly understood field. The few known major bacterial RNA-chaperones were mainly characterized in Enterobacteria but none of them is conserved or proven functional in cyanobacteria, suggesting there must be other proteins to fulfill these functions.

To identify a protein with this functionality, the Grad-Seq approach is applied (developed by Smirnov et al. (2016), *PNAS*). This method provides a tool to infer RNA-protein complexes by applying whole cell lysates to a sucrose density gradient and fractionation after ultracentrifugation. The fractions are subject to mass-spectrometry and RNA-sequencing analyses, giving information on the co-distribution of sRNAs with a limited number of candidate proteins from the total proteome of *Synechocystis* to identify putative major RNA-chaperones.

DUF1127 protein RSP_6037 is a RNA-binding protein

Julian Grützner[1], Gabriele Klug[1]

[1]Institute of Micro- & Molecularbiology, Giessen

The facultative phototrophic bacterium Rhodobacter sphaeroides is able to adapt its life style to changing environmental conditions. In the past our group elucidated in R. sphaeroides the role of protein regulators and small, regulatory RNAs (sRNAs) in the stress responses, particularly to oxidative stress. The four homologouse sRNAs CcsR1-4 (conserved CCUCCUCCC motif stress-induced RNA), which are expressed by the alternative sigma-factors RpoHI and RpoHII, modulate the C1 metabolism under various stress conditions (Berghoff et al. 2009). These sRNAs are derived from the 3´ UTR of the RSP_6037 mRNA. The RSP_6037 gene encodes a small protein of 70 amino acids and the amino acids 23-62 constitute a domain of unkown function (DUF1127), which is structcurally related to eukaryotic RNA binding proteins (Billenkamp et al. 2015). Small proteins with the DUF1127 domain are widely distributed among Alpha- and Gammaproteobacteria and more than 11000 bacterial sequences with DUF1127 proteins are listed in InterPro.

Our previous data show that the small protein RSP_6037 influences the amount of the CcsR sRNAs and that the uncharacterized DUF1127 domain is sufficient for the function of this small protein. By a Co-immunoprecipitation we could show binding between the sRNA CcsR1 and the DUF1127 protein. We also test the hypothesis that the stability of the sRNAs CcsR1 is influanced by binding of the DUF1127 domain of RSP_6037. So the small DUF1127 protein RSP_6037 may plays an important role in the stability and maturation of non-coding RNAs.

Posttranscriptional regulation of ribosomal genes by an antibiotic-dependent complex of the leader peptide peTrpL and the attenuator sRNA rnTrpL

Hendrik Melior[1], Siqi Li[1], Maximilian Stötzel[1], Sandra Maaß[2], Konrad U. Förstner[3], Saina Azarderakhsh[1], John Ziebuhr[1], Christian H. Ahrens[4], Dörte Becher[2], Elena Evguenieva-Hackenberg[1]

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[3]University of Cologne, Germany
[4]Agroscope Switzerland

Ribosome-mediated transcription attenuation in bacteria is an important regulatory mechanism that is controlled by the translation of a short upstream ORF (uORF). Efficient uORF translation causes transcription termination downstream of the uORF. The resulting leader peptide and small attenuator RNA are generally considered nonfunctional. Here, we show that, upon exposure to translation-inhibiting antibiotics, the leader peptide peTrpL (14 aa) of a Sinorhizobium meliloti tryptophan (Trp) biosynthesis operon acts together with its cognate attenuator RNA rnTrpL to destabilize rplUrpmA mRNA which encodes ribosomal proteins. Under these conditions, rnTrpL is produced independently of Trp availability by transcription termination at the attenuator. The sRNA rnTrpL, the leader peptide peTrpL and their target mRNA rplUrpmA were co-purified in an antibiotic-dependent complex. Currently we are analyzing the effect of the purified ribonucleoprotein complex on synthetic RNA in vitro. The surprising role of peTrpL and rnTrpL in antibiotic-triggered posttranscriptional regulation is conserved in other bacteria.

Global examination of bacterial translatomes using ribosome profiling

Dr. Lydia Hadjeras[1], Sara L. Svenson[1], Ann-Janine Imsiecke[1], Elisabetta Fiore[1]
Kathrin Froschauer[1], Cynthia M. Sharma[1]

[1]Institute of Molecular Infection Biology, University of Würzburg

RNA-sequencing technology (RNA-seq) has revealed an unexpected complexity of bacterial transcriptomes and has exposed extensive production of small transcripts, typically in the range of 50–350 nt, often encoded in intergenic regions between protein coding genes. These small transcripts, including antisense RNAs, often do not encode protein products and are thus commonly known as non-coding small RNAs. However, in the last years it has become clear that some smaller transcripts do encode protein products that were missed during genome annotation. Some are even dual-function sRNAs with both coding and regulatory characteristics. Consequently, the necessity of the development of new approaches complementary to the RNA-seq is important in order to clarify which of the identified small transcripts are coding or non-coding.

The recently established ribosome profiling (Ribo-seq) approach for global translatome analysis, based on deep sequencing of mRNA fragments protected from RNase digestion by the translating ribosome, has proven to be a powerful technique, which has revealed much about mRNA translation and detection of ORFs and their boundaries. In particular, ribosome profiling has revealed an emerging number of small ORFs and their translation products, so-called small proteins <50 amino acids. Moreover, Ribo-seq analysis of the translatome cannot only identify sRNAs with coding potential, but also mRNAs whose translation might be affected by sRNA by analyzing the translatome of sRNA mutant strains. Here, we have established and performed Ribo-seq for the Epsilonproteobacteria Campylobacter jejuni and Helicobacter pylori and have generated a first catalog of candidate sRNAs, potentially dual-function sRNAs and small proteins. We have also started to implement translation initiation site profiling (TI-seq), where sequencing of footprints from initiating ribosomes stalled with antibiotics provides start codon information. Ribo-seq combined with TI-seq will help to refine the translatomes and the gene annotation of sRNAs, dual function sRNA and small proteins in other bacteria.

Temperature-dependent modulation of the sRNA CyaR

Stephan Pienkoß[1], Marcel Holler[1], Mara Klöhn[1], Franz Narberhaus[1]

[1]Ruhr University Bochum

The bacterial regulation of gene expression consists of a diverse and complex regulatory network. In addition to the regulation at DNA level, RNA-mediated regulation represents a mechanism that ensures the rapid adaptation of bacteria upon changing environmental conditions e.g. temperature, osmotic stress and nutrient uptake. Regulatory small non-coding RNAs (sRNAs) are versatile RNA elements that mostly regulate target mRNAs posttranscriptionally by base-pairing in a positive or negative manner. On the one hand mRNAs are stabilized or the binding of the ribosomal subunit is facilitated, on the other hand the sRNA-mRNA-complex leads to the degradation of the mRNA or prevents the binding of the ribosomal subunit (1,2).

We focused on the gram-negative foodborne pathogen Yersinia pseudotuberculosis and its temperature dependent RNA-mediated gene expression under environmental (25 °C) and infection-relevant conditions (37 °C). Based on PARS profiling (1), the sRNA CyaR was identified as an sRNA that undergoes a structural change of the secondary structure in response to an increasing temperature. This led us to the hypothesis of a temperature-dependent regulation of gene expression by CyaR. Due to the high homology of CyaR in Y. pseudotuberculosis and E. coli we also investigated the temperature-dependent regulation of CyaR in E. coli. For Y. pseudotuberculosis, a CyaR deletion mutant was constructed and ompX was identified as an mRNA target. Furthermore, a possible temperature-dependent regulation of the target mRNAs ompX, yqaE and luxS was demonstrated in E. coli via northern blots.

