Riboregulation in Neisseria meningitidis
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Chapter 9

Summary, general discussion, and future perspectives

Robert Huis in 't Veld
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Summary

Exploration of the $\sigma^E$ regulon in *Neisseria meningitidis*

The first regulatory system in *N. meningitidis* unraveled in chapters 2 and 3 is that of the alternative $\sigma$ factor E ($\sigma^E$). Bacteria live in an ever-changing environment and must alter protein expression to adapt to these changes and survive. The RNA polymerase associates with $\sigma$ factors to aid in the recognition of target promoter regions that initiate transcription of coding sequences (CDSs) that produce the required proteins to mount an adequate response to these changing environments. In exponentially growing bacteria, transcription is initiated by RNA polymerases carrying the housekeeping $\sigma$ factor, known as $\sigma^{70}$. Specific response genes that are regulated by a subset of alternative $\sigma^{70}$-like transcription factors have evolved in response to changing environments. In a wide variety of bacterial species the sigma factor $\sigma^E$, also known as extracytoplasmic factor (ECF), is essential in mounting responses to environmental challenges such as; oxidative stress, heat shock, and misfolding of membrane proteins\(^1\). In addition, $\sigma^E$ is of importance for virulence of bacterial pathogens. The regulon size of $\sigma^E$ varies widely among bacterial species studied ranging from $\sim$100 unique $\sigma^E$ controlled transcription units in *Escherichia coli* and related bacteria to a surprisingly small regulon of 5 genes in *Neisseria meningitidis* and *gonorrhoeae*\(^2\).

In order to more extensively explore the meningococcal $\sigma^E$ regulon we employed recently developed techniques in molecular biology. In chapter 2, we used next-generation 454-pyrosequencing whole genome sequencing (WGS) to obtain the genome sequence of the *N. meningitidis* strain H44/76. *N. meningitidis* H44/76 was originally isolated from a patient from an outbreak in Norway in 1976 is widely used in molecular genetics studies in laboratories around the world. In 2010, only seven complete *N. meningitidis* genomes had been sequenced of which only one was serogroup B (strain MC58). The addition of the whole genome sequence of H44/76 helps future research that involves this laboratory strain.

In chapter 3 we consequently employed whole transcriptome analysis (WTA) using the Sequencing by Oligonucleotide Ligation and Detection (SOLiD) next-generation sequencer to sequence and compare all RNA molecules in wildtype (wt) meningococci to a strain that overexpresses $\sigma^E$ after deletion of the anti-$\sigma^E$ factor MseR (for Meningococcal SigmaE Regulator)\(^2\). The newly sequenced H44/76 genome was used as a backbone for mapping the resulting RNA reads and ensures a 100% match with the H44/76 strain used for WTA. Almost $1 \times 10^8$ RNA
read sequences per strain were obtained. Roughly one third of the reads mapped to tRNA or rRNA genes, one third mapped to either CDSs or intergenic regions (IGRs) on both DNA strands, and the remaining third could not be mapped to the genome. Differential expression analysis showed a surprisingly small number of genes regulated by the $\sigma^E$ factor. WTA confirmed the differential expression of the $\sigma^E$ operon and MsrA/MsrB genes as demonstrated previously by proteomics and confirmed with reverse transcriptase PCR (RT-PCR)$^2$. Additionally, 4 other genes were identified which were hitherto not associated with $\sigma^E$. Of special interest, small non-coding RNA (sRNA) transcribed in an intergenic region (IGR) was identified. This novel sRNA was subsequently called $\sigma^E$sRNA for $\sigma^E$ induced sRNA. Small non-coding RNAs are involved in the ribo-regulation of target mRNAs and are the main topic of chapters 4 through 8.

In summary, by employing next-generation sequencing of both the genome and the transcriptome we investigated the regulon of the alternative sigma factor E. Its small regulatory repertoire compared to E. coli and its lack of regulating proteins involved in the outer membrane stress response is a prime example of the divergent evolution of superficially similar regulatory systems in these two gram-negative bacteria. It confirms the necessity to perform independent experimental validation in non-canonical organisms with a focus on their specific niche and pathogenicity.

