

Control Mechanism of Gene Expression During Development of Lambdoid Bacteriophages

Sylwia Bloch

Lambdoid bacteriophages form a group of viruses from the *Siphoviridae* family. Their name is after phage λ that is a model organism in molecular biology. Phages clustered in this group share a common scheme of genome organization and lifecycle. Some of lambdoid bacteriophages can play crucial roles in creating the pathogenic profiles of many feared bacterial strains. Such bacteria can be exemplified by Shiga toxin-producing *Escherichia coli* (STEC). The STEC strains acquired *stx* (*stx1* and/or *stx2*) genes coding for Shiga toxins, responsible for their high pathogenicity, by lysogenization with lambdoid bacteriophages, called Shiga toxin-converting bacteriophages (Stx phages), that occur in these bacteria as a prophages.

STEC strains are responsible for severe infections with relatively high level of morbidity and mortality among children and elderly persons. Many epidemiological surveys have revealed that STEC strains are prevalent in gastrointestinal tracts of cattle or other domestic animals. Humans become infected with this pathogen by consuming contaminated food (beef, vegetables, fruits) or drinking contaminated water or raw milk. The main epidemiological problem with STEC is a wide spectrum of phenotypes and clinical symptoms, which is probably associated with exchangeable genetic elements during a number of horizontal gene transfer events. The consequence of this process is creation of new atypical bacterial strains. An interesting example of this phenomenon is a novel Shiga toxin-producing *E. coli* O104:H4 serotype that caused the largest outbreak in Germany (May 2011) and worldwide with over 4000 cases of diarrhea, over 830 cases of hemolytic uremic syndrome (HUS), and 54 deaths (reviewed in article [1] of the series presented as this PhD thesis).

The characteristic feature of λ and Stx phages is their capability to choose one of two alternative pathways of development, lytic or lysogenic, upon infection of the host cell. The decision whether to propagate lytically or to lysogenize *E. coli* depends on environmental conditions and physiology of bacteria. During the lysogenic development, which is one of the two replication strategies, phage DNA is incorporated into the host chromosome, forming a prophage. At this stage, the viral genome is replicated together with bacterial DNA, and majority of phage genes are silenced. The molecular explanation of this phenomenon is

repression of the early lytic promoters, p_R and p_L , by the cI protein. However, the lysogenic stage is not permanent, because when the host cell is endangered by stress conditions, phage developmental switch to the lytic cycle is observed. In this process, phage DNA is excised from the chromosome and replicated separately. This leads to synthesis of phage-encoded regulatory and structural proteins, and as a consequence, to production of progeny virions that are released due to the lysis of the host cell.

The switch from lysogenic to lytic lifecycle is achieved by prophage induction. This process usually takes place when a bacterial cell is stimulated to express genes of stress responses. One common signal that induces lambdoid prophages is bacterial DNA damage. The molecular event responsible for this process is the cleavage of the cI repressor, the regulatory protein which otherwise inhibits phage transcription during lysogeny. The proteolysis of the cI protein is a RecA-dependent process, and occurs after activation of the bacterial SOS response as a result of appearance of single-stranded DNA regions near DNA lesions. It is worth mentioning that prophage induction can be provoked by many different factors like low pH, iron ions, UV irradiation, antibiotics (e.g. mitomycin C) or hydrogen peroxide. The consequence of this process is initiation of phage lytic development through transcription at the early p_L and p_R promoters.

As the *stx* genes are located in the “late” region of the phage genome (downstream of the p_R promoter), their expression occurs in the next step of the lytic cycle. The effective expression of *stx* genes leads to production of large amounts of Shiga toxins that are released to intestine, where they attack eukaryotic cells and block protein synthesis, leading to the cell death. The consequences of these processes are bloody diarrhea with severe complications, like hemolytic uremic syndrome.