References:

[1] Gottesman S, Storz G (2010) Cold Spring Harb. Perspect Biol, 10.1101/cshperspect.a003798.
[2] Gottesman S (2004) Annu Rev Microbiol 58:303-328.
[3] Righetti et al., (2016) Proc Natl Acad Sci USA 113(26):7237-7242.

Role of sRNA 5’-phosphorylation state for target regulation

Alexandra Schilder[1], Boris Görke[1]

[1]MFPL, Vienna Biocenter (VBC), Austria

In Enterobacteriaceae RNA degradation is initiated by endoribonuclease RNase E. The 5’-end-dependent mechanism of RNase E cleavage relies on the conversion of 5´-triphosphate (5’-PPP) groups of primary transcripts to 5’-monophosphate (5’-P) groups by the pyrophosphohydrolase RppH. 5’-Terminal-monophosphates can allosterically activate RNase E by contacting the catalytic domain (Bandyra et al., 2018, Mol Cell 72:1-11). Some base-pairing small RNAs (sRNAs) trigger degradation of their target RNAs by recruiting RNase E. In a previous in vitro study it was shown that the regulatory potential of MicC sRNA towards its target mRNA is higher when MicC is present in the 5’-P form as compared to the 5’-PPP form (Bandyra et al., 2012, Mol Cell 47:943-953). Thus, an RNA silencing pathway was suggested, where 5’-P sRNAs accelerate target turnover by allosteric activation of RNase E. Moreover, recently an RNA aptamer was discovered that provides so far the only tool to generate 5’-P RNA species in vivo on demand (Göpel et al., 2016, NAR 44:824-837).

In the current study, the aptamer was used to establish a conditional cleavage system allowing the release of 5’-P sRNAs to study their impact on target RNA degradation in vivo. sRNAs that are generated via primary transcription (e.g. MicC, RyhB) are highly unstable in their monophosphorylated forms in vivo. Accordingly, the 5’-PPP forms exerted a stronger or comparable regulatory effect on their targets as compared to the 5’-P variants. In contrast, the 5’-phosphorylation state has no impact on stability of sRNAs that are naturally monophosphorylated (e .g. CpxQ, SroC), as they are generated via processing. The latter sRNA class yielded comparable effects on its target RNAs, regardless of their 5’-phosphorylation status and the presence of RppH. Based on our results, it appears that 5’-P sRNA-stimulated target degradation by RNase E does not represent a mechanism operating in vivo.

Revising RNA Architecture - Characterization of NAD-capped RNA in bacteria

Dr. Katharina Höfer[1], Florian Abele[1], Andres Jäschke[1]

[1]Heidelberg University

The complexity of the transcriptome is triggered by the specific interplay of transcription initiation, termination and enzymatic RNA processing and decay. The selective degradation of RNA is a crucial component within the regulation of intracellular RNA levels, thus enabling the cell to respond quickly to changing environmental conditions. Especially the removal of a 5'-RNA cap structure is essential for the initiation of RNA decay. Besides the canonical 5'-N7-methyl guanosine cap in eukaryotes, the redox cofactor nicotinamide adenine dinucleotide (NAD) was identified as a new 5'-RNA cap structure in prokaryotic as well as in eukaryotic organisms.

Recently, two classes of NAD-decapping enzymes have been identified, that remove the NAD-cap using different mechanisms.

We identified the Nudix hydrolase NudC as the first bacterial NAD-RNA decapping enzyme which converts NAD-RNA into 5'-monophosphorylated-RNA in vitro and in vivo. Crystal structures of E. coli NudC in complex with NAD reveal the catalytic residues lining the binding pocket and principles underlying the specific molecular recognition of NAD-RNA. Using biochemical mutation analysis tools, we identified an RNA-binding platform that specifically interacts with the RNA, triggering the hydrolysis of the NAD-cap. These studies demonstrate clearly that NAD-RNA is NudCs primary biological substrate.

Moreover, we chemically synthesized fluorescent NAD-RNA analogs to identify and characterize novel NAD-RNA decapping enzymes in real-time. This approach is suitable for enzymatic reactions which result in the release of the quencher, either nicotinamide (Nam) or nicotinamide mononucleotide (NMN), triggering a fluorescence turn-on.

To show the feasibility of the system we applied the fluorescent-NAD-RNA to a library of different NAD processing enzymes and discovered that the eukaryotic enzyme CD38 processes NAD-capped-RNA into ADP-ribose-modified-RNA. Our findings indicate that the decapping of cofactor-modified-RNAs like NAD-RNA is a highly regulated process in the cell performed by different enzymes.

CRISPRtracr: identification and classification of the tracrRNAs

Omer Alkhnbashi[1] and Rolf Backofen[1]

Chair of Bioinformatics, University of Freiburg, Freiburg, Germany

The CRISPR-Cas (Clustered regularly interspaced short palindromic repeats – their associated proteins) system is an RNA-guided adaptive immunity that protects archaea and bacteria from invading mobile genetic elements (MGE). The type II CRISPR-Cas systems employ trans-encoded small RNA (tracrRNA) and Cas9 protein to scan for viral DNA targets.

Here, we develop a CRISPRtracr tool which predicts tracrRNAs and provides a full sequence and structure classification of the tracrRNAs. We also characterize a diversity of crRNAs (CRISPR-repeat), tracrRNAs and Cas9 proteins, and show a variety of sequences and structures.

Ribosome profiling in the alpha-proteobacterium S. meliloti

Saina Azarderakhsh[1], Adithi R. Varadarajan[2], Christian H. Ahrens[2], Elena Evguenieva-Hackenberg[1]

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Ribosome profiling is a global method to determine mRNA sites bound by ribosomes, thus leading to the identification of actively translated mRNAs. Ribosome profiling is also used for genome-wide detection of translational start sites [1]. It could also be useful for characterization of regulatory 5' untranslated regions (UTRs), in particular the regulatory binding of ribosomes in these regions [2]. Since increasing numbers of small regulatory sRNAs with a dual function (as a riboregulator and a small mRNA) are described, ribosomal profiling can also help to identify sRNAs harboring small ORFs. In a previous inventory study of our model organism Sinorhizobium meliloti, hundreds of sRNAs were identified [3,4]. We are interested in ribosome profiling of S. meliloti. For this, optimization of polysome isolation from S. meliloti was performed and a 6-frames protein data bank was created. The combination of data from previous transcriptome analyses by others [3,4] and our results will help to determine the translational start sites in this model organism, to define the position of regulatory ribosomes, and to identify new dual function sRNAs. In addition, novel small proteins might be detected and verified. Most recent results for ribosome profiling of S. meliloti will be presented.

