Molecular mechanisms controlling development of Shiga toxin-encoding lambdoid bacteriophages

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Shiga toxin is one of the most potent bacterial toxins and is produced by the bacteria naturally occurring in the environment. For the first time this toxin was identified in the 19th century as associated with *Shigella dysenteriae* infections. Nowadays, the most serious threat is ascribed to strains included in the group of Shiga-toxigenic *Escherichia coli* - STEC, and the production of toxins is their main virulence factor. Further studies led to a detailed description of the relevant factors and classification as exotoxins type A_1B_5. It consists of two major subunits, an effector A subunit that binds noncovalently to a pentamer of five identical B subunits that attach to the cellular receptor (Gb3), found primarily on endothelial cells. Binding to this receptor leads to the transport in a retrograde way resulting in the delivery of the active A subunit into the cytosol. The A subunit exhibits N-glycosidase activity and causes the cleavage the N-glycosidic bond of the adenosine residue at position 4324 in 28S RNA. This specific modification leads to the disruption of the elongation factor binding in translation process. The proper functions of eukaryotic cells are then impaired leading to their death.

Environmental reservoir of STEC strains are pigs, cattle and other farm animals that may be asymptomatically colonized by bacteria. STEC have been assigned as causative agents in food poisoning outbreaks dangerous for humans. Contact with infected agricultural products originating from animal husbandry is the main route of infection. These usually relate to contaminated meat, unpasteurized milk, fruit, vegetables or even water. STEC food-borne infections typically occur in the form of mild diarrhea. However, in a small proportion of patients with immune deficiency, particularly young children and the elderly, infection with EHEC can lead to a far more serious illness. The most severe symptoms are caused by strains of the *enterohemorrhagic E. coli* EHEC (belonging to STEC group) including bloody diarrhea, thrombotic thrombocytopenic purpura and hemolytic uremic syndrome (HUS). These complications are the direct threat to the health and lives of patients with the mortality within a group of patients who developed HUS syndrome up to 10%. It should be emphasized that not sufficiently described ecology and etiology of STEC strains infections is still a huge challenge at an epidemiological and economic field. The last large outbreak in Germany in 2011 is a significant example with more than 4,000 infected people. 830 among them had

developed complications such as HUS and 54 fatal cases were reported. So high extent of infection cases has resulted also in economic loss related both to the necessity of sanitary-epidemiological support, as well as the loss of food producers, whose costs were estimated at 3-6 billion Eur.

Shiga toxin genes (*stx*) transfer across pathogens are due to the Stx-encoding lambdoid bacteriophages (Stx), which are classified within *Caudovirales* genus (belonging to *Siphoviridae* or *Podoviridae* families, long- or short-tailed respectively). Their life cycle, morphology and genome arrangement are similar to model in molecular biology, λ phage. Infection of bacterial cells is initiated by binding to the surface receptor BamA and then phage DNA is injected into host cell. These bacteriophages are able to integrate into bacterial chromosome in the form of prophages. At this stage of development - called the lysogeny, expression of almost all phage genes is strongly inhibited (except cl repressor). It is worth mentioning that BamA protein, evolutionary highly conserved among *Enterobacteriaceae*, is an universal molecular target for the development of phage-mediated infection. This important feature promotes a horizontal gene transfer, including the transfer of virulence factors, not only in STEC strains. The most common strain associated with EHEC infection is O157:H7, responsible for first large outbreak reported in 1982 in USA. Nowadays, new serotypes of *E. coli* capable of Shiga toxins synthesis frequently appear, exhibiting higher pathogenicity and resistance to environmental factors including low pH, high and low temperature as well as multidrug resistance. The *stx* genes are located in the genomes of all analyzed to date Stx bacteriophages in the lysis genes region, thus, they are under control of the late phage promoter p_R' and toxin production is closely correlated to the virus lytic development. Effective toxin production occurs only after prophage induction and phage genome synthesis in DNA replication process. Subsequently, it leads to the expression of phage structural elements and assembling the progeny virions. Finally, the propagation of the virus in the environment is associated with cell lysis and the host death. Along with the release of viral particles, the toxin also comes out into the intestinal lumen.

These temperate lambdoid phages lack proteins required to express their own genes and replicate their own genomes. Therefore, they require the host cell machinery for expression of phage genes, and further synthesis of new phage particles in the lytic cycle. The choice between lytic or lysogenic development is affected by metabolic and physiological cell conditions, environmental signals such as pH, temperature, nutrients availability. To coordinate processes taking place in varying environments, bacteria have developed a number of control mechanisms that assure stability of metabolic and genetic functions. Current state

of the knowledge inadequately explains how the development of lambdoid bacteriophages in host cells is regulated by these processes.