The Hfq regulon of N. meningitidis
Small RNAs are important in many cellular processes and prominent in those involving adapting to physiological changes such as functioning in posttranscriptional regulators of gene expression to orchestrate stress responses and metabolism$^{3-7}$. Many sRNAs are synthesized upon nutritional stresses encountered by bacteria. In invasive pathogens, this is most relevant as they usually encounter harsh conditions upon interaction with the host. sRNAs often regulate expression of target mRNAs that form part of a nutritional regulatory circuit or network. sRNAs usually act by occupying or freeing-up ribosomal entry sites of target transcripts as well as by regulating accessibility of transcripts for RNases in an antisense fashion.

The RNA chaperone protein Hfq is frequently involved in ribo-regulation, enhancing these processes$^{8,9}$. It has multiple regulatory roles within the prokaryotic cell, including promoting stable duplex formation between small RNAs and mRNAs. Deletion of hfq leads to pleiotropic phenotypes.
proteome and transcriptome studies of *N. meningitidis* have generated limited insight into differential gene expression due to Hfq loss\(^{10,11}\). Understanding the differential gene expression influenced by Hfq facilitates finding *bona fide* mRNA targets of yet undiscovered or uncharacterized sRNAs.

In *chapters 4 and 5* we discovered which proteins are responsive to the loss of Hfq. This differential protein expression may be the result of: 1) a direct interaction between Hfq, sRNA and the mRNA encoding the differentially expressed protein, 2) more indirectly from the interaction between Hfq, sRNA and a mRNA encoding other regulatory proteins, and 3) downstream effects from these directly and indirectly Hfq regulated proteins. In *chapter 4*, a *hfq* deletion strain of *N. meningitidis* was created. This strain formed small colonies after overnight growth on rich solid media compared to the wildtype. In addition, the strain exhibited a growth deficiency even in nutrient enriched GC broth specifically designed to support growth for fastidious gonococci. We consequently applied traditional 1- and 2-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) protein separation followed by mass spectrometry (MS) to identify excised proteins. Utilizing this method, we identified 28 proteins to be significantly affected upon deletion of *hfq*. These proteins were mainly involved in general (carbohydrate) metabolism and oxidative stress providing a plausible explanation for the observed diminished growth characteristics.

SDS-PAGE protein separation has its limitations in being laborious, having restricted physical resolution, and difficulty in detecting hydrophobic integral membrane proteins and low-copy number proteins\(^{12}\). Statistical analysis of identified differentially expressed proteins is hampered by its semi-quantitative results. Therefore, only a limited amount of proteins can be reliably differentiated and subsequently identified. Reversed-phase liquid chromatography (LC) prior to analysis by data independent alternate scanning mass spectrometry (MS\(^5\)) allows for the absolute quantification of hundreds of proteins in a complex mixture. LC-MS\(^5\) can be optimized for a standardized workflow that has a stable performance and efficiency throughout the experiment, facilitating in high reproducibility\(^{13,14}\).

In *chapter 5* we applied LC-MS\(^5\) and rigorous False Discovery Rate (FDR) control to further identify which proteins are differentially expressed due to the loss of *hfq*. In conjunction with previous transcriptomic and proteomic studies, a comprehensive network of Hfq regulated proteins was constructed. Differentially
expressed proteins were found to be involved in a large variety of cellular processes. Potential gaps in the Hfq dependent sRNA repertoire of the meningococcus were identified in either the direct or indirect regulation of outer membrane proteins (OMPs), the methyl-citrate and tricarboxylic acid (TCA) cycles, iron and zinc homeostasis, and the assembly of ribosomal proteins.

The chaperon protein Hfq plays a remarkable role in regulating fundamental cell biological processes in *N. meningitidis*. The pleiotropic effects of a deletion mutant have become more evident by studying its regulatory proteome in depth and comparing it with results of independent experiments performed in other laboratories on both the proteomic and transcriptomic level. A key role of Hfq is to facilitate the RNA-RNA interaction between a sRNA and its mRNA target(s). By removing Hfq from the meningococcal cell these interactions are abrogated. Hence, many proteins that are differentially regulated in a *hfq* deletion mutant would be similarly differentially regulated in a sRNA deletion mutant as well. This strategy will be pursued in chapters 6, 7 and 8. The results of chapter 5 convincingly show the reliability and usability of LC-MS to further elucidate the sRNA regulon of *N. meningitidis*.