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The host-encoded RNase E endonuclease as the crRNA maturation enzyme in a CRISPR–Cas subtype III-Bv system

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The maturation of crRNAs critically depends on RNA endonucleases such as Cas6 (most subtype I, III-A, III-B and III-D systems), Cas5 (subtype I-C), RNase III/tracrRNA (type II) or the recently identified single effector proteins of type V and VI systems. However, some subtype III-B systems lack obvious RNA endonuclease candidates for the crRNA maturation[1]. Here we studied the subtype III-B system in the cyanobacterium Synechocystis sp. PCC 6803 lacking any Cas6 endoribonuclease for the maturation of crRNA. Furthermore, this system encodes a peptidase for an unknown purpose and harbors an unusual Cmr6-Cmr1 fusion protein characterizing it as a subtype III-B variant (III-Bv) system. Its mature crRNAs are characterized by unusually long 5' handles of 13 and 14 nt, both in vivo and in vitro[2]. We identified the major housekeeping RNase E to be responsible for the maturation of these transcripts. In vitro cleavage assays revealed a high level of processing robustness tolerating various modifications near the cleavage site. UV-crosslinking of the enzyme and its substrate and subsequent site-directed mutagenesis identified four amino acid residues that are involved in RNA binding from which two are essential for catalysis. Overexpression of RNase E in vivo led to the overaccumulation of the respective crRNAs suggesting the stabilization by the increased formation of RNP complexes or that the maturation was rate limiting in their biogenesis. In contrary, a dCas9/sgRNA knock-down approach reduced the abundancy of mature crRNA. The results open a new view on the adaptation of the CRISPR machinery and suggest rapid sequence evolution within a flexible region of an otherwise very conserved protein to accommodate new substrates.

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Antibiotics and depolarizing toxins trigger processing of ribosomal RNAs at their 3’ ends in *Escherichia coli*

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In Escherichia coli, the type I toxin-antitoxin systems tisB/istR-1 and dinQ/agrB are part of the SOS response. Transcription of the toxin genes tisB and dinQ is repressed by the SOS master regulator LexA under normal growth conditions, but is induced as soon as DNA damage occurs. Both systems display complex regulation on the post-transcriptional level by mRNA 5’ UTR structures and RNA antitoxins IstR-1 and AgrB. Toxins TisB and DinQ are small hydrophobic proteins that target the inner membrane and cause depolarization, which in turn favors ATP depletion, growth stasis and formation of multidrug tolerant persister cells. However, the physiological state of cells expressing these toxins is poorly understood.

Here, we show that the β-lactam antibiotic ampicillin causes degradation of the 16S and 23S rRNAs, with the occurence of truncated forms of both molecules. Differential probing revealed that both rRNAs are processed at their 3’ ends. In cells treated with the SOS-inducing antibiotic ciprofloxacin, this rRNA processing was not detected, unless TisB synthesis was increased by deletion of antitoxin istR-1 and the inhibitory 5’ UTR structure in the tisB mRNA (strain Δ1-41 ΔistR). We therefore tested whether antibiotic-independent expression of TisB was sufficient to cause rRNA processing. Intriguingly, ectopic expression of both TisB and DinQ reproduced the processing pattern. We hypothesized that rRNA processing indicates a defect in the translational machinery, which we tested upon cirpofloxacin treatment in strain Δ1-41 ΔistR. Indeed, ciprofloxacin-dependent activation of the SOS response was not detected on the protein level, although mRNAs of SOS response genes were clearly induced. We conclude that depolarizing toxins indirectly affect the translational machinery to corrupt protein biosynthesis, which might favor formation of dormant cells (e.g. persisters). The factors that are required for the rRNA processing still await identification.

Lead-seq: In vivo RNA structure probing on genome-wide scale

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Deciphering the dynamic conformation of RNA molecules within living cells is imperative to understand the structure-function relationship of RNAs. Recent advances in probing the in vivo structurome, including the use of SHAPE (Selective 2'-Hydroxyl Acylation analyzed by Primer Extension) reagents or DMS (dimethyl sulfate), provided unprecedented insights into the architecture of RNA molecules in the living cell (1). Here, we report the establishment of lead probing in a global RNA structuromics approach. In order to elucidate the transcriptome-wide RNA landscape in the enteric pathogen Yersinia pseudotuberculosis, we combined lead(II) acetate-mediated cleavage of single-stranded RNA regions (2) with high-throughput sequencing. This approach, termed "Lead-seq", provides structural information on all four nucleotides and is sensitive to secondary as well as tertiary interactions of RNA molecules. The method recapitulates single RNA secondary structures, but also reveals global structural features of coding and non-coding RNAs. Moreover, the application of Lead-seq to Y. pseudotuberculosis cells grown at two different temperatures uncovered thermo-responsive structural dynamics of RNA. Overall, this study establishes Lead-seq as complementary approach to determine intracellular RNA structures on a global scale.

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A 3'UTR derived small RNA in *Staphylococcus aureus*, RsaG, regulates redox homeostasis in response to glucose-6-phosphate

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Staphylococcus aureus is an opportunistic pathogen that causes multiple infections by colonizing a large number of tissues and organs. This bacterium has developed a wide range of strategies that allow its quick adaptation in response to various stresses and changes in the environment. In addition to transcription factors and two-component systems, the non-coding RNAs (sRNAs), which are often induced upon stress, regulate in a dynamic manner the expression of numerous genes, some of them encode virulence factors, through direct basepairings with their target messenger RNAs (mRNAs). Around 90 sRNAs have been identified in S. aureus although their functions are still poorly explored. The primary aim of the project is to characterize the function of one peculiar sRNA, called RsaG. This sRNA is located in the 3' untranslated region (3'UTR) of the uhpT gene encoding a glucose-6-phosphate (G6P) transporter expressed in response to extracellular G6P, a major carbon source. The transcript uhpT-RsaG undergoes a rapid degradation from 5' to 3' end by the action of two exoribonucleases, which are blocked by a particular structure at the 5' end of RsaG leading to its accumulation. A combination of in vitro and in vivo approaches allowed the identification and validation of 22 RNA targets of RsaG including rex, a mRNA encoding a transcriptional repressor, which regulates the synthesis of proteins involved in the anaerobic and nitrogen metabolism, and a second sARN, RsaI. We will describe the molecular mechanism by which RsaG activates Rex synthesis, and the functional consequences of these regulations during infection. This work should ultimately reveal new strategies and/or targets to fight this pathogen.

Functional study of RNase E and RNase J in the Cyanobacterium *Synechocystis*

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mRNA levels result from an equilibrium between transcription and degradation. Ribonucleases (RNAses) facilitate the rapid turnover of mRNA, which is an important way of controlling gene expression, allowing the cells to adjust transcript levels to the surrounding environment. In contrast to the heterotrophic bacteria Escherichia coli and Bacillus subtilis, model Gram-negative and Gram-positive bacteria, respectively, processing/decay of mRNA has not been studied in much detail in photosynthetic cyanobacteria. Synechocystis sp. PCC6803 is a genetically amenable model organism for cyanobacteria and it has a multicopy-genome. It encodes orthologs of both E. coli and B. subtilis RNAses, including RNase E and RNase J, respectively. Both nucleases appear to be essential and their genes cannot be deleted to homozygosis in Synechocystis. We characterized the activity of both nucleases in vitro by comparing their cleavage specificity respect to their orthologs from E. coli and B. subtilis as well as to the chloroplast RNAse J of Chlamydomonas reinhardtii. Both RNase E and RNase J showed a similar cleavage pattern in vitro. However, like the B. subtilis counterpart, RNAse J displays a robust 5' exoribonuclease activity and a weaker endonuclease activity. In order to study the in vivo enzymatic activity of both nucleases, we applied an RNA-seq analysis to measure changes of mRNA steady-state levels in partially depleted strains of RNAse E and RNAse J compared to a wild-type. The resulting transcriptome analysis allowed to observe effects on specific transcripts, with RNase E affecting the expression of more genes compared to RNAse J. In order to obtain a stronger deletion phenotype for subsequent RNA-seq analysis we will construct strains conditionally expressing RNAse E and RNase J from a plasmid in order to completely segregate the knock-out deletions.