Induction of lytic cycle from the prophage state depends on activation of SOS response in host cell. Factors causing such a response are related to DNA damage including: hydrogen peroxide (as a natural agent in gastrointestinal tract) or some antibiotics, which mechanism of action leads to damage of the genetic material in a direct or indirect way. Activation of the lytic cycle takes place by disabling CI repressor protein by the bacterial RecA factor and consequently starts the production of progeny phage particles. Due to this mechanism of lytic cycle induction, using antibiotic therapy to combat STEC infections is substantially limited and may be potentially hazardous for the patient.

Lambdoid bacteriophages encoding Shiga toxin, despite their considerable similarities to λ bacteriophage, are not a homogeneous group. They exhibit some differences in genetic sequences, both in individual genes and their arrangement. This can have a significant impact on the regulation mechanisms at various stages of phage development, as well as affect the level of toxin production. Furthermore, the lack of effective and safe treatment to combat infections caused by STEC strains points to the need of the research on the regulation of fundamental processes of bacteriophages life cycle in order to solve the problem of pathogenic bacteria associated with Stx phages.

In the light of facts described above, the aim of the studies presented in my PhD thesis was to identify the molecular mechanisms underlying the regulation of the development of lambdoid bacteriophages bearing Shiga toxin genes and to examine the possibility to use them for controlling the expression of STEC virulence factors.

First, I decided to employ into my research the representatives of lambdoid bacteriophages: Φ24B, 933W, P22, P27, P32 (Stx) and λ bacteriophage to examine how the changes in the host cells can affect their life cycle. Considering the fact that the replication of the genetic material is an important step affecting further stages of phage development, the plasmids containing an origin of replication derived from these viruses were also used in my studies. The diverse set of representatives of lambdoidal viruses, phage derived plasmids as an independent replicons (capable of independent replication in *E. coli* cells) was used to verify the hypothesis: whether the development of bacteriophages depends on the regulation via global regulatory mechanisms of gene expression in a host cell?

One of the major cellular processes coordinating the response to environmental changes and stress factors is the stringent metabolism control. It was reported that in nutrient deficiency phage development is slowed down but under starvation conditions it can be

completely inhibited. The results of previous studies have shown that the stringent response and overproduction of its alarmone, guanosine tetra- and pentaphosphate, (p)ppGpp (synthesized by RelA/SpoT proteins) effectively inhibits growth of the representatives of lambdoid bacteriophages. The *relA* gene product, ppGpp synthetase I, is associated with ribosomes and is activated under amino acids starvation, while the product of *spoT* gene, ppGpp synthetase II, is activated in response to other environmental factors as pH changes, metal ions, osmotic stress, temperature, carbon and nitrogen limitation. Moreover, SpoT enzyme exhibits the ability to hydrolyze guanosine tetraphosphate, hence it is essential for the metabolism of these molecules. Induction of the stringent response leads to the inhibition of the energy-consuming processes (synthesis of stable RNA, DNA replication). ppGpp alarmone acts primarily on RNA polymerase activity resulting in the inhibition of transcription from numerous promoters. In *relA* mutant strain the synthesis of ppGpp under amino acid starvation does not occur which leads to e.g. continuous synthesis of stable RNAs (this is called relaxed response). Moreover, alarmone molecules can affect bacteriophage development through the impairment of DNA synthesis by inhibition of transcriptional activation of the origin of replication. At the initial step of experiments, I constructed phage lysogens of *E. coli* isogenic strains: wild type, *relA* mutant and *relA spoT* ($ppGpp^0$) mutant or their equivalents transformed with the plasmid derived from relevant bacteriophage. My further analyses showed that even a basic physiological concentrations of alarmone in host cell can affect the development of all five tested Stx bacteriophages. The control occurs at the lytic stage of virus development, and ppGpp negatively influences DNA replication of both Stx bacteriophages and phage-derived plasmids. This exceptional sensitivity to adjustment by low concentrations of ppGpp distinguish Stx phages from λ phage. Moreover, I demonstrated that overproduction of guanosine tetraphosphate leads to significant reduction of the expression from late phage promoters and thereby synthesis of green fluorescent protein (GFP) expressed under the control of a toxin gene promoter. This information seems to be particularly important, as it can be assumed that at natural conditions the stringent response can lead to the reduction of Stx toxin production, and thus also to the impairment of STEC strains virulence [1].