**sRNA identification and characterization in *N. meningitidis***

In chapter 6, we explored two structurally nearly identical sRNAs tandemly arranged in *N. meningitidis*. Growth analysis of deletion and overexpression mutants shows similar growth in nutrient rich medium. However, replication was abrogated in a nutrient poor medium with glucose as the sole carbohydrate source. In consideration of the niches encountered by the meningococcus in the human host, we hypothesized that the human equivalent of nutrient rich and poor niches are blood and CSF respectively. The overexpression of both sRNAs abrogated growth in CSF but not in blood. Differential protein analysis of a deletion mutant strain revealed the upregulation of several proteins of the methyl-citrate and TCA-cycle. Several of these proteins were shown to be part of the Hfq regulon chapters 4 and 5 and predicted in silico to form a duplex with the sRNA at the ribosomal entry site. We used a green fluorescent protein (GFP) reporter system in *E. coli* to assess whether these proteins are bona fide targets15. In this heterologous system both the sRNA and the 5’UTR of the target protein fused to a GFP gene are expressed. When the sRNA forms a duplex with the ribosomal binding site of the target protein green fluorescence is diminished. This interaction can be further confirmed by inserting mutations in the predicted interaction site of the sRNA after which green fluorescence is restored. In this *E.*
coli model, the interaction of the sRNA and 2 methyl-citrate and 4 TCA cycle proteins was confirmed. In meningococcal cells 5 of these genes were shown to be controlled by the sRNAs by using RT-PCR to detect transcript levels of target mRNAs which show downregulation upon overexpression of the sRNAs.

In bacteria, the stringent response is a stress response in reaction to limited availability of nutrients like amino-acids, iron or fatty acids or because of cold and heat shock. This response controls the expression of a large part of the genes in the cell, diverting energy away from resource-consuming cell processes like replication, transcription, and translation. In Neisseria, this response is mediated by the protein RelA\textsuperscript{16}. Upon deletion of this gene, transcript levels of both sRNAs were substantially increased. Consequently, when this relA mutant is grown in nutrient poor medium, four sRNA target mRNA transcripts were downregulated. This downregulation was abrogated in a double mutant where relA and both sRNAs are absent. These results confirmed relA-mediated down-regulation of the sRNAs.

In the conclusion of chapter 6, we identified two highly conserved sRNAs, designated sibling Neisseria metabolic switch regulators (NmsRs) which are functionally involved in the regulation of TCA cycle activity by antisense mechanisms. These novel sibling sRNAs extend the stringent response in meningococci, thereby connecting metabolic status to colonization and possibly, virulence.

In chapter 7, we identified novel sRNAs by combining traditional techniques like RT-PCR and northern blotting with our WTA analysis from chapter 3. Next, we applied LC-MSE\textsuperscript{5} as described in chapters 5 and 6 to identify differentially expressed proteins of 2 novel and 1 previously described sRNAs. We assessed the proteome of strain overexpressing σ\textsuperscript{Es}sRNA discovered in chapter 3 and further explored the NmsR regulon of chapter 6. In silico target prediction was used to discover potential interaction between the sRNA and the 5’UTR of target mRNA transcripts. One of the novel sRNAs is shown to be iron-regulated and differential proteome and bioinformatic analysis indicate it regulates an immunogenic outer membrane protein. Protein expression profiling of a strain overexpressing σ\textsuperscript{Es}sRNA revealed relatively few targets of unknown significance. To further elucidate which proteins are differentially expressed in the presence or absence of the NmsRs we created two strains in which NmsR-A or B is overexpressed. The protein profiles of these strains were combined with those of wildtype and NmsR-
A&B deletion strains. Three of the six targets from chapter 6 were confirmed and an additional 9 proteins were identified, greatly expanding the repertoire of the NmsRs regulated proteome. In conclusion of chapter 7 the combined approach of WTA and LC-MS has proven successful in characterizing the role of some but not all sRNA candidates. A more optimized approach is necessary to discover the regulatory roles of the latter sRNAs which will be discussed in the last section of this chapter.