Regulating the regulator – The LysR-type transcriptional regulator LsrB controls multiple sRNA genes

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Small non-coding RNAs are ubiquitous post-transcriptional regulators in all kingdoms of life. Despite the ever-growing number of identified and characterized bacterial sRNAs, little is known about upstream regulators that mediate transcriptional regulation of sRNA genes. The few well-established examples are mostly derived from E. coli and Salmonella and the combination of these sRNAs and their corresponding transcriptional regulators often results in mixed regulatory circuits [1].

In the plant pathogen Agrobacterium tumefaciens, which is known for its unique ability of interkingdom gene transfer, many sRNAs exhibit a growth phase-dependent expression pattern [2, 3]. We report studies of the LysR-type transcriptional regulator LsrB, which is highly conserved among alphaproteobacteria. LsrB is required for wild-type growth, exopolysaccharide synthesis and stable transformation of the plant host. Moreover, LsrB regulates the expression of at least four growth phase-dependent sRNA genes. Our results suggest that LsrB can act as both transcriptional activator and repressor, controlling a vast number of sRNA and protein-coding genes in A. tumefaciens.

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sRNA scr5239 – an important regulator of the C- metabolism in *Streptomycetes*

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We are interested in the identification and characterization of small non-coding RNAs (sRNAs) in Streptomyces coelicolor. Using RNAseq we identified a 159 nt transcript called scr5239. It has a high degree of sequence and structure conservation. Interestingly its expression is dependent on the global transcriptional regulator DasR. DasR is the major regulator of phosphosugar metabolism in Streptomyces and directly and indirectly controls the expression of more than 100 genes. To identify targets of scr5239 we performed an iTRAQ analysis comparing the wild type to sRNA overexpression and deletion strains. 32 of ~1800 identified proteins showed a significant sRNA dependent change in protein expression. Among them were transcriptional regulators, enzymes of central metabolism but also hypothetical proteins of unknown function. Analysis of the mRNA level of all 32 proteins showed that only six of them show the same behaviour of mRNA and protein implying cleavage and digestion of the mRNA upon sRNA binding or sRNA dependent changes of transcriptional regulation of these genes.

GO-term analysis showed that more than one third of the identified proteins are metabolic enzymes. One of these is the Phosphoenolpyruvat-Carboxykinase (PEPC). PEPC is a key enzyme of the metabolism as it connects the glycolysis with the TCA cycle. It catalyses the irreversible carboxylation of phosphoenolpyruvate to oxaloacetate and maintains the continuity of the TCA cycle carbon fluxes. Overexpression or deletion of scr5239 leads to repression or induction of protein expression respectively. We used the native promotor vs. a synthetic promotor to show posttranscriptional regulation. After the investigation of the interaction between the sRNA and PEPC mRNA we are currently characterizing the regulation network of the sRNA. Therefore, we are using different carbon sources and studying the connection between DasR and the sRNA to understand how scr5239 affects the central carbon metabolism.

RNase E substrate affinity in *Synechocystis* sp. PCC 6803

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RNase E is an essential endoribonuclease in E. coli. The enzyme is involved in RNA maturation and degradation and its C-terminus acts as the scaffold of the degradosome. Furthermore, it plays an important role in the maturation and action of several sRNAs.

Until now, two different ways of target recognition by RNase E were identified. Firstly, RNase E shows a high affinity to 5'-mono-phosphorylated RNAs. This affinity is based on several amino acids forming a shallow pocket and specific hydrogen bonds to 5'-phosphates. Mutating one of those amino acids (T170V) abolishes the enzyme's high affinity to 5'-mono-phosphorylated RNAs without strongly affecting the overall catalytic activity. Secondly, the 5'-bypass pathway recognises RNAs' secondary structures. Bandyra et al. [1] identified eight amino acids which seem to be responsible for this interaction.

RNase E homologues with different structural properties were found in many bacteria. However, its target affinity was mainly investigated in the closely related species Salmonella and E. coli.

Synechocystis sp. PCC 6803 is the best studied model organism for cyanobacteria. Synechocystis RNase E is homologous to the N-terminal half of E. coli RNase E. The amino acids involved in 5'-mono-phosphate coordination are conserved in Synechocystis. In a mutant strain carrying the mutation homologous to E. coli T170V, 5S rRNA maturation is impaired. This is in concordance with similar findings for E. coli. However, the eight amino acids identified by Bandyra et al. for the 5'-bypass pathway seem to be only weakly conserved. By performing in vitro cleavage assays, we want to verify potential homologous amino acids identified by homology modeling.

Using RNA-Seq and iCLIP, we plan to elucidate the role of both substrate recognition pathways on a genome-wide level and identify potential new sRNAs and their targets.

[1] Bandyra et al., 2018, Molecular Cell 72, 275-285

Exploring the role of small RNAs in virulence control of the food-borne pathogen *Campylobacter jejuni*

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Campylobacter jejuni is a human pathogen and currently the leading cause of bacterial food-borne gastroenteritis worldwide (WHO). Bacterial virulence factors or mechanisms contributing to its pathogenesis in humans compared to its commensal lifestyle in chickens are still not fully elucidated. To study Campylobacter infection we have established a novel intestinal 3D tissue infection model. This model is based on an acellularized extracellular matrix scaffold, which is reseeded with human intestinal epithelial cells (Pusch et al., 2011), recapitulating aspects of the micro-environment present in the human intestine. Using this model, we have observed that C. jejuni shows infection phenotypes that are not apparent in standard in-vitro cell culture systems, but resemble in-vivo observations. To further study the underlying molecular mechanisms of Campylobacter-host interactions, we employed a dual RNA-seq approach (Westermann et al., 2012) to simultaneously follow the transcriptomic output of both host and bacterial cells upon infection of Caco-2 cells in 2D monolayer assays as well as in the 3D tissue model. Initial focus was laid on the changes occurring in the bacterial transcriptome and several C. jejuni sRNAs (Dugar et al., 2013) were found to be significantly upregulated, which might point towards their involvement during the infection process. One upregulated sRNA candidate was shown to be dependent on RNase III as well on RNase Y for proper 5´- and 3´-end processing, respectively. We have constructed deletion mutants of selected sRNA candidates to further study their potential role in infection assays. Besides validating the involvement of the sRNAs during C. jejuni pathogenesis with complementation strains expressing the respective sRNAs in-trans, we further aim to identify their cellular targets via RNA-seq of deletion mutants. This might help to uncover their molecular functions also with regard to host-pathogen interactions.