In the light of the facts described above, it is obvious that detailed understanding of the regulations of expression of phage genes during development of lambdoid viruses is crucial for both basic knowledge about the pathogenicity of STEC bacteria and a putative further work on treatment of infections by these pathogens. Therefore, the aim of my work was to determine specific regulatory mechanisms of genes' expression that influence the lambdoid phage lysis-versus-lysogenization decision and its further development.

My research was focused on nonpathogenic phage λ and phage $\Phi 24_B$ that represents Shiga toxin-converting bacteriophages. As both phages belong to lambdoid viruses, they reveal many similarities in organization of their genomes and viral development. Nevertheless, taking into consideration that the differences in many crucial processes and in

virions structures were also reported, it was decided that both phages should be used as research models in this work.

In the first step of my work, I analyzed impact of two chemical induction agents, mitomycin C and hydrogen peroxide, on expression of phage genes during the lytic development. Both agents act as inducers by interfering with DNA. In contrast to mitomycin C, hydrogen peroxide appears to be a natural prophage inducer, as both protist predators and mammalian neutrophils excrete this compound when hunting for bacteria and responding to bacterial infection, respectively.

When selecting genes for quantitative real-time reverse transcription PCR (qRT-PCR) analysis, my attention was attracted towards genes and open reading frames (ORFs) localized in region between *exo* and *xis* genes (called the *exo-xis* region). In the case of λ phage, this region consists of two recognized genes: *ea22* and *ea8.5*, and five additional uncharacterized ORFs, named: *orf60a*, *orf63*, *orf61*, *orf73* and *orf55* of largely unknown functions. Comparatively, the *exo-xis* region of $\Phi24_B$ phage contains additional ORFs, but there is no homolog of the *ea8.5* gene which encodes the λ Ea8.5 protein, potentially having a regulatory function. In spite of the differences in composition of both λ and $\Phi24_B$ *exo-xis* regions, attention needs to be paid to highly conserved sequences of four open reading frames (*orf60a*, *orf63*, *orf61*, *orf73* in the case of λ). Considering the evolutionary tendency to preserve the most necessary genes unchanged or just slightly changed, it appears unlikely that lambdoid phages, including Stx phages, maintain such highly conserved sequences for no reason. To test the hypothesis that the *exo-xis* region may have influence on λ and $\Phi24_B$ development, I have examined phage lytic growth under conditions of its overexpression. I have used derivatives of a ColE1-like plasmid bearing the whole *exo-xis* region of λ (pGAW3775) or $\Phi24_B$ (pSBe.x.r. $\Phi24_B$) [2]. My initial studies demonstrated that more efficient induction of λ and $\Phi24_B$ prophages, caused by mitomycin C and hydrogen peroxide, was observed in cultures of host cells bearing a plasmid with the *exo-xis* region relative to those containing only the plasmid vector. Moreover, after prophage induction, an increase in phage DNA amount was significantly higher in lysogenic *E. coli* cells containing plasmid-borne *exo-xis* region, while survival rate of such bacteria was lower [3].

Keeping in mind these observations, I used qRT-PCR to analyze the expression profiles of phage genes and ORFs (including those located within the *exo-xis* regions) during the prophage induction process. One of unexpected results of my research indicated that even in the case of the same phage, the nature of tested agent (mitomycin C or hydrogen peroxide) has influence on the level and profile of gene expression after prophage induction.

This conclusion was mostly true for phage $\Phi 24_B$. I have found that during prophage induction with mitomycin C, some of ORFs, especially *orf60a*, *orf63* and *orf73* homologues, were expressed as efficiently as the *N* gene. Surprisingly, when hydrogen peroxide was used as an inductor, levels of mRNAs for homologues of *orf73* and *ea22* gene were significantly higher than other tested genes or ORFs, which was considered as the main difference in comparison to mitomycin C-induced lysogens. The patterns of genes' and ORFs' expression, obtained for phage λ after treatment of the host cells with the same two induction agents, were quite similar. I have noticed that the *ea22* gene and all tested ORFs were highly expressed, while the level of mRNA for the *ea8.5* gene was as low as those for *N*, *cro*, and *Q* genes [3].