Finally in chapter 8, we first show with differential protein analysis that NrrF is involved in the regulation of proteins involved in iron uptake, iron-dependent metabolic processes and the oxidative stress response. Using the heterologous gfp-reporter system we further characterized in detail the interaction between Nrrf and PetA, an iron-containing protein that is part of the cytochrome bc complex. This novel target of NrrF is functionally involved in respiration. Translational repression of the operon PetABC results from direct interaction between NrrF and the 5’UTR of petABC.

In contrast to ‘classic’ sRNA translational repression of mRNAs the nucleotides that form the ribosomal binding site (RBS) and/or start codon do not seem to be directly involved in this interaction. However, duplex formation between NrrF and petA may cause indirect stereochemical interference. Previously, the requirement of Hfq for the interaction between NrrF and SdhC has been contested. In the gfp-reporter system Hfq is dispensable for the interaction between NrrF and petA. These two characteristics are in stark contrast with the strictly Hfq-dependent NmsRs of Chapter 7 of which the known target interaction site fully covers the RBS and interaction is dependent on Hfq. By extending the experimentally validated NrrF regulated network of N. meningitidis we provide important insights into the mechanism by which an essential component of the respiratory chain is indirectly controlled by iron availability. Adaptation of expression of components of the cytochrome bc complex in response to iron limitation is mediated at the post-transcriptional level through the action of the small regulatory RNA NrrF.

In Chapters 6 and 8 we described the regulatory role of two sRNAs, showing their complex and extensive involvement in the fundamental cell biological processes of carbohydrate metabolism and oxidative phosphorylation (Figure 1). The sRNAs regulate these processes by simultaneously downregulating multiple enzymes involved in these pathways. Small RNAs like the NmsRs and NrrF can be
transcribed quickly, have a relatively low biological cost and can act immediately on an already existing cellular pool of mRNA targets\(^7\). These factors make them ideally suited to adopt different metabolic pathways in response to changes in the environment.

**Figure 1. Schematic representation of targets regulated by NmsRs and NrrF.** Red lines indicate inhibition of targets. TCA, tricarboxylic acid, OXPHOS, oxidative phosphorylation.
Overview and update of sRNAs in *N. meningitidis*

**NrrF**

*NrrF*, Neisserial regulatory RNA responsive to iron (*Fe*), was the first sRNA described in *N. meningitidis* and a result from a search for sRNAs involved in iron homeostasis\(^{18}\). The level of bound and unbound iron is tightly controlled in most prokaryotes. It is both essential for numerous biological processes and toxic in catalyzing the formation of reactive oxygen species (ROS)\(^{18}\). The human host of *Neisseria meningitidis* and *N. gonorrhoeae* controls free iron levels by regulating dietary intake and uptake, storage in hepatocytes and macrophages, and sequestering by iron-binding proteins like ferritin, transferrin and lactoferrin. During a host infection, the local free iron is further restricted and together with the targeted release of ROS they form a powerful tandem of the innate immunity defense\(^{19}\). More than 10% of the protein coding capacity of pathogenic *Neisseria* is dedicated to the response to host iron restriction. These include producing transferrin binding proteins and ferric binding proteins to scavenge free iron, increasing the number of intracellular oxidoreductases to protect from ROS, and shift away from iron dependent biological processes like the TCA cycle and oxidative phosphorylation and enzymes containing iron-sulfur clusters\(^{20-23}\). Host invasion and immune response evasion mechanisms like antigenic variation of pili and phase variation of iron-binding receptors are also influenced by iron availability\(^{24}\).

The regulatory response to intracellular iron in many bacteria is largely mediated by the iron (*Fe*) uptake regulator (Fur)\(^{25}\). This protein primarily acts as a classic repressor; when loaded with iron it dimerizes and binds to a specific DNA sequence (the Fur box) in promoter regions of target CDSs, blocking the initiation of transcription by RNA polymerases. When intracellular iron-levels drop iron disassociates from the Fur protein dimer, Fur monomers release from the Fur box, and RNA polymerase can initiate transcription.