RsaC sRNA balances two defensive responses in *S. aureus*

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The human pathogen Staphylococcus aureus produces numerous small regulatory RNAs (sRNAs) for which functions are still poorly understood. Here, we focused on an atypical sRNA called RsaC. Its length varies between different isolates due to the presence of repeated sequences at the 5' end, ranging up to 1,116 nt in HG001 strain. The 3' part of RsaC is highly conserved and contains C-rich sequences, which are characterized as regulatory motifs in other staphylococcal sRNAs. Using MS2-affinity purification coupled with RNA sequencing (MAPS) and quantitative differential proteomics, we identified sodA mRNA as a main target of RsaC sRNA. SodA is a Mn-dependent superoxide dismutase involved in oxidative stress response. We demonstrated that in presence of RsaC, S. aureus cells were less resistant to oxidative stress, in relation with lower activity of SodA enzyme. Remarkably, rsaC gene is co-transcribed with the major manganese ABC transporter MntABC and, consequently, RsaC is mainly produced in response to Mn starvation. This 3'UTR-derived sRNA is released from mntABC-RsaC precursor after cleavage by RNase III which presumably recognizes a duplex formed by RsaC and its antisens RNA. By negatively regulating non-essential Mn-containing enzymes such as SodA, RsaC reduces the needs for Mn. SodM, an alternative Sod enzyme using either Mn or Fe as co-factor, replaces SodA to response to oxidative stress. Thus, RsaC may counteract the sequestration of Mn by the host organism, strategy used to limit the virulence of S. aureus.

The regulatory role of the non-coding RNA YFR2 and YFR1 in *Prochlorococcus* MED4

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In cyanobacteria, especially Prochlorococcus that lacks many protein regulators, gene regulation by non-coding RNAs (ncRNAs) and antisense RNAs (asRNAs) plays an important role. There are currently more than 20 regulatory small RNAs (sRNAs) that have been identified in the model strain Prochlorococcus MED4. While most of these sRNAs are ecotype-specific, sRNA homologs of Yfr1 and of the Yfr2 family are widely found throughout the cyanobacterial phylum. The sRNA Yfr1 and members of the Yfr2 sRNA family are almost universally present within cyanobacteria. The conserved motifs of these sRNAs are nearly complementary to each other, suggesting their ability to participate in crosstalk. The conserved motif of Yfr1 is shared by members of the Yfr10 sRNA family, members of which are otherwise less conserved in sequence, structure, and synteny compared to Yfr1. Unlike most studied regulatory sRNAs, Yfr1 gene expression only slightly changes under the tested stress conditions and is present at high levels at all times. In contrast, cellular levels of Yfr10 increase during the course of acclimation to darkness, and levels of Yfr2 increase when cells are shifted to high light or nitrogen limitation conditions. We investigated the targetomes of Yfr2, Yfr1, and Yfr10 in Prochlorococcus MED4, establishing Cell-free RNA Affinity Pull-down (CRAFD-Seq) as a new method for identifying direct targets of these sRNAs that is applicable to all bacteria, including those that are not amenable to genetic modification. The expression of some Yfr2 homologs that possess CGRE1 (cyanobacterial GntR family transcriptional regulator responsive element 1) in their promoter is regulated by the cyanobacterial GntR transcriptional regulator PMM1637.

Differential regulation of multiefflux operon genes by an antibiotic-triggered antisense RNA and the leader peptide peTrpL

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The sRNA rnTrpL is an attenuator-derived, dual-function sRNA encoding the leader peptide peTrpL in the plant symbiont Sinorhizobium meliloti [1]. Constitutive ectopic overexpression of rnTrpL from a pRK plasmid (that harbors a tetracycline (Tc) resistance gene) resulted in apparently lower Tc-effect on the S. meliloti 2011 transcriptome. This was explained by a peTrpL-dependent increase of the activity of the multidrug efflux pump SmeAB. We found that induced expression of peTrpL in strain 2011ΔtrpL destabilizes the smeR segment of the tricistronic mRNA smeABR. Since smeR encodes the TetR-type repressor SmeR of the smeABR operon, this results into higher smeAB expression, increased multidrug efflux and multiresistance. This posttranscriptional pathway for differential expression of co-transcribed genes is induced by several SmeR effectors like the antibiotics tetracycline, erythromycin, chloramphenicol, rifampicin and the plant flavonoid genistein. These SmeR effectors also induce the transcription of an antisense RNA (asRNA) that is complementary to smeR. An effector-dependent ribonucleoprotein complex consisting of peTrpL, smeR mRNA and its asRNA was purified from S. meliloti. The molecular mechanism of the smeR destabilization by peTrpL in a complex with a triggering antimicrobial compound is under investigation. The role of peTrpL in bacterial multiresistance is conserved in other Alphaproteobacteria.

[1] Melior et al NAR

A small RNA in *Haloferax volcanii* associated with the CRISPR locus

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H. volcanii is an archaeal model organism possessing a type I-B CRISPR-Cas system. We have previously determined the characteristics of the interference activity in detail, revealing the requirements for an efficient interference (Maier et al., 2013). During these studies we detected a small RNA gene encoded between one of the CRISPR loci and the cas gene cluster. This RNA is transcribed into a precursor RNA that is subsequently processed into a 51 nt long mature RNA comprising the conserved crRNA 5´ handle.

We want to investigate the biogenesis as well as functions of this sRNA in the halophilic archaeon H. volcanii. For our first goal we will analyse whether this sRNA is also processed by the Cas6 endonuclease, which is responsible for processing the neighbouring CRISPR locus. In addition, we want to deduce whether the 5' handle sequence allows for the incorporation of the sRNA into the Cascade complex. If so, we want to identify the genes targeted by the small RNA to learn about functions of Cascade beyond immunity. To that end, we want to employ the RILseq method, which was established by Melamed et al. (2016) [2]. RILseq allows to identify the sRNA associated target RNAs by copurification via a sRNA binding protein. To purify the sRNA and its target RNAs we will use a FLAG-tagged Cas7 protein. Since the sRNA might not be incorporated into the Cascade complex, but bound by the Lsm protein instead we will also use a FLAG-tagged Lsm protein for the RILseq approach. The biological functions of the sRNA will also be examined with the help of a strain that has the sRNA gene deleted.

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[2] Melamed et al., 2016; Molecular Cell 63: 884–897

The redox-sensitive DEAD-box RNA helicase CrhR interacts with the transcribed leader of a subtype III-D CRISPR-Cas system

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CrhR is a redox-sensitive DEAD-box RNA helicase from *Synechocystis* sp. PCC 6803. It is important in *Synechocystis* for its normal growth at low temperatures. CrhR catalyzes both double-stranded RNA unwinding and annealing and may be involved in the rearrangement of the secondary structure of RNA. Direct RNA targets of CrhR and interaction partners are remaining unknown. In order to monitor genomewide binding of CrhR and identify yet unknown functions, we applied several different approaches to pulldown RNA interacting with the RNA helicase. One of the RNAs, that was co-purified together with the helicase, was the transcribed leader of a subtype III-D CRISPR-Cas system. We show that CrhR interacts with this RNA *in vitro*.

