“The role of small, non-coding RNA molecules in regulating the development of bacteriophage Φ24B and its host, the Shiga toxin–producing bacterium Escherichia coli”

Natalia Lewandowska, M.Sc.

Shiga toxin–producing *Escherichia coli* (STEC) are highly pathogenic bacteria responsible for severe gastrointestinal diseases and life-threatening complications in humans. The virulence of STEC is directly associated with the presence in their genomes of Stx bacteriophages, which carry the genes encoding the Shiga toxin, a dangerous threat to human health. Stx phages belong to the family of lambdoid phages and exhibit significant similarities in genome organization and life cycle to bacteriophage λ, which is the best characterized temperate phage. One of the most studied representatives of this group of bacterial viruses is bacteriophage $\Phi 24_B$, which serves as the model system in this doctoral dissertation.

During infection, a temperate phage injects its linear double–stranded DNA into the bacterial host cell and chooses one of the two alternative developmental pathways: the lysogenic or the lytic cycle. During lysogeny, the phage genome integrates into the host chromosome, maintaining in the form of a prophage and replicating passively along with the bacterial genome. At this stage, most phage genes (including those encoding Shiga toxin) remain transcriptionally inactive, and toxin production is effectively suppressed. Prophage induction, triggered by various external factors, initiates the lytic cycle. At this point, phage DNA is excised from the host chromosome, replicates independently apart from the bacterial genome, and is next transcribed, serving as a template for the synthesis of structural phage proteins and lytic enzymes. This process ultimately leads to lysis of the host cell and the release of progeny phage particles, along with large amounts of Shiga toxin, into the intestinal lumen. Prophage induction may be triggered by a variety of environmental and physiological factors, including UV radiation, hydrogen peroxide, and certain antibiotics, therefore, they should not be used in the treatment of these infections. The mechanism of Shiga toxin action involves inhibition of protein synthesis through blockade of translation at the elongation stage, leading to cell death and tissue damage. Clinically, STEC infections manifest as bloody diarrhea, abdominal pain, and vomiting, and in 15–20% of cases, they can develop to severe complications such as hemorrhagic colitis, hemolytic uremic syndrome (HUS), or thrombotic thrombocytopenic purpura

(TTP). Children under five years of age and elderly individuals are particularly sensitive to these complications, with reported mortality rates in these groups reaching even 87%. Currently, available therapeutic strategies targeting STEC bacteria have significant limitations, and antibiotic therapy is not recommended, as it may stimulate prophage induction and toxin release, thereby worsening the infection.

The choice between lysogenic and lytic development in lambdoid phages is tightly regulated and depends both on the physiological state of the host cell and on regulatory factors encoded within the phage genome. The main factor controlling the switch between phage life cycles is the CI repressor – a DNA–binding protein that inhibits transcription from the major phage promoters (p_L and p_R), thereby preventing the expression of most phage genes, including those encoding lysis–associated proteins and Shiga toxin. The entrance of the phage into the lytic cycle requires inactivation of the CI repressor. One of the best characterized mechanisms of this process is activation of the bacterial SOS response following DNA damage. This leads to RecA–dependent autocatalytic cleavage of both the host LexA repressor and the phage CI repressor. In addition to SOS–dependent regulation, antirepressors encoded in the phage genome (such as Cro, Ant, Roi, and D_ant) can antagonize CI activity by competition for binding at operator sites within the major phage promoters, thereby enabling the expression of genes whose protein products are responsible for bacterial cell lysis. Ultimately, the dominance of repressors or antirepressors within the cell determines the developmental pathway of the phage.

In addition to regulatory proteins, small non–coding RNAs (sRNAs) have emerged as important controllers of gene expression not only in bacteria but also in bacteriophages. Prokaryotic sRNAs are typically 50 to 550 nucleotides in length and regulate gene expression at the posttranscriptional level, either by base pairing with target mRNAs or by interacting with proteins to modulate their activity. The largest group of bacterial sRNAs functions through complementary binding to target mRNAs. Within this group, two subtypes can be distinguished: *cis*–encoded and *trans*–encoded sRNAs. Genes encoding *cis*–acting sRNAs are located in the genome in the same region as their target genes but on the opposite strand. These molecules pair to target RNA with full complementarity that results in highly specific regulation, often through inhibition of translation or induction of mRNA degradation. On the other hand, genes encoding *trans*–

acting sRNAs, are located in genomic regions distinct from their target binding sites. Besides, these molecules typically pair through shorter sequences with limited complementarity. For stable interaction, they often require the support of RNA chaperone proteins such as Hfq or ProQ. These sRNAs generally act as global regulators, influencing multiple mRNA targets whose gene products are involved, for example, in stress responses and cellular adaptation to environmental conditions.