Taking above results into consideration, despite of high similarity between λ and $\Phi 24_B$ *exo-xis* regions, it is very difficult to propose a common (for both analyzed phages) mechanism of genes expression regulation in the prophage induction process. This machinery is probably more complicated and depends on the mode of lytic cycle initiation, however, high levels of mRNAs of tested genes and ORFs located within the *exo-xis* regions suggest that they can play significant regulatory roles in lifecycle of lambdoid viruses [3].

In the next part of my work, I analyzed the impact of UV irradiation on stabilities of lambdoid virions. I have studied λ and $\Phi 24_B$, and focused on mechanisms that impaired their infectivity after UV treatment. I present evidence that phage $\Phi 24_B$ is significantly more sensitive to UV light (at the dose of 50 J/m²) relative to virions of phage λ . I proposed that this phenomenon may be a consequence of damage of large proportion of $\Phi 24_B$ bacteriophage capsids, which was confirmed using electron microscopic analysis. Moreover, infection of *E. coli* cells with UV-treated lambdoid phages resulted in modulation of expression of genes that are crucial for the lytic development. I observed that in the case of population of bacterial host cells infected with $\Phi 24_B$ phages treated with UV light, the level of expression of both tested genes, *N* and *cro* (transcribed from the early promoters *p_L* and *p_R*, respectively), was significantly lower relative to *E. coli* population infected with non-irradiated Stx phages. Interestingly, despite the membership to lambdoid group, in the case of bacteriophage λ , UV irradiation of virion particles prior to infection did not have influence on the level of these mRNAs. The presented findings indicate that Stx phages are more sensitive to UV light than λ phage, and shed a new light on differences in regulation of genes' expression of lambdoid phages [4].

In the next stage of my studies, I focused on testing the impact of the RNA polyadenylation process on expression of λ and $\Phi 24_B$ genes during the lytic cycle. In *E. coli* bacteria, poly(A) polymerase I (PAP I), encoded by the *pcnB* gene, is the major enzyme

capable of a post-transcriptional modification based on addition of poly(A) tail to RNA strand. In prokaryotic cells, this process causes destabilization of RNA. I investigated the effects of *pcnB* gene deletion on the level of expression of crucial viral genes (*xis*, *cIII*, *N*, *cI*, *cro*, *cII*, *oop*, *O*, *Q*, *R*) after prophage induction. I observed that shortly after treatment of lysogenic cell with mitomycin C, the amount of all tested transcripts of both phages, λ and $\Phi24_B$, was highly increased in *pcnB* mutants relative to wild type host cells. Nevertheless, different results were evident at later time points, when the level of expression of most tested genes remarkably increased in wild-type strains contrary to polyadenylation-deficient cells. This was also true for two genes, *N* and *O*, that play significant roles in the lytic pathway of tested bacteriophages. Interestingly, these disproportions were pronounced mainly after induction of the $\Phi24_B$ prophage, while in the case of phage λ the differences were moderate. The proposed mechanism by which RNA polyadenylation affects the abundance of mRNAs for many crucial viral genes at different times after prophage induction in wild-type strains, is associated with the decrease of the bacterial growth rates in response to stress conditions, and resultant transiently enhanced polyadenylation, occurring shortly after addition of mitomycin C; a decreased level of PAP I occurs at later times [5].