A new role for CsrA: Promotion of complex formation between an sRNA and its mRNA target in *Bacillus subtilis*

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SR1 is a dual-function sRNA from Bacillus subtilis that acts as base-pairing regulatory RNA on ahrC mRNA encoding the transcriptional activator of the arginine catabolic operons rocABC and rocDEF and as peptide-encoding mRNA on the gapA operon. Previously, we found that the abundant RNA chaperone Hfq bound both SR1 and ahrC mRNA, but did neither stabilize either RNA nor promote the interaction between both complementary molecules. Using DRaCALA we discovered that CsrA bound efficiently SR1. EMSA revealed that SR1 and ahrC mRNA were bound by CsrA and identified GGA motifs important for CsrA binding. In addition, EMSAs with labeled SR1 and unlabeled ahrC RNA and vice versa demonstrated that CsrA increased complex formation between both complementary RNAs significantly. Footprinting analysis showed that CsrA induces slight structural alterations in ahrC mRNA which open region G' that is complementary to SR1 region G, thus facilitating the initial contact with SR1. CsrA did neither stabilize SR1 or ahrC mRNA nor directly affect ahrC translation. The in vitro effects of CsrA were confirmed in vivo using transcriptional procABC- and procDEF-lacZ fusions and growth experiments with minimal medium and L-arginine as main C-source. Mutations corroborated the importance of CsrA binding motifs M1 and M2 in ahrC RNA in vivo. Translational lacZ fusions excluded a direct influence of CsrA on translation of rocA, rocD, the first genes of the operons, or CcpN, the repressor of sr1 transcription. Based on EMSAs with mutated SR1 and ahrC mRNA species we suggest a mechanism of action of CsrA. Future research will reveal if the new role of CsrA in the promotion of sRNA/target RNA pairing is confined to the SR1/ahrC case.

Synteny based network analysis

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Small RNAs are relevant regulators of bacterial metabolism. Therefore, they modulate protein expression by mRNA interaction. Also, the number of known expressed sRNAs with unknown function increases rapidly due to improved sequencing techniques. As it is likely that a critical sRNA is conserved, an essential step in identifying the task of an sRNA is to search for homologs. However, as some sRNAs show a more conserved structure instead of conserved sequence, it is not easy to find such. One standard tool helping to predict sRNA homologs is GLASSgo, which can find them from a single sequence input. Although sequences predicted by GLASSgo are highly trustable, the workflow might not find some homologs with very low sequence conservation. To overcome this issue, an additional synteny analysis independent from the sequence and structure might gain information about potential homologs and improve the GLASSgo prediction.

Here, we present a synteny network build from the GLASSgo hits to test questionable sequences. Therefore we make use of Google´s PageRank to rate nodes in the Network and are considering how to assess trustworthiness of candidate sequences that are joined to the network.

The involvement of the antisense RNA RSaspufL in regulated formation of photosynthesis complexes in *Rhodobacter sphaeroides*

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The facultative phototrophic model bacterium Rhodobacter sphaeroides is known for its metabolic versatility. Under microaerobic conditions it synthesizes intracytoplasmic membranes harbouring the pigment protein complexes needed for anoxygenic photosynthesis. For fast adaption to varying environmental conditions the transcription of genes in the photosynthetic gene clusters is tightly regulated. The puf operon comprises genes which encode proteins of the light harvesting complex I (LHI) and the reaction centre (RC). RNA-Seq and Northern blot analysis of transcripts derived from the puf operon unveiled that also certain small RNAs are transcribed. Up to date two different puf operon associated sRNAs were characterized. The Hfq-dependent sRNA aspufL was detected antisense to the 5´ region of the pufL gene extending into the pufA-pufL intercistronic region. Northern blot results confirmed the presence of aspufL (~ 180 nt) under microaerobic and phototrophic conditions. An artificial increase in the amount of the aspufL by plasmid driven over-expression led to a reduction in the amount of LHI/RC-complexes. In vivo reporter assays showed that aspufL influences pufL in an Hfq-dependent manner. Additionally, we could show that the over-expression also influences the half-live of the polycistronic puf mRNA. Taken together aspufL is the second non-coding RNA which is associated with the puf-operon and plays an important role in processing and degradation of its target mRNA.

Ribonucleases with novel catalytic features in plant symbiotic bacteria

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Extensive work in model enterobacteria has evidenced that several endoribonucleases, such as RNase E or RNase III, serve pivotal roles in small RNA-mediated posttranscriptional silencing of gene expression. Characterization of these protein hubs commonly provide global functional and mechanistic insights into complex sRNA regulatory networks. The nitrogen-fixing legume endosymbiont Sinorhizobium meliloti is a non-classical model bacterium with a very complex lifestyle in which riboregulation is expected to play important adaptive functions. Here, we have characterized for the first time two ubiquitous endoribonucleases present in S. meliloti: RNase III, the prototypical double-strand bacterial RNase, and YbeY. Both enzymes showed catalytic features distinguishable from those of their Escherichia coli orthologs. Purified S. meliloti RNase III (SmRNase III) behaves as a strict metal cofactor-dependent double-strand endoribonuclease, degrades endogenous RNA substrates of diverse biogenesis with different efficiency, and is involved in the maturation of the 23S rRNA. SmRNase III loss-of-function neither compromises viability nor alters morphology of S. meliloti cells, but influences growth, nodulation kinetics, the onset of nitrogen fixation and the overall symbiotic efficiency of this bacterium on the roots of its legume host, alfalfa, which ultimately affects plant growth. On the other hand, the recently discovered endoribonuclease SmYbeY exhibits unprecedented catalytic versatility for the cleavage of single- and double-stranded RNA molecules (ssRNA and dsRNA), influencing turnover of bulk and sRNA-regulated mRNAs in S. meliloti. We have also observed that SmYbeY exhibits SmRNase III-like metal-dependent catalytic activity on dsRNAs. This similarity and the affinity of SmYbeY for asRNA–mRNA duplexes led us to speculate with a major role of SmYbeY in antisense regulation.

Posttranscriptional regulation of SAM in the alpha-proteobacterium *Sinorhizobium meliloti*

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Methionine is used together with ATP to synthesize S-adenosylmethionine (SAM), the main methyl donor in the cell. We have shown that in the nitrogen fixing plant symbiont Sinorhizobium meliloti, RNases E and J are needed for homeostasis of SAM [1]. In the S. meliloti 2011 mutants with mini-Tn5 insertions in the corresponding genes rne and rnj the SAM level was increased. In bacteria the SAM biosynthesis is posttranscriptionally regulated by SAM-binding riboswitches. In several alpha-proteobacteria SAM-II riboswitch was predicted in the 5'-UTRs of particular methionine genes [2]. In S. meliloti SAM-II riboswitches were predicted in the 5'-UTRs of the genes metA and metZ. To address the mechanisms of posttranscriptional SAM regulation in S. meliloti, we analyzed metA and metZ by qRT-PCR and the corresponding liberated (sRNA) riboswitches by Northern blot hybridization. We expect under high methionine conditions increased levels of liberated riboswitches and lower mRNA levels when compared to low methionine conditions. In line with this, in the wild type strain 2011 we observed increased levels of the two liberated riboswitches in rich TY medium when compared to minimal GMX medium. However, the levels of the corresponding mRNAs did not differ under both conditions. Currently we are analyzing transcriptional and translational metZ and metA reporter fusions to study the regulation of these met genes and their impact on SAM regulation.