In eukaryotes, a distinct class of very short regulatory RNAs, known as microRNAs (miRNAs), has been identified. Similar to prokaryotic sRNAs, these molecules play a key role in posttranscriptional regulation. Typically, only 19–25 nucleotides in length, microRNAs are produced from longer precursor transcripts and have been extensively characterized in terms of both their biogenesis and functional roles. Importantly, viruses that infect eukaryotic cells also encode microRNAs in their genomes. These molecules may regulate the expression of both viral and host genes, thereby facilitating infection and promoting viral stability within the host cell.

While most prokaryotic sRNAs that have been described till now are relatively long (above 50 nucleotides) and do not undergo a maturation process, recent reports indicate that microRNA–type small RNAs may also function in bacterial systems, and their genes might be located in both bacterial and phage genomes. Importantly, these very short molecules constitute only a small fraction of the sRNAs identified so far in bacteria and bacteriophages. Moreover, phage–encoded sRNAs remain less well characterized than those encoded in bacterial genomes, so these molecules were reviewed in this doctoral dissertation in the frame of **article no. 1**. In this paper, we performed a detailed analysis of phage–derived sRNAs described in the literature and proposed their classification based on the biological processes they regulate. This approach enabled the identification of sRNAs involved in the regulation of, among others: (i) phage DNA replication, (ii) bacterial lysogenization, (iii) cell division, as well as sRNAs influencing (iv) cellular metabolism, (v) stress response, and even (vi) pathogenicity [**article no. 1**].

Among the regulatory sRNAs described in **article no. 1**, the phage–derived molecule 24B_1 was also mentioned. Its discovery in 2015 set a significant turning point in studies of prokaryotic sRNAs resembling eukaryotic microRNAs. 24B_1 is a 20–nucleotide sRNA molecule originating from an 80–nucleotide precursor transcript produced by the Shiga toxin–encoding bacteriophage $\Phi 24_B$. This molecule represents the

first functional sRNA of microRNA–like size identified in bacteriophages. Its role, significance, and functions were widely characterized by us in **article no. 2**. In this study, we investigated the impact of 24B_1 on the development of the phage and its host, *E. coli*, under conditions of its overexpression. An additional copy of the 24B_1 gene was incorporated into cells on a plasmid. Through a series of experiments analyzing physiological aspects of phage development, we demonstrated that, functionally, 24B_1 promotes maintenance of the prophage state in *E. coli* cells. Notably, overexpression of the phage–encoded sRNA 24B_1 from its natural promoter (p_{24B_1}) significantly affected phage progeny. During infection with wild-type $\Phi 24_B$, elevated levels of 24B_1 sRNA resulted in reduced efficiency of progeny phage production in the lytic cycle, while phage adsorption to the host cell surface was significantly increased at early stages of infection. Despite increased adsorption efficiency, bacterial cells overexpressing 24B_1 exhibited reduced lysis efficiency and increased survival compared to control strains. These effects were coupled with a higher proportion of lysogens among cells that survived infection, indicating that an increased level of 24B_1 in the cell promotes phage development toward lysogeny. This trend was observed across different multiplicities of infection (m.o.i.) and under conditions that favored both lysogenic and lytic development. Although bacterial survival rate was lower under nutrient–rich conditions favoring the lytic cycle, nevertheless, lysogens constituted the majority of cells that survived infection.

Consistent with the results of the physiological experiments, additional RT–qPCR analyses demonstrated that overexpression of the 24B_1 molecule leads to changes in phage gene expression. While the expression levels of the analyzed genes at early stages of infection were initially comparable between bacterial cells with normal and elevated 24B_1 production efficiency, in the overexpression variant, at later stages of infection, genes associated with lysogeny – such as *cI*, *cII*, and *cIII* – showed markedly higher expression levels. These genes encode key regulatory proteins of the lysogenic pathway, thereby confirming earlier observations that 24B_1 promotes the establishment of lysogeny.