Using a bioinformatic approach, Mellin and co-workers\(^{18}\) screened the genome of *N. meningitidis* for intergenic regions (IGRs) for a consensus Fur-box region followed by \(\rho\)-independent transcription terminator within 250 nucleotides (nts), the average length of sRNAs described in canonical gram-negative bacteria. From 19 candidate sRNAs only NrrF showed up-regulation upon iron restriction. Electrophoretic Mobility Shift Assay (EMSA) analysis confirmed the binding of Fur to the NrrF promoter. Putative targets of NrrF where identified using a
bioinformatic tool called TargetRNA\(^{26}\). Among the results were \(sdhA\) and \(sdhC\) which were particularly interesting as in \(E. coli\) these proteins are post-transcriptionally regulated by the Fur-regulated sRNA RyhB. Wildtype and a NrrF deletion mutant grown in iron rich and restricted conditions were compared showing the iron-restriction induced repression of \(sdhA/sdhC\) was abrogated in the NrrF deletion mutant. These target genes belong to the operon encoding the succinate dehydrogenase complex (\(sdhCDAB\)). This complex generates fumarate from succinate during the TCA cycle, and concomitantly feeds electrons to the respiration chain\(^{27}\). The expression of NrrF and target regulation of \(sdhA/sdhC\) was confirmed independently in both \(N. meningitidis\) and \(N. gonorrhoeae\) with additional targets remaining elusive\(^{17,23,28}\).

In **Chapter 8** we expanded upon the known regulatory repertoire of NrrF. We show definitive proof of direct Hfq-independent interaction of NrrF with PetA using a combined approach of bioinformatic prediction, target validation in a \(E. coli\) gfp-reporter system and mass proteomic analysis of a \(N. meningitidis\) strain overexpressing NrrF. For the first time the interaction between NrrF and a target is interrogated at the nucleotide level giving precise insight into NrrF/target duplex formation. PetA is known as the Rieske iron-sulfur protein and is 1 of the 3 respiratory subunits of the cytochrome c reductase complex. In \(N. meningitidis\), upon iron-restriction Fur derepresses NrrF which downregulates both the succinate dehydrogenase and the cytochrome c reductase complexes. This shuts down essential parts of the iron-dependent TCA cycle and oxidative phosphorylation shifting metabolism and energy generation away from iron utilization.

**AniS**

Both commensal and pathogenic *Neisseria* live in an environment in which the availability of oxygen varies considerably due to the limited perfusion of epithelial tissue and heterogeneous occupation by other oxygen-respiring microorganisms\(^{29}\). Denitrification is an alternative respiratory pathway genetically available for *Neisseria* when oxygen is limited. In response to low oxygen the transcriptional regulator Fumarate and Nitrate Reductase (FNR) induces the expression of nitrate reductases. These enzymes catalyze the reduction of nitrite to nitrous oxide and accept electrons from the respiratory chain\(^{30}\). FNR also induces the transcription of the Anaerobically induced sRNA (AniS), a sRNA serendipitously discovered in a *hfq* mutant\(^{31}\). By combining the results of microarray experiments of FNR overexpression and AniS deletion mutants two
hypothetical genes (NMB0214 and NMB1468) were identified as putative targets of AniS. These interactions are enhanced by the protein chaperone Hfq. Bioinformatic analysis predicted the interaction between AniS and the ribosomal binding site on the 5′UTR of the two hypothetical genes. In the gfp-based plasmid system in E. coli overexpression of AniS reduced the expression of a GFP-gene fused to the 5′UTR of NMB1468. The expression of AniS has been confirmed in gonococci but no further targets have been identified, which leaves the contribution of AniS in the Neisserial response to anaerobiosis yet unresolved.