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The phosphotransferase protein EIIANtr regulates AcnB aconitase activity in a phosphorylation-independent manner in *Escherichia coli*

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Bacteria constantly adapt their metabolism to the availability of carbon and nitrogen sources. An important system for carbohydrate utilization is the canonical PEP-dependent phosphotransferase system (PTS). In parallel, many Proteobacteria possess a nitrogen related PTS, which exclusively exerts regulatory functions and consists of the three proteins EINtr, NPr and EIIANtr (encoded by ptsN). EIIANtr regulates various cellular processes, such as phosphate and potassium uptake, depending on its phosphorylation state 1. However, not all phenotypes of E. coli lacking ptsN can be explained by the known targets of EIIANtr. For instance, reduced flux through the tricarboxylic acid (TCA) cycle was observed in a ΔptsN strain, while it was not affected in ΔptsP or ΔptsO strains, in which EIIANtr is mainly present in its non-phosphorylated form 2. These findings suggest that the flux through the TCA cycle is regulated by EIIANtr levels rather than its phosphorylation state.

In this study, the aconitase AcnB was identified as a novel interaction partner of EIIANtr. This interaction is independent of the EIIANtr phosphorylation state. Furthermore, higher aconitase activity was measured in a ΔptsN strain as compared to wildtype, but not in a ΔptsP strain, suggesting a regulation of AcnB depending on EIIANtr levels, but independent of its phosphorylation state. In order to find factors, whose inactivation leads to an up-regulation of ptsN, we carried out a transposon mutagenesis screen. In addition to Hfq, a key player in post-transcriptional regulation, we identified several genes with described roles in cell envelope homeostasis. Taken together, these findings suggest that EIIANtr amounts are modulated at the post-transcriptional and potentially transcriptional level in response to cell envelope stress, thereby affecting basic metabolic pathways.

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Generation of bacterial sRNAs from sRNA-mRNA cotranscripts

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During the past decades different types of non-coding RNAs (ncRNAs) were detected in prokaryotes. Within this class the small RNAs (sRNAs) soon became a main focus of interest. These sRNAs play a major role during gene regulation on a post-transcriptional level and can act in different ways to either facilitate or to impede translation of the targeted mRNAs.

RNA-Seq data of the model organism Rhodobacter sphaeroides indicated the presence of several sRNA-mRNA cotranscripts. They consist of the coding sequence itself and an additional sRNA which is located in the 5' untranslated region (UTR) or in the 3'UTR. We are elucidating the mechanisms of maturation and aim to identify the RNases involved in this processing. The involvement of RNase E in the maturation of several sRNAs was already demonstrated (Förstner et al., 2018), but the role of other RNases needs further investigation. As a facultative photosynthetic alpha-proteobacterium Rhodobacter sphaeroides needs a tight regulation of photosynthesis genes to avoid photooxidative stress. We are investigating the regulation of processing during cellular stress, i.e. in response to reactive oxygen species (ROS). Moreover the aim is to analyze the processing of sRNAs during different growth phases as well as the regulation of the amount and activity required RNases.

Transcriptional control of *Prochlorococcus* MED4 RNase E

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The ecologically important cyanobacterium Prochlorococcus is infected by ecotype-specific cyanophages. While most host genes are down-regulated during infection, RNase E is induced on mRNA and protein level. Long antisense RNAs protect the phage transcriptome and parts of the host transcriptome from enhanced RNase E degradation. Furthermore, during phage infection an alternative promoter P2 is predominantly utilized leading to a shorter version of the RNase E protein. We found that the phage Gp0.7 protein, which possesses a MarR trancriptional regulator DNA-binding domain, possibly regulates RNase E promoter recognition. In the heterologous E. coli reporter system expression of Gp0.7 induces a general induction of the GFP reporter irrespective of the possible operator sequences. However, the downstream region of the alternative RNase E promoter P2 shows enhanced induction of the GFP reporter. We detected an inverted repeat in the RNase E downstream promoter P2 region, which occurs in several host and in two phage downstream promoter regions. All of these promoters show enhanced induction of the GFP reporter.

A framework for the biocomputational prediction and analysis of sRNAs in microbial environmental populations and their experimental validation

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Small regulatory RNAs and antisense RNAs have important roles in the regulation of gene expression in bacteria but are underexplored, especially in natural populations. The cyanobacterium Prochlorococcus is an important primary producer in the oligotrophic ocean. Starting from a metatranscriptomic differential RNA-Seq (mdRNA-Seq) dataset, we identified abundant sRNAs in environmental populations, tracked their homologs in laboratory isolates and provide a framework for detailed further characterization.

Several of the experimentally validated sRNAs responded to meaningful changes in the cultivation conditions. Expression of the sRNA Yfr28 was highly stimulated in low-nitrogen conditions and predicted targets encode a sigma factor and several enzymes and transporters, suggesting its pivotal role in the coordination of primary metabolism.

The presented work-flow will be useful also for other environmentally relevant microorganisms, for which the capabilities for experimental validation are frequently limiting while there is abundant sequence information available.

Transcriptional and post-transcriptional control of a *trans*-acting small RNA regulator of virulence in *Campylobacter jejuni*

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Global transcriptome mapping has identified many candidate small RNAs (sRNAs) in Campylobacter jejuni, a major foodborne pathogen, as well as the related gastric pathogen Helicobacter pylori. Functional characterization has revealed first mechanisms and functions of these sRNA canidates. For example, H. pylori uses a small RNA called RepG sRNAs to repress targets via base-pairing of C/U-rich loops with G-rich sequences (Pernitzsch et al. 2014). Recently, we have identified a potential functional homolog of RepG in C. jejuni. CJRepG represses the flagellin modification factor ptmG, and both the sRNA and target influence host interactions. While both CJRepG and HPRepG can repress the target ptmG in C. jejuni, it is unclear if these sRNAs are true homologs. Unlike HPRepG, CJRepG (CJnc190) is processed from longer precursors into a mature sRNA by RNase III, and this maturation is required for target ptmG repression. Furthermore, while HPRepG is encoded as a stand-alone sRNA, CJRepG is encoded antisense to a second non-coding RNA, CJnc180. Here, we have investigated the processing of the sRNAs and the role of the antisense RNA in more detail. CJRepG precursors are expressed from two promoters that produce several primary transcripts with different 5'-ends, but all of these transcripts are processed to the same 3'-end-derived mature sRNA. Surprisingly, processing of the asRNA by RNase III, but not CJRepG, requires its antisense partner in vitro and in vivo. We also show that overexpression of the asRNA can antagonize CJRepG repression of its target, ptmG. Together, our data suggest that CJRepG is a member of a growing class of trans-acting sRNAs that arise from complex biogenesis pathways. This confers the potential for multiple transcriptional and post-transcriptional inputs for their own regulation in order to fine-tune the physiology of bacterial pathogens.

An RNA thermometer controls the flagellar regulator TviA from *Salmonella enterica* serovar Typhi

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RNA thermometers (RNATs) are cis-regulatory RNA elements, which control translation of bacterial genes by temperature-dependent occlusion of the Shine-Dalgarno sequence. These RNA-based thermoregulators allow an immediate and precise adjustment of gene expression to fluctuating temperature during the infection cycle of bacterial pathogens (1).

Salmonella enterica serovar Typhi (S. Typhi) is an enteric pathogen that causes a severe systemic infection, namely typhoid fever. A global in silico approach predicted a potential RNA thermometer in the 5'-untranslated region of the tviA gene encoding the TviA transcriptional regulator. TviA steers expression of several virulence-related genes: For instance, TviA reduces the synthesis of flagellin or the production of the invasion associated type III secretion system (T3SS-1), but positively regulates biosynthesis of the so-called Vi capsule (2). The Vi capsule is a polysaccharide polymer that prevents S. Typhi from complement-mediated clearance.

