„The role of genes and open reading frames from the exo-xis region of lambdoid phages in the development of these phages"

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In recent years, there is a lot of reports about infection of Shiga toxin-producing *E. coli* (STEC) strains. The occurrence of EHEC is global. This is evidenced by food poisoning detected in various countries of the world, including in the USA, Canada, Japan, and European Union countries. One of the largest epidemics caused by EHEC strains was reported in Japan in 1996, where over 10,000 patients were infected , especially primary school children (Watanabe, 1999). In Europe, the largest epidemic in recent years occurred in 2011, in Germany, where 54 fatalities were reported. It is known that the source of infection were fenugreek seeds contaminated with *E. coli* O104: H4 that had been imported to Europe from Egypt (Frank *et al.,* 2011; Bloch *et al*., 2012). The main reservoir of EHEC is cattle, but they also occur in the digestive tracts of other farmed animals, like sheep, goats and poultry. Importantly, EHEC strains do not cause disease symptoms among them. Bacteria excreted with animal feces, get into the farmland, from where they can contaminate water, fruit and vegetables, which are a threat to human health (Gyles, 2007).

The Shiga toxins, produced by bacteria are responsible for the symptoms associated with EHEC infections. These virulence factors are encoded by *stx* genes that are located in the genomes of bacteriophages called Shiga toxins-converting bacteriophages (Stx phages), which occur in bacteria as prophages. These phages belong to lamdoid phage family, in which bacteriophage λ is the best investigated member. All lambdoid phages reveal high similarities in the genomic organization and lifecycle (Campbell, 1994). It is worth to mention that the effective production of Shiga toxins occurs only upon prophage induction and its further lytic development. The toxin released in the intestines, penetrates into the lumen of blood vessels and is spread with blood along the human body to organs such as the kidneys or the brain (Muniesa *et al*., 2012). In most cases, the effect of the infection is food poisoning, manifested as acute, abdominal pain and bloody diarrhea. In some patients, such infection may result in different complications and development of chronic diseases such as hemolytic uremic syndrome, which can lead to the death of the patient, especially if the infection affects children or elderly persons (Karakulska, 2002; Serna & Boedeker, 2008).

Stx phages, belong to the group of temperate phages, like bacteriophage λ. Depending on the intracellular conditions, bacteriophages may develop according to one of two pathways, lysogenic or lytic. The decision depends primarily on the physiological state of the host cell and the number of viral particles infecting bacteria. The lytic pathway leads to phage proliferation,

production of large amounts of Shiga toxin and lysis of the host cell. In turn, at the lysogenic stage, the bacteriophage genome is integrated with the bacterial chromosome as a prophage and replicated with it. Lysogeny is a transitional stage in the development of phages. The prophage induction and further lytic development are results from the action of various factors causing bacterial S.O.S response, such as UV rays, antibiotics or reactive oxygen species (Kobiler *et al.,* 2005; Węgrzyn & Węgrzyn 2005). Interestingly, It appears that hydrogen peroxide is a natural induction factor occurring in the human body, which is produced by neutrophils appearing during bacterial infection (Łoś *et al*., 2013).

It is known, that at the molecular level, the choice of the appropriate development pathway by bacteriophage λ depends on the presence of specific phage proteins. The competition between the Cro protein, important for lytic development, and the main lytic repressor, the cI protein, is crucial here. The cI protein, inhibits transcription from two early phage promoters: *p*L and *p*R, thereby allowing phage to be maintained as a prohage. Importantly, autoproteolysis of the cI occurs during the bacterial S.O.S. response. The third protein important at this stage is cII, which activates three phage promoters *p*E, *p*I and *pa*Q (Węgrzyn *et al*., 2005). Activation of these promoters leads to the formation of "pro-lysogenic" products (Łoś *et al.*, 2008). The biochemical bases of the lysis vs. lysogenization decision have been relatively well known, but available reports indicate that regulation of this process is still far from completeness (Łoś *et al*., 2008; Węgrzyn *et al*., 2012; Bloch *et al*., 2013). Moreover, significant differences in the development of phages λ and Stx have been also shown (Nejman *et al.,* 2009, 2011). Due to this, the knowledge resulting from research on bacteriophage λ should not be extrapolated to Stx phages without experimental verification. In the light of this problem, the detailed understanding of regulation of Stx phage development, especially at the stage of choosing a specific pathway and, the broad knowledge about factors involved in the lytic development of these phages, are crucial for learning the principles of EHEC strain pathogenicity.