G4-associated sRNA and AS RNA

NrrF and AniS are examples of sRNAs that are transcribed in IGRs and affect the translation of mRNA targets at different genomic loci. This class of sRNAs are called in trans acting sRNAs. Another group of sRNAs are transcribed directly antisense to the coding sequence they influence and are called in cis acting sRNA. Two of these cis acting sRNAs are involved in pilin antigenic variation in pathogenic Neisseria. In bacteria, pili are thin appendages consisting of proteins that protrude out of the cell wall. They are involved in the exchange of genetic information (DNA conjugation), attachment to host surfaces during colonization and infection, and the formation of biofilms. Some pili can generate motile forces which enable jerky movements called twitching motility. In pathogenic Neisseria spp., genes that encode for these type IV pili (tfp genes) undergo antigenic variation (Av) in response to host immunogenic pressure. The pilin expression locus (pilE) is interchanged with silent loci (pilS) using enzymes that participate in general recombination and repair pathways. This homologous DNA recombination leads to new variants of the tfp pilin protein that can differ in levels of functionality, expression, colony morphology and immune evasion. Despite this clear role in virulence, regulation of pilin expression and variation in N. meningitidis is still not well understood. A transposon-based genetic screen led to the discovery of several intergenic loci upstream of pilE that are necessary for pilin Av. Among these loci essential for Av is a 16 base pair (bp) guanine (G)-rich sequence that forms a parallel tertiary single strand DNA structure. Exactly how this structure influences pilin Av is still unknown. It was originally postulated that this tertiary DNA structure was stabilized on both strands by unknown proteins. It turned out that in N. gonorrhoeae a sRNA is transcribed covering the (G)-rich strand that stabilized the opposing (C)-rich strand by forming a DNA/RNA hybrid. Disrupting the promoter of this sRNA (but leaving the guanine quartet (G4) intact) blocks pilin Av. Expressing the sRNA in trans on
an expression vector did not restore Av, indicating the G4-associated sRNA is strictly cis-acting. The G4-associated sRNA was later described in *N. meningitidis*, as well as another antisense RNA called AS RNA which covers the entire opposite strand of the *pilE* locus. Expression of AS RNA did not change *pilE* transcript or protein level, but influences antigenic variation of pilin38.

**CRISPR/CAS**

Meningococci, unlike gonococci, contain functional clusters of regularly interspaced short palindromic repeat (CRISPR) loci. Several CRISPR associated small RNAs responsible for target specificity (crRNAs) and target processing (tracrRNA) have been identified as well39. CRISPR pathways have been revealed as RNA-directed immune systems that protect prokaryotes from phage infection and horizontal gene transfer (HGT)40. There are three forms of HGT; phage transduction, conjugation and natural transformation. *Neisseria* spp. are constitutively transformation competent. Frequent HGT is thought to promote antigenic and phase variation as well as homology-based DNA-repair. In a recent study39 several features are described that make the *N. meningitidis* CRISPR/CAS system exceptional. First, *N. meningitidis* uses CRISPR/CAS to limit HGT through natural transformation. Second, compared to other CRISPR/CAS systems, the number of proteins required for its function is considerably streamlined. The system itself only contains the CRISPR array, 3 CAS proteins and a tracrRNA. Its activity is independent of processing by bacterial RNase III or endogenous eukaryotic processing alternatives when used for eukaryotic genome editing. In most systems, a large CRISPR RNA (crRNA) is transcribed from promoters within “leader” sequences outside the CRISPR array. These crRNAs contain “protospacers” that need to be processed before the spacers can direct specific cleavage of target sequences. In *N. meningitidis*, these spacers are transcribed from independent short promotors and do not require further processing. The identification of a streamlined and processing-independent CRISPR/CAS system may increase the range of applications in eukaryotic genome editing41.

**Global analysis of sRNAs**

Several global transcriptomic approaches have been undertaken to identify sRNAs differentially expressed in a variety of environmental models that resemble *in vivo* compartments during pathogenesis42-46. Using microarray experiments and bioinformatic analysis of meningococci incubated in *ex vivo* human blood the presence of 91 differentially expressed putative sRNAs were
discovered, six of which (including NrrF) were confirmed by 5’-3’ RACE\textsuperscript{46}. In a later study, 98 sRNAs were discovered in a multiple infection-relevant conditions. Eight new sRNAs and three sRNAs from the previous study were confirmed by northern blot\textsuperscript{44}. One of the validated sRNAs, Bns1, is differentially induced by glucose in experimental medium and under ex vivo exposure to glucose rich human blood. In an infant rat model the deletion strain showed decreased survival. Microarray analysis of a Bns1 deletion strain shows substantive differential regulation of genes involved in metabolism but its true targets await experimental validation. Recently, a random transposon insertion library of \textit{N. meningitidis} was created and the fitness of mutations during routine growth and that of colonization of endothelial and epithelial cells were assessed using transposon insertion site sequencing (Tn-seq) analysis\textsuperscript{45}. 8 intergenic regions containing sRNA candidates were found to be essential for growth and 33 were found to be specifically required for host cell colonization.