Results from immunofluorescence microscopy as well as flow cytometry demonstrated higher capsule synthesis at 37 °C compared to 25 °C. Reporter gene studies confirmed the regulatory potential of the tviA 5'-UTR. Moreover, enzymatic structure probing in combination with toe printing analysis revealed the RNA-based sensor to melt open at elevated temperatures leading to increased ribosome binding. Finally, insertion of stabilizing and destabilizing point mutations into the 5'-UTR impair thermometer functionality. We postulate that the tviA RNAT allows adequate regulation of immune evasion at virulence relevant conditions.

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Analysis of the post-transcriptional regulation by antisense RNA for controlling the ratio of CpcL-/CpcG-phycobilisome under nitrogen starvation conditions.

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A light-harvesting antenna system is important for efficient photosynthesis. Phycobilisomes (PBS) are the major light-harvesting antennae in cyanobacteria. Previously, we have reported the presence of a supercomplex comprising the photosystem (PS) I tetramer and a specific type of the phycobilisome, CpcL-PBS, in the heterocyst-forming cyanobacterium Anabaena sp. PCC 7120 (Watanabe et al 2011 PCP, Watanabe et al 2014 PNAS). Normally, PBS is composed of several rods and core cylinders connected through specific linker proteins (Watanabe and Ikeuchi 2013 Photosyn. Res.). In contrast, CpcL-PBS is a rod-type PBS that lacks the core components. The specific linker protein CpcL directly connects the PBS rod with PSI to form PBS-CpcL-PSI supercomplexes. Under nitrogen starvation, the amount of the PBS-CpcL-PSI supercomplex was increased and even larger supercomplexes were detected in cell extracts from whole filaments. The protein amount of CpcL was higher, whereas a rod-core linker of normal PBS, CpcG, was lower in heterocysts than in vegetative cells. These results suggest that an increased ratio of CpcL/CpcG facilitates the formation of the CpcL-PBS rather than CpcG-PBS. It is suggested that transcription of the cpcL is up-regulated under nitrogen starvation conditions by RNA-seq data (Mitschke et al 2011 PNAS). We found a possibility of the post-transcriptional regulation by an antisense RNA of cpcG4 from RNA-seq data. The transcription of asRNA of cpcG4 was induced under nitrogen starvation conditions. The asRNA of cpcG4 may down-regulate the translation of cpcG4 to avoid the assembly of CpcG-PBS under nitrogen starvation conditions. Now we are investigating the correlation between the transcriptional levels of cpcL, cpcG4 and the asRNA of cpcG4 and the assembly of CpcL- and CpcG-PBS under nitrogen starvation conditions.

Global Analysis of Changing Transcript Stability in the Acclimation to Low Iron in a Photosynthetic Cyanobacterium

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The regulation of RNA stability is crucial in the post-transcriptional regulation of gene expression in bacteria. Here, we determined RNA half-lives in the model cyanobacterium Synechocystis sp. PCC 6803 during standard conditions and iron-deplete growth, a condition that poses substantial physiological challenges to a photosynthetic organism and involves well-studied transcriptional and post-transcriptional regulatory mechanisms. We found 3200 transcripts with half-life detected in iron stress conditions while 2567 were in the standard conditions. In total, 2315 different transcripts were detectable under both conditions and subjected to comparative analysis. The median half life was around 2 minutes in both conditions. The results allow for the first time to distinguish between the effects of transcriptional and post-transcriptional regulation in the iron starvation response. Statistical and clustering analyses will aid our understanding of mRNA decay in iron stress condition and further illuminate more about the complexity in the iron regulatory network of cyanobacteria.

Global transcriptional control by the 6S RNAs and the phenotypes of their knockout derivatives in the *Bacillus subtilis* wild type strain NCIB 3610

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Bacterial non-coding 6S RNAs act as global transcriptional regulators. By mimicking the open complex state of promoter DNA, they bind to the active site of RNA polymerase (RNAP). Mostly mild phenotypes have been observed for 6S RNA deletion mutants in the few model organisms studied so far. This contradicts the remarkable abundance, conservation and dissemination of 6S RNA among bacteria. Interestingly, *B. subtilis* possesses two 6S RNA paralogs, named 6S-1 and 6S-2 RNA.

A genome-wide comparison of the laboratory *B. subtilis* strain 168 and its undomesticated ancestor wild type strain NCIB 3610 revealed many mutations and predicted losses of gene function in the former strain. This prompted us to construct 6S RNA knockouts of the NCIB 3610 wild type strain to obtain more biologically relevant information on the regulatory function of the two 6S RNAs in *B. subtilis*. For the first time, we observed a strong phenotype of a 6S-2 RNA knockout strain, that is, increased biofilm formation on solid media, as well as a delayed swarming activity and the ability of surface-attached biofilm formation in liquid culture in a temperature-dependent manner.

It was reported for *E. coli* that DNA promoter sequence plays an important role for the sensitivity of genes to be inhibited by 6S RNA (1). We have begun to address this issue for *B. subtilis* as well by analyzing the influence of 6S RNAs on the expression of selected genes, regarding different sigma factors, by comparative qRT-PCR experiments, using the wild type and 6S RNA knockout derivative strains. Preliminary results suggest that the effects of 6S RNA single and double knockouts are complex and growth phase-dependent. Based on these findings, we plan RNA-Seq experiments for the NCIB 3610 wild type strain and its 6S RNA knockout mutants at different growth states to trace the dynamic transcriptional reprogramming in the presence and absence of the 6S RNAs. Subsequently, more qRT-PCR experiments for selected genes are planned to validate the results of the RNA-Seq analysis. Thereby, we hope to understand the key features of gene regulation by 6S RNAs and the physiologically relevant specifity of both 6S RNA paralogs in *B. subtilis.*

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Natural functions of CRISPR-Cas systems in cyanobacteria

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Cyanobacteria are major players in the global ecosystem, they are the only bacteria that able to produce oxygen during photosynthesis, which allowed them to change the atmosphere of the Earth by producing a huge amount of oxygen 3.5 billion years ago. In present time, cyanobacteria can be found in almost every habitat.

The unicellular freshwater cyanobacterium Synechocystis sp. PCC 6803 was the first phototrophic organism whose genome was completely sequenced. To date, no bacteriophages are known to infect this strain allowing large-scale production strategies. Therefore, it is surprising that the strain still contains three highly transcribed CRISPR-Cas immunity systems (CRISPR1-3) which begs the question of a function of these systems beyond defense.

One possible function of the CRISPR system is the regulation of host gene expression. A part of the 5`UTR of the chromosomally-encoded gene cycI shows 100% similarity to the 5` handle of CRISPR3 crRNAs. The cycI gene product was described as the catalytic subunit of the Mg-protoporphyrin IX monomethylester cyclase which synthesizes the chlorophyll intermediate protochlorophyllide. It was demonstrated that the housekeeping enzyme RNase E recognizes repeats of the CRISPR3 locus and is responsible for the processing of pre-crRNA (Behler et al., 2018).

We are going to test whether cycI mRNA is a substrate for RNase E cleavage and how does it impact on the protein expression and chlorophyll biosynthesis. Also, we would like to reveal whether cleavage of the 5`UTR by RNase E is essential for transcript stability or expression of the cyclase enzyme.

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