The available literature and research of the team I joined at the beginning of my doctoral studies indicated that products encoded in the poorly characterized region located between the *exo* and *xis* genes in genomes of Stx phages may be involved in the lytic development of these phages. In the case of λ phage, this region consists of two genes *ea22* and *ea8.5* and five open reading frames (ORFs): *orf60a, orf63, orf61, orf73* and *orf55*. However, in bacteriophage $\Phi24_B$, a representative of Stx phages, this region contains additional open reading frames and is devoid of the *ea8.5* gene. The first report about the role of the *exo-xis* region in the regulation of lytic development of phage λ appeared in 2002. It was demonstrated that this region influences the

regulation of the bacterial cell cycle, causing temporary stopping of cell division and inhibiting the initiation of DNA replication. As suggested, this effect may stimulate the efficiency of DNA phage replication during the lytic cycle, due to the higher availability of bacterial replication proteins which are required at this stage of phage development (Sergueev *et al.,* 2002). Subsequent reports in this topic indicated that the *exo-xis* region decreases the level of transcription from cII-stimulated (and thus relevant for lysogenic cycle) promoters *p*I, *pa*Q and *p*E (Łoś *et al.,* 2008). Interestingly, efficiency of lysogenization and survival of *E. coli* bacteria after infection of phages λ and $\Phi 24_B$ were impaired when the *exo-xis* region was overexpressed. Under similar conditions, induction of these prophages was found to be more effective (Łoś *et al.,* 2008; Bloch *et al.,* 2013). Additionally, it has been proven that the expression profiles of particular phage genes, as well as genes and open reading frames from the *exo-xis* region, during the infection and induction processes were considerably different (Bloch *et al*., 2014).

This information undoubtedly demonstrated the importance of this region in the regulation of the development of λ and Stx phages. It is therefore justified to examine the functions of individual open reading frames and genes from the *exo-xis* region, especially that only one gene from this region, *ea8.5*, has been previously characterized in detail. The *ea8.5* gene has been shown to encode a regulatory protein with a specific structure, containing a homeodomain and a zinc finger motif, whose presence indicates its ability to bind DNA, and consequently the ability to form nucleoprotein complexes (Kwan *et al*., 2013). Moreover, the involvement of Ea8.5 at the stage of the "lysis vs. lysogenization" decision was indicated and its negative effect on the activity of *p*I and *pa*Q promoters was demonstrated (Łoś *et al.,* 2008). Due to the fact that the observed effects of inhibition of the promoters' activities under overexpression of *ea8.5* gene were not as spectacular as in the case of the presence of many plasmid-born copies of the entire *exo-xis* region (Łoś *et al.,* 2008), it has been suggested that also other products from this region participate in this regulation. In this light, I decided to carry out research aimed at analyzing the biological significance of sequences from the *exo-xis* region, well preserved in the genomes of lambdoid phages. Due to the fact that earlier studies on the *exo-xis* region were conducted in the presence of additional plasmid-born copies of the whole *exo-xis* region, a new approach was proposed in this work. This approach was based on the construction of phage deletion mutants lacking the sequence of the entire region, as well as individual open reading frames. Phage mutants were constructed by homologous recombination using the λRed system (Nejman-Faleńczyk *et al.,* 2015) and the main goal of my research was to clarify the role of individual genetic elements from the *exo-xis* region in the development of

lambdoid phages. In my research, I have used phage λ, as a model organism in molecular biology, and $\Phi24_B$ phage, a representative of Stx bacteriophages.