\textbf{\textit{σ}}\textsuperscript{EsRNA and NmsRs}

In chapter 3 we have shown that transcription of \textit{σ}\textsuperscript{EsRNA} is induced by the alternative sigma factor E. In a recent transcriptome analysis in a variety of physiologically relevant stress signals \textit{σ}\textsuperscript{EsRNA} was up-regulated during growth in minimal media and under heat-shock treatment\textsuperscript{44}. In Chapter 6 we analyzed the proteome of meningococcal cells overexpressing \textit{σ}\textsuperscript{EsRNA} and grown in nutrient rich medium. In this experiment no differentially expressed proteins were detected that indicated a specific role of this sRNA. Further experiments of a deletion vs. an overexpression mutant in minimal media or under heat shock might provide a clearer picture of the role of this sRNA.

The regulon of the NmsRs described in chapters 6 and 7 dramatically increases the known sRNA regulatory repertoire of \textit{N. meningitidis}. For six targets, extensive experimental evidence on both the transcriptional and translational level was provided \textit{in vivo} and strong proteomic evidence was given for another 9 targets. This multitude of targets is unprecedented in \textit{N. meningitidis} and rare in sRNAs in the wider prokaryotic world. The methods developed and employed in this thesis lay the groundwork for characterizing even more sRNAs and their targets.
Outlook and future recommendations

The research performed in this thesis has contributed to the understanding of the fundamental biology of *Neisseria meningitidis*. However, the number of yet uncharacterized sRNAs discovered in this thesis and those discovered by others is rapidly increasing\(^{44-46}\). The discrepancy between discovery and actual characterization of sRNAs and their targets is a consequence of the ‘big data’ approaches enabled by the development of next-generation sequencing in the last decade. The labor time required both in the ‘wet lab’ and the ‘dry lab’ to investigate the individual sRNAs and their regulatory role is outpaced by the relative ease to generate mass amounts of ‘omics’ data\(^{47}\).

Part of this discrepancy can be overcome by the very same advances in molecular biology. For example, creating gene deletion mutants has long been limited to naturally competent bacteria. When forced to grow under antibiotic pressure, these bacteria readily swap out a non-essential gene for a resistance gene by homologues recombination. However, most bacteria do not show natural competence and this method is too laborious to perform for the hundreds of discovered sRNA candidates. Introducing an antibiotic resistance gene into a genome may interfere with the expression of surrounding genes, this is known as the polar effect. It also introduces the need for antibiotic pressure which possibly interferes with the physiology of the cell under investigation. This is especially true in case of plasmid borne overexpression of genes.

Two major developments have vastly improved the ease of creating mutant strains. The first is the use of transposon mutagenesis\(^{48}\) and subsequent sequencing of the randomly inserted transposons (Tn-seq\(^{49}\)) to create a library of strains with known disrupted coding and non-coding genes. Recently this approach led to the discovery of many sRNAs essential for growth and colonization in *N. meningitidis*\(^{45}\). The second development followed the initial discovery of CRISPRs in *E. coli*\(^{50}\). More than 30 years later it was revealed that in *Streptococcus thermophilus* CRISPR together with CRISPR associated (CAS) genes functions as innate and adaptive defense against invading bacteriophages and other foreign DNA species\(^{40,51}\). This discovery has taken the biomolecular community by storm, opening the way to limitless possibilities in genome editing in all clades of life. The CRISPR/CAS toolbox dramatically facilitates precise genome editing of bacteria. The resulting “CRISPR-craze”\(^{52}\) will almost certainly ensure a future Nobel price.
The deletion mutants generated by these methods can then be efficiently interrogated using transcriptome sequencing as used in chapter 3 and mass proteomic analysis as used in chapter 4. Based on our statistical analyses of proteomic data generated by LC-MS in chapters 5 through 8 a minimum of 4 biological replicates (representing > 10⁶ bacterial cells each) is recommended. These experiments are preferably performed in the same growth medium as used to identify the upregulation of a candidate sRNA of interest. For example, if a sRNA candidate is upregulated in iron-limited conditions, the transcriptomic and proteomic analyses should be performed in the same iron-limited environment. Human blood and CSF are two physiological niches that are particularly relevant to study pathogenesis of N. meningitidis. The first represents significant problems as human blood is a complex mixture of proteins, carbohydrates, and nucleotides vastly outnumbering those of the bacterial cell. The blood-brain barrier poses a significant barrier to these molecules, leaving the CSF a relatively devoid of above molecules. CSF is easily accessible by lumbar puncture and therefore particularly suitable to serve as infection model to study meningitis in vivo. With the ever increasing sequencing power of successors of the ‘first-generation’ next-generation sequencers dual RNA-seq of host-pathogen interaction will be within reach.53