As the greatest success during realization of my PhD thesis, I can indicate the identification of the first functional microRNA-size molecule in bacterial cells. This RNA is derived from phage $\Phi24_B$, and regulates expression of phage genes during $\Phi24_B$ development in *E. coli* cells. The small RNA (20-nt long), named 24B_1, is encoded in the *lom-vb_24B_43* region of the $\Phi24_B$ (but not λ) phage genome, and probably it is created by the cleavage of a longer transcript (80-nt long). *In silico* analysis showed that 24B_1 can presumably recognize two sites, one located upstream of the *S* gene, and second within the *d_ant* gene that encode a potential anti-repressor protein. After discovery of the 24B_1 molecule, I asked whether this small RNA plays role(s) in physiological processes. To answer this question, I employed $\Phi24_B$ bacteriophage bearing deletion mutation of the 24B_1. Surprisingly, this modification resulted in faster production of phage progeny after prophage induction with mitomycin C and more efficient lytic development relative to wild-type phage $\Phi24_B$. Moreover, the phage $\Phi24_B\Delta24B_1$ revealed a decreased efficiency of lysogenization, lower survival rate of bacteria after infection, and impaired adsorption on the host cell. Subsequently, I determined the pattern of expression of genes (*xis*, *cIII*, *N*, *cI*, *cro*, *cII*, *O*, *Q*, *cat*, *S*, *R*, *d_ant*, *R1*, *Rz1*) following prophage induction with mitomycin C and phage infection, using qRT-PCR. Interestingly, after prophage induction, I noticed only slight differences in the levels of mRNAs between $\Phi24_B$ and $\Phi24_B\Delta24B_1$. However, during

infection, the efficiency of expression of all tested genes drastically increased in $\Phi 24_B \Delta 24B_1$ -infected *E. coli* cells. The proposed mechanism of this phenomenon indicate that 24B_1 acts as a negative regulator of the *d_ant* gene expression, which in turn has influence on the level of the cI protein (the repressor of major phage promoters). As a consequence of such regulation, 24B_1 indirectly influence the control of lysis-versus-lysogenization decision of Stx phage and expression of various phage genes [6].

Results presented in this work indicate that despite of significant similarities within sequence, genome organization and development of lambdoid phages λ and $\Phi 24_B$, the regulatory mechanisms controlling expression of phages' genes during their lifecycles are quite different and depend on both extracellular and intracellular factors. This knowledge may shed a new light on designing of prevention and treatment strategies of STEC infections.

References

- [1] Bloch S, Felczykowska A, Nejman-Faleńczyk B. *Escherichia coli* O104:H4 outbreak - have we learnt a lesson from it?; *Acta Biochimica Polonica*; 2012; 59(4): 483-488.
- [2] Bloch S, Nejman-Faleńczyk B, Łoś JM, Barańska S, Łeppek K, Felczykowska A, Łoś M, Węgrzyn G, Węgrzyn A. Genes from the *exo-xis* region of λ and Shiga toxin-converting bacteriophages influence lysogenization and prophage induction; *Archives of Microbiology*; 2013; 195: 693-703.
- [3] Bloch S, Nejman-Faleńczyk B, Dydecka A, Łoś JM, Felczykowska A, Węgrzyn A, Węgrzyn G. Different expression patterns of genes from the *exo-xis* region of bacteriophage λ and Shiga toxin-converting bacteriophage $\Phi 24_B$ following infection or prophage induction in *Escherichia coli*; *PLoS One*; 2014; 9(10): e108233.
- [4] Bloch S, Nejman-Faleńczyk B, Topka G, Dydecka A, Licznarska K, Narajczyk M, Necel A, Węgrzyn A, Węgrzyn G. UV-Sensitivity of Shiga Toxin-Converting Bacteriophage Virions $\Phi 24_B$, 933W, P22, P27 and P32; *Toxins*; 2015; 7(9): 3727-3739.
- [5] Nowicki D, Bloch S, Nejman-Faleńczyk B, Szalewska-Pałasz A, Węgrzyn A, Węgrzyn G. Defects in RNA polyadenylation impair both lysogenization by and lytic development of Shiga toxin-converting bacteriophages; *Journal of General Virology*; 2015; 96(7): 1957-1968.
- [6] Nejman-Faleńczyk B, Bloch S, Licznarska K, Dydecka A, Felczykowska A, Topka G, Węgrzyn A, Węgrzyn G. A small, microRNA-size, ribonucleic acid regulating gene expression and development of Shiga toxin-converting bacteriophage $\Phi 24_B$; *Scientific Reports*; 2015; 5:10080.