In addition to its impact on phage development and gene expression, overexpression of the 24B_1 molecule altered host cell metabolism, contributing to the reprogramming of bacterial energy pathways toward conditions that are more favorable for maintenance of the lysogenic state. Elevated levels of 24B_1 resulted in a significant

reduction in intracellular ATP concentration, with no effect on bacterial growth rate or cell viability. Metabolomic analysis under conditions of 24B_1 overexpression further revealed disruptions in central carbon metabolism, characterized by decreased levels of fumarate and accumulation of NAD^+, key metabolites of the tricarboxylic acid cycle. To investigate the observed decrease in intracellular fumarate levels, we analyzed the sequences of genes encoding subunits of succinate dehydrogenase Sdh, the enzyme responsible for the conversion of succinate to fumarate. Bioinformatic analysis identified a potential 24B_1 binding site within the 5' region of the *sdhB* gene mRNA. *In vitro* RNA–binding assays, based on differential mobility of molecules and their complexes in a gel, demonstrated that the mature 24B_1 molecule, contrary to its precursor, is capable of binding to this mRNA fragment, although the interaction was relatively weak. This suggests that maybe additional factors or specific intracellular conditions may be required for efficient binding *in vivo*. Despite its low affinity, this interaction may indicate a direct role of 24B_1 in regulating *sdhB* expression, potentially leading to reduced levels of the enzyme and disruption of the Krebs cycle at the point of succinate to fumarate conversion. As a consequence, NAD^+ accumulates (due to its reduced conversion to NADH), and cellular ATP levels decrease as a result of impaired energy metabolism. It is hypothesized that reduced intracellular ATP levels may disturb the activity of ATP–dependent proteases, thereby allowing repressors of the lytic cycle to remain active and directing the phage toward the lysogenic development pathway.

Further structural analyses demonstrated that the mature 24B_1 molecule shows a less complex secondary structure than its precursor, which likely increases its accessibility for intermolecular interactions *in vivo*. On the other hand, the following studies revealed that the RNA chaperone protein Hfq binds the precursor form of 24B_1, but does not interact with the mature molecule. This observation suggests that Hfq plays a role in precursor stabilization or in the biogenesis process, rather than in the functional activity of the mature sRNA itself.

In summary, the key findings of **article no. 2** indicate that 24B_1 functions as a regulatory RNA that controls both host cell metabolism and phage gene expression, thereby promoting maintenance of the phage in the lysogenic developmental pathway.

Further investigation of the $\Phi 24_B$ phage genome, combined with transcriptomic analyses, led to the identification of another phage–encoded, very small RNA molecule –

UpRoi1, containing only 30 nucleotides in length. In **article no. 3**, we performed a detailed characterization of this molecule and demonstrated that, in contrast to 24B_1, UpRoi1 promotes the lytic development of the phage. We identified potential binding sites for this molecule in both the phage and bacterial genomes and experimentally confirmed that UpRoi1 directly interacts with both phage and bacterial transcripts. These interactions are stabilized by the RNA chaperone protein ProQ.

The UpRoi1 molecule is encoded by the *uproi1* gene, located within the regulatory region of the phage genome between the *ant* and *roi* genes, but on the opposite strand, and contains identified p_N promoter and T2 terminator sequences. Quantitative analyses using RT–qPCR confirmed the production of the UpRoi1 transcript in lysogenic cells and demonstrated that its levels increase significantly after prophage induction, with the highest expression observed during transcription from its natural p_N promoter. Even under conditions of spontaneous induction, the UpRoi1 overexpression variant exhibited a significantly elevated level of this transcript in the cell, confirming its efficient production in the absence of the exogenous inducer. Further experiments showed that overexpression of UpRoi1 significantly reduced bacterial survival after infection and enhanced cell lysis in culture under both spontaneous conditions and mitomycin C–induced prophage activation. This effect was coupled with an approximately tenfold increase in the number of phage progeny particles, as well as a decrease in the percentage of viable host cells in the culture, as confirmed by flow cytometry analyses.