After elucidating the differential transcriptomic and proteomic profiles of the sRNA candidates, the next step is matching the sRNA with their target mRNAs. Bioinformatic target predictions have seen steady improvements and an increasing library of experimentally confirmed sRNA/target combination serves as a source of prediction model training.54 In silico target prediction is however still far from perfect, perhaps advances in the field of (cloud-based) machine learning might improve its accuracy in the near future. Predicted targets of sRNAs, especially in non-canonical bacteria like N. meningitidis, can only be taken as a starting point for further investigations. For all but E. coli, target validation in the native environment of the bacterial cell of the species of interest remains elusive.

In chapters 6 and 7 we identified the NmsRs as the simultaneous regulator of multiple targets proteins in the methyl citrate and TCA cycle. Overexpression of the NmsRs effectively shut down the generation of essential carbohydrate intermediates and consequently halted replication of the meningococcus in human CSF. β-Lactam antibiotics like penicillins and cephalosporins inhibit the biosynthesis of peptidoglycan, an abundant component of the gram-positive cell wall. In gram-negative bacteria like N. meningitidis, an outer layer of
lipopolysaccharides (LPS) slows down penetration of penicillins through the outer cell membrane, and only partially strips the inner cell membrane leaving the bacteria as fragile but metabolically active spheroplasts. Adequate bioavailability of β-lactams in the CSF requires frequent or extended high IV dosing available only in a clinical setting. A novel antimicrobial agent that can provide rapid and specific inhibition of meningococcal metabolism and replication and preferably can be supplied orally may improve clinical outcome, especially in the developing world. To date, no therapeutic drugs for bacterial infections have been developed to specifically interfere with small non-coding RNAs or make use of it to inhibit its targets. However, RNA interference (RNAi) using microRNAs or CRISPR/CAS have been actively pursued in the treatment of the hepatitis C virus (HCV) and the human immunodeficiency virus (HIV)\textsuperscript{55,56}. The stability and propagation of HCV is dependent on a functional interaction between the HCV genome and liver-expressed microRNA-122 (miR-122). Miravirsen\textsuperscript{®} (Santaris Pharma) is a locked nucleic acid (LNA) modified DNA phosphorothioate antisense oligonucleotide that sequesters mature miR-122 in a highly stable heteroduplex, thereby inhibiting its function. LNA DNA or RNA nucleotides are protected from enzymatic degradation and can be injected intravenously or intramuscularly. Miravirsen contains 15 nucleotides, significantly smaller than the 89 nucleotides of the NmsRs, therefore the toxicity profile, bioavailability both in the human body and intracellularly of the bacterium, and ultimately clinical efficacy of this larger molecule remains to be investigated. The NmsRs comprises two different and individually active sRNAs targeting the essential Shine Dalgarno (SD) sequence of multiple mRNAs involved in carbohydrate metabolism. This makes it potentially difficult for \textit{N. meningitidis} to develop resistance as it requires the simultaneous development of multiple and potentially essential mutations. Developing the means of expulsion of artificial NmsRs out of the cell would likely expulse naturally transcribed NmsRs as well which will disrupt the natural function of these sRNAs. The NmsRs are widely conserved among both \textit{N. meningitidis} and \textit{N. gonorrhoeae} which may lead to the development of new treatment options for both human pathogens.
Summary, general discussion, and future perspectives

References


Edwards, J., Quinn, D., Rowbottom, K. A., Whittingham, J. L., Thomson, M. J. & Moir, J. W. Neisseria meningitidis and Neisseria gonorrhoeae are differently adapted in the regulation of denitrification: single nucleotide polymorphisms