My studies began with an assessment of the impact of removing the entire *exo-xis* region or its individual elements on prophage induction with use of hydrogen peroxide or UVirradiation **[article no. 1]**. The obtained results indicate that the deletion of the *exo-xis* region from the $\Phi24_B$ phage genome resulted in a drastic impairment of the prophage induction process in *E. coli* bacterial cells after treatment with hydrogen peroxide, but doid not cause such an effect when UVirradiation was used as an inductor. The detected number of phage particles released after hydrogen peroxide induction of the $\Phi24_B\Delta$*exo-xis* mutant corresponded to the level of spontaneous induction, which is the value determined from the control sample without the addition of an inductor. Interestingly, the deletion of the *exo-xis* region from the genome of phage λ, resulted in only a slight delay in the induction process with hydrogen peroxide. In addition, compared to wild-type phage, the deletion of the *exo-xis* region negatively influenced the expression of genes from the S.O.S. regulon and regulatory genes of $\Phi24_B$. A similar, but not so spectacular effect was caused by the removal of the sequence of *exo-xis* region from the λ phage genome. In turn, the removal of individual open reading frames or genes located in the *exo-xis* region from λ and $\Phi24_B$ phage genomes, caused a delay of induction process with hydrogen peroxide only in the cases of the use deletion mutants of $\Phi24_B$ phage. Curiously, UV light did not cause such effect in any of the analyzed deletion mutants **[article no. 1]**. On this basis it was concluded that the products encoded in the *exo-xis* region are important in the hydrogen peroxide-induced S.O.S. response. So, I decided to do a functional characteristics of three selected open reading frames. Bearing in mind, that well-preserved evolutionary sequences may have significant biological significance, I decided to examine in detail three subsequent ORFs (*orf60a, orf63* and *orf61*) located at the beginning of the *exo-xis* region and having very highly conserved sequences in lambdoid phage genomes.

First, we analyzed the *orf63*. In the **article no. 2**, we showed that the *orf63* open reading frame is a functional gene encoding a small protein containing α-helical structures. I studied the effect of removing the sequence coding for Orf63 at the various stages of the development of λ and $\Phi24_B$ phages. First, I analyzed the effect of this deletion on the process of prophage induction after treatmentd with hydrogen peroxide. To test efficiency of this process, I have estimated the number of progeny phages appearing after prophage induction. I observed that both λΔ*orf63* and Φ24BΔ*orf63* mutations decreased the efficiency of lytic development and $\Phi24_B$ mutant additionally revealed a delay inthe release of the first progeny phages, in comparison to wild-type phage. Interestingly, during analyzing the kinetics of lytic

development of these phages (in one step growth experiment), I observed only a slight increase in phage burst size of the mutants. This undoubtedly indicates the impaired efficiency of prophage induction in the absence of functional Orf63 protein. Additionally, analysis of expression of phage genes during the process of induction of wild-type and *orf63*deletion prophages showed a decrease in the levels of gene expression in both mutants. In turn, analysis of genes expression performed in a complementation experiment, applying the host cells lysogenic withdeletion mutant and additionally carrying a plasmid with a copy of the deleted gene: pUC18_λ_*orf63* or pUC18_ Φ24B_*orf63*, allowed to observe the restoration of the expression of the analyzed genes to their original level only in the case of the λΔ*orf63* mutant. Since, this results are similar to those obtained for wild-type bacteriophage that has no changes in its genome, it can be assumed that the functions of the *exo-xis* region have been fully restored in this case. In contrast, in the experiment of complementation of the $\Phi 24_B$ *orf63* deletion , this effect was not observed. This might suggest that specific amount of Orf63 protein is required for accurate regulation of Stx phage lytic development. In this case, the amount was not reached. Aside from the effects mentioned above, the removal of the *orf63* sequence from λ and $\Phi 24_B$ phage genomes also resulted in an increase in efficiency of lysogenization. Moreover, survival rates of bacterial cells in populations infected with analyzed mutant phages were higher than those in experiments with wild-type phages. These results are consistent with those described above and definitely indicate the important function of Orf63 in the lytic development of lambdoid phages **[article no. 2]**. Interestingly, experiments carried out by other scientists (Blasche et al., 2013) using the yeast two-hybrid system showed that Orf63 interacted with the YqhC protein. This protein is a transcription factor, enabling the production of the enzyme (YqhD) responsible for decreasing the level of reactive oxygen species formed in the cell under oxidative stress conditions. It appears that Orf63 directly inhibits activity of YqhC, and indirectly inhibits YqhD, thus preventing the silencing of the cellular oxidative stress response. As a result, the level of reactive oxygen species increases in the cell, and the S.O.S. response is activated, stimulating prophage induction and phage lytic development **[article no. 2]**.