The genomic localization of UpRoi1, together with its high level of complementarity to the potential target sequence, suggested that it may function as a *cis*–acting RNA molecule, interacting with the non–translated 5'UTR region of the *roi* gene transcript. *In vitro* RNA–binding analysis demonstrated direct binding of UpRoi1 to a 100 nucleotide long fragment of the 5'UTR of *roi* gene mRNA. The RNA chaperone protein ProQ was identified as a stabilizing factor for this complex, whereas Hfq showed no affinity to it. Spectroscopic analyses further confirmed this interaction. Observed changes in UV absorbance, as well as alterations in circular dichroism (CD) spectra, indicated structural reorganization upon complex formation. The calculated binding constant (K_{bind} = 3.81 × 10^6 M^{-1}) indicates high binding affinity, while the negative Gibbs free energy (ΔG = −37.52 kJ/mol) reflects a spontaneous, thermodynamically favorable, and stable interaction. CD spectral analysis additionally revealed that although UpRoi1 alone exhibits limited

structural stability, its interaction with the structured 5'UTR of *roi* mRNA promotes the formation of a highly ordered conformation enriched in helical structures.

Furthermore, confirmed by us experimentally, the binding of the SOS response regulator, the LexA protein, to the natural p_N promoter region suggests that UpRoi1 expression is integrated with the host SOS response system. In turn, RNA–seq and RT–qPCR analyses revealed increased expression of phage antirepressor genes (*cro*, *roi*, *ant*, and *d_ant*) in cells that overexpressed UpRoi1, while the transcript level of the CI repressor remained unchanged. This indicates that the regulatory mechanism is based on antirepressor activity, instead of the direct effect on the *cI* gene mRNA level in the cell.

Based on these findings, we proposed an UpRoi1–dependent mechanism of the bacteriophage $\Phi 24_B$ life cycle switch [**Fig. 9 in the article no. 3**]. In this regulatory model, LexA protein, acting as a repressor in the bacterial SOS response system, binds to the p_N promoter sequence of the *uproi1* gene and inhibits its transcription. In response to DNA damage (for example, caused by UV radiation or other stress factors), single–stranded DNA (ssDNA) accumulates in the cell, leading to activation of the DNA repair protein RecA. RecA activation simultaneously initiates two mechanisms of CI inactivation (the repressor responsible for maintaining lysogeny): direct autocleavage of the CI protein and an indirect pathway resulting from LexA degradation, which is also mediated by RecA activity. Degradation of the LexA repressor relieves transcriptional repression of the *uproi1* gene. As a consequence of this, the UpRoi1 molecule is produced and binds to the 5'UTR mRNA of the *roi* antirepressor gene, specifically within the T1 terminator sequence that is located upstream of the *roi* coding sequence and overlaps with its putative promoter p_{946}. The co–localization of the T1 terminator and p_{946} promoter likely serves as a safeguard against premature transcription of the *roi* gene. In the presence of UpRoi1, whose interaction with the 5'UTR mRNA of the *roi* gene likely destabilizes the T1 terminator structure, the increased expression of *roi* from the p_{946} promoter can occur. This results in the production of the Roi antirepressor, which can compete with CI for binding to operator sites of the major phage promoters p_R and p_L. Finally, both direct and indirect mechanisms lead to the abolition of CI–mediated repression, enabling transcription of early phage genes located under the control of the p_R and p_L promoters and triggering the switch of the phage life cycle toward lytic development.

In **article no. 3**, we also demonstrated that, in addition to regulating phage gene expression, UpRoi1 modulates the expression of host genes located in *trans* position relative to its encoding locus. Transcriptomic analysis revealed 158 genes with increased expression level and 83 genes with decreased expression level in cells overexpressing UpRoi1. Notably, there was a marked upregulation of genes (*cheA*, *cheW*, *tar*, *tap*, *tsr*, *motA*, *motB*, *fliC*, and *flgL*) involved in chemotaxis and flagellar assembly. Proteomic profiling further showed elevated levels of proteins associated with transcriptional and translational activity, alongside a significant decrease in the level of DnaA, the initiator of chromosomal DNA replication. This suggests a potential redistribution of host cellular resources in favor of phage genome replication during the lytic cycle.

Further bioinformatic analyses, combined with the transcriptomic data, enabled the identification of *flgL* mRNA as a direct bacterial target of UpRoi1. This gene encodes a hook filament junction protein essential for flagellar structure. Mobility gelshift binding assay demonstrated that UpRoi1 RNA and an 85-nucleotide-long fragment of *flgL* mRNA formed a high-affinity complex, stabilized by the RNA chaperone protein ProQ. Spectroscopic measurements confirmed a strong and spontaneous interaction (K_{bind} = 4.71 × 10^7 M^{-1}; ΔG = −43.75 kJ/mol). From a physiological perspective, UpRoi1 overexpression increased bacterial motility, as shown by increased dispersion of cells in agar under conditions of spontaneous induction, which correlated with elevated level of *flgL* expression. We propose that this positive, UpRoi1–dependent regulation may reflect a phage strategy favoring lytic development in nutrient–rich environments, potentially directing lysogenic host cells toward conditions conducive to such growth.