The use of the antibiotic treatment to combat STEC infections may have undesirable effects on patients health. This is due to the uncontrolled induction of prophages associated with the mechanism of action of most antibiotics, leading to activation of the SOS response and resulting in the toxin proteins production in the gastrointestinal tract. The lack of treatment alternatives for STEC/EHEC infections prompted the next stage of my work where

I investigated antimicrobial properties of the plant derivatives, isothiocyanates (ITC). ITCs are intermediate metabolites commonly synthesized in plants from cruciferous family. These compounds are currently an important research subject due to their potential chemopreventive or anti-oxidant properties that can be used to fight carcinogenesis processes. In my work, I checked the activity of compounds naturally occurring in the human daily diet for their use in the treatment against EHEC. In addition to determine the antibiotic properties of ITC compounds, I attempted also to explain their molecular mechanism of action. This basic knowledge was required to determine the possibility and effectiveness of these compounds to inhibit EHEC virulence. I reported a potent antibiotic properties of certain isothiocyanates: phenethyl (PEITC), benzyl (BITC), allyl (AITC) and sulforaphane (SFN). The detailed observations led to the conclusion that ITCs lead to the inhibition of nucleic acids synthesis in bacterial cell. It is particularly woth noticing that the biological activity of the compounds did not induce prophage. The prophage induction impairment by ITC was observed also in the presence of strong inducers such as mitomycin C and hydrogen peroxide. Hence I was interested in the mechanism underlying this phenomenon. I demonstrated that the presence of the ITC leads to metabolic stress and ppGpp synthesis. The experiments employing *relA* and *spoT* mutants showed that ITC-induced production of ppGpp is RelA-dependent indicating the involvement of amino acid starvation. In further studies I showed that specific amino acids can reverse the effect of tested ITCs. This observation may indicate the potential interactions of ITCs with the individual amine groups of free amino acids or binding to the specific sites of protein domains. Because the effectiveness of the therapy depends on the efficient inhibition Shiga toxin synthesis, model eukaryotic cell lines capable of expressing Gb3 receptor and thus sensitive to the toxin was employed in my studies. Assessment of cell viability (MTT) using human and simian (HeLa and Vero lines) cells is an efficient and accurate test to obtain toxicological data. The ITC treatment of EHEC leads to a significant reduction in the toxicity of the bacterial cell lysates, indicating inhibition of the toxin synthesis by these compounds. These results were confirmed by the observation of GFP expression (the marker protein) under the control of toxin promoter using fluorescence microscopy. SOS response can be also activated by genotoxic effect of reactive oxygen species, leading thus to the prophage induction. Therefore I assessed the potential ITC effect on the oxidative stress in EHEC cells, and their impact on the cell membrane integrity. These analyzes showed no elevated levels of reactive oxygen species in the cells exposed to the compounds. In addition, membrane damage caused by ITCs seem to be the secondary effects of the induction of stringent response rather than the main mechanism of ITC action. These

findings led to the understanding of the mode of action of the antibacterial compounds from the ITC group, include the overproduction of ppGpp alarmone in *E. coli* cells. This indicates a high potential of ITC for the treatment alternatives of infections caused by Shiga-toxigenic bacteria [2, 3].

In next part of my work I analyzed the impact of polyadenylation, one of the most important control mechanism of RNA cellular metabolism, on lambdoid bacteriophages development. This process is associated with the activity of RNA polyadenylase (PAP I), *pcnB* gene product. Previous studies have shown that mutations in *pcnB* gene affect both the development of host cells and λ phage. In this study we determined the impact of PAP I gene dysfunction on the development of lambdoid phages representatives, Φ24B, 933W, P22, P27, P32 and λ. I presented the evidence that in PAP I deficient cells the lytic development of Stx viruses is significantly less efficient comparing to the wild type bacteria. This is manifested by impaired ability of prophage induction, and by a lower yield of phage particles after infection. More importantly, I found that after induction of lytic cycle, phage DNA synthesis efficiency is reduced. Further, the detailed studies showed severely impaired ability of phage genetic material integration into the host chromosome. It is interesting to note that the observed phenomenon is common to all tested bacteriophages, which may indicate a similar regulatory mechanism. These findings suggest that in PAP I deficient cells, impairment of the post-transcriptional modification of RNA may affects the metabolism of these molecules and subsequently influence cell physiological condition through the reduced efficiency of RNA degradation. Moreover, an impairment of specific activity of regulatory small RNAs such as *oop* phage transcript could explain these observations. A better understanding of the processes underlying this type of regulation requires further research, which could determine the molecular targets of poly(A) modification [4].

The original reports which have become the basis for my PhD thesis, present important knowledge of the fundamental biological processes and exhibit application potential. These results indicate that bacterial gene expression systems may be molecular targets in controlling the development of lambdoid bacteriophages harboring *stx* toxin genes. The differences in genetic structure of phages may have significant impact on the effectiveness of control of the bacteriophages development by host cell machinery. Furthermore, I have identified natural anti- STEC compounds, isothiocyanates, and presented their molecular mechanism of action leading to the activation of the stringent control by amino acid starvation.

References:

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