In the **article no. 3**, I showed that two other open reading frames from the *exo-xis* region, named *orf61* and *orf60a*, are also responsible for promoting the lytic development of phages λ and Stx. The analysis of their nucleotide sequences, as well as the analysis of the putative amino acid sequences of their potential products, indicated that they are highly preserved in the genomes of lambdoid phages (they show over 90% similarity). In this light, I decided to study their biological significance. For this purpose, I used recombinant prophages with the deletion of either *orf61* or *orf60a*. I observed that a lack of one of these sequences in the $\Phi 24_B$ phage

genome caused a delayed prophage induction and reduced the efficiency of lytic development after treatment with hydrogen peroxide as an inductor. Interestingly, I did not notice the delay of prophage induction after removing any of the analyzed sequences from the λ phage genome. Otherwise at certain times after induction of all the tested mutant prophages with hydrogen peroxide, I noticed higher survival of host cells. Next, I tested the significance of the introduced deletions on the infection process of bacterial cells by the studied phages. During the analysis of the intracellular life cycle of phages during the one infective cycle, I did not observe the significant differences between mutants and wild-type phages. However, I noticed an increase in bacterial cell survival upon infection with phages lacking the *orf60a* or *orf61* sequence, when compared to the wild type bacteriophage infection. In order to investigate the cause of this effect, I checked the effectiveness of the lysogenization of *E. coli* bacteria by mentioned phages. Interestingly, the lysogen formation process was more efficient for all analyzed mutants. On this basis, it was concluded that *orf61* and *orf60a* play a regulatory function, especially at the lysis-versus-lysogenization decision of development of lambdoid bacteriophages. Surprisingly, the efficiency of adsorption of $\Phi 24_B$ mutants lacking *orf60a* and *orf61* was lower compared to wild-type phage. This allows to speculate that the products encoded by these sequences are involved in the formation of phage virions, and their absence leads to the formation of virions unable to effectively adsorb on the host cell surface. In this light, the contribution of products encoded by *orf61* or *orf60a* in control of expression of genes coding for capsid proteins or their direct participation in intermolecular interactions during the virion formation process, is suspected. Although the detailed functions of these products are not yet known, the obtained results suggest that the analyzed open reading frames encode functional proteins important in the development of lambdoid phages. This conclusion was also supported by the confirmation of the presence of *orf60a*- and *orf61*-derived transcripts with the use of RT-qPCR (Bloch *et al.,* 2014). In addition, experiments using the ribosome profiling technique, performed to identify the network of proteins produced by bacteriophage λ, showed the presence of protein products encoded by *orf60a* and *orf61* in *E. coli* cells after prophage induction (Liu *et al.,* 2013). The presence of the Orf61 protein may also be indicated by the interaction of the *orf61* product with the *int* and *orf-314* genes' products, detected in the yeast two-hybrid system (Rajagopala *et al*., 2011). Unfortunately, attempts of our research team to purify the Orf60a and Orf61 proteins failed **[article no. 3]**.

The results presented in this work leads not only to the extension of theoretical knowledge about lambdoid phages (including phages carrying Shiga toxin genes), but also provide important information about the functions of the analyzed genes from the *exo-xis*

region. The obtained data confirm the important role of the *exo-xis* region and products encoded within it, in the regulation of the development of lambdoid phages, especially at the stage of 'lysis or lysogenia' decision. The three genetic elements analyzed by me*: orf63, orf61* and *orf60a* play important roles in the lytic development of lambdoid phages. Importantly, despite the high similarities between the analyzed sequences, detailed analysis of their significance in the development of phages λ and Stx showed some differences. This is another proof that the knowledge obtained as part of research carried out on the model bacteriophage λ, should not be directly extrapolated to Stx phages and it's experimental verification is required. In addition, this work evidence that, despite many years of research on the λ phage, it's genome still has uncharacterized regions of sequence that may have important biological significance. Determining of their function may provide relevant knowledge , and in the case of Stx phages, may also have practical benefits allowing to identify new molecular targets that could be important in the development of therapy against EHEC infections. The lack of effective drugs against EHEC means that the implementation of alternative therapy is currently extremely desirable.

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Articles included in the doctoral dissertation:

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