As a result of our studies on phage–derived sRNA molecules, conducted in collaboration with researchers from the Faculty of Chemistry at the University of Gdańsk and the Faculty of Biology at Adam Mickiewicz University in Poznań, we developed an additional important interdisciplinary approach [**article no. 4**]. This approach includes a procedure for analyzing the binding of small sRNA molecules to their target transcripts, combining *in vitro* RNA–binding assays based on electrophoretic mobility shift analysis with chemical spectroscopic analyses, including measurements of changes in UV absorbance and circular dichroism spectra. The integration of these three techniques enabled the development of a potent and reliable tool for estimating the strength, stability, and spontaneity (thermodynamic favorability) of sRNA–mRNA interactions.

In light of the results obtained so far, we can point to the opposing activities of the 24B_1 and UpRoi1 molecules and draw the main conclusion that phage–encoded sRNAs of sizes comparable to eukaryotic microRNAs can function as regulatory factors controlling the switch of the phage life cycle. Similar roles are played by microRNAs of viruses infecting eukaryotic cells, such as herpesviruses, which exhibit notable similarities in their life cycles to temperate tailed bacteriophages, including Stx phages. Both groups of these viruses are capable of switching between a latent state (latency in herpesviruses, lysogeny in phages) and lytic development. During latency (or lysogeny), the majority of viral genes remain inactive, while a limited number of expressed genes ensure maintenance of the viral genome integrated into the host cell, and avoidance of host defense mechanisms. Viral reactivation, or prophage induction, initiates excision followed by replication of viral DNA and entry into the lytic cycle, finally leading to host cell lysis and release of progeny virions. In herpesviruses, microRNAs play a crucial role in regulating the switch between life cycles. Most characterized microRNAs promote latency by repressing the expression of lytic genes, although some also facilitate lytic development. These viral microRNAs are often encoded on the antisense strand and regulate the expression of both viral and host genes involved in processes such as apoptosis, metabolism, and cell migration.

The results described above clearly indicate that microRNA–like phage–encoded sRNAs form an additional mechanism of gene expression regulation and control bacteriophage development and phage–host interactions. By modulating the expression of phage repressors, antirepressors, and host genes, these small RNAs contribute to the precise regulation of the phage life cycle. Understanding the mechanisms of action of phage–encoded sRNAs not only expands fundamental knowledge of phage biology but also opens new perspectives for therapeutic strategies aimed at reducing Shiga toxin production by maintaining the latent state of Stx phages in pathogenic *E. coli* strains. In light of these findings, it can be concluded that Stx phages encode sRNA molecules functionally analogous to eukaryotic microRNAs. The lysogeny–promoting molecule 24B_1 represents the first evidence of a regulatory mechanism resembling that observed in viruses infecting eukaryotic cells. This discovery was further extended by the identification and characterization of another phage–encoded sRNA, UpRoi1, which also plays a key role in phage developmental switch. Beyond its role in regulating phage gene

expression and promoting lytic development, UpRoi1 also controls host gene expression through direct binding to the mRNA of the *flgL* gene. This interaction may facilitate the dissemination of viral particles by stimulating bacterial cell motility towards more favorable environments, thereby ensuring a balance between phage production and host population survival. Similar to herpesvirus microRNAs that enhance host cell migration and invasiveness, UpRoi1 appears to control viral spreading through the regulation of essential bacterial life processes.

These observations strongly support the hypothesis that temperate bacteriophages carry genes encoding regulatory sRNAs that, in many aspects, resemble microRNA molecules functioning in eukaryotic cells. Phage–encoded sRNAs emerge here as key regulators of phage–host interactions, activated by environmental signals, triggers, and changes in cellular metabolic state or fluctuations in bacterial population density, that direct the phage toward the appropriate development pathway. Therapeutic intervention involving such sRNAs, or their interactions with target transcripts, may represent a novel strategy for controlling prophage induction and limiting Shiga toxin production during STEC infections.