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Role of *orf73* in the development of lambdoid bacteriophages during infection of the *Escherichia coli* host

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Shiga toxin-producing *Escherichia coli* (STEC) is a group of pathogenic strains responsible for human infections that result in bloody diarrhea and hemorrhagic colitis, often with severe complications. The main virulence factors of STEC are Shiga toxins encoded by the *stx* genes located in genomes of Shiga toxin-converting bacteriophages (Stx phages). These bacterial viruses are clustered in the lambdoid bacteriophage family represented by phage λ. Here, we report that expression of *orf73* from the *exo-xis* region of the phage genome promotes the lysogenic pathway of development of λ and $\Phi 24_B$ phages. We demonstrated that the mutant phages with deletions of *orf73* revealed higher burst size during the lytic cycle. Moreover, survival rates of *E. coli* infected with mutant bacteriophages were lower relative to wild-type viruses. Additionally, *orf73* deletion negatively influenced the lysogenization process of *E. coli* host cells. We conclude that *orf73* plays an important biological role in the development of lambdoid viruses, and probably it is involved in the network of molecular mechanisms of the lysis-*vs*.-lysogenization decision.

Key words: Lambdoid bacteriophages, Shiga toxin-producing *Escherichia coli* (STEC), *exo-xis* region, lysis-*vs*.-lysogenization decision

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Abbreviations: EHEC, enterohemorrhagic *Escherichia coli*; HC, hemolytic colitis; HUS, hemolytic-uremic syndrome; m.o.i., multiplicity of infection; ORF, open reading frame; *p*, promoter; PFU, plaque-forming unit; RT-qPCR, Reverse Transcription Quantitative Polymerase Chain Reaction; STEC, Shiga toxin-producing *Escherichia coli*; Stx phages, Shiga toxin-converting bacteriophages

INTRODUCTION

Bacteriophages, also known as phages, are bacterial viruses that exist in the environment as free biological molecules. They are found in every environment (e.g. water, air, soil or waste water) and are considered the most abundant organisms on Earth (Clokie *et al*., 2011; Weitz *et al*., 2012). Interestingly, these bacterial killers, due to their specificity, have many potential applications not only in the fields of genetic and molecular biology (Węgrzyn & Węgrzyn, 2005; Kirsch & Comeau, 2008; Kutter and *et al*., 2015), but also in such areas as human therapy (Górski *et al*., 2018), veterinary (Squires, 2018), agriculture or food and industry control (Gutierrez *et al*., 2016; Svircev *et al*., 2018; Zachary *et al*., 2018). However, bacteriophages also have "the dark side". It is widely known that many bacterial virulence factors are encoded within phage genomes. Moreover, these viruses are classified as mobile genetic elements and for this reason they can play crucial roles in the evolution of microorganisms and creation of pathogenic profiles of many feared bacterial strains (Tinsley *et al*., 2006; Navarro and Muniesa, 2017). Such bacteria can be exemplified by Shiga toxin-producing *Escherichia coli* (STEC), with the most dangerous subset of these strains called enterohemorrhagic *E. coli* (EHEC) (Hunt, 2010). This group is exemplified by the highly pathogenic *E. coli* O157:H7 strains, and a new atypical *E. coli* O104:H4 serotype that caused the largest outbreak in Germany and worldwide in 2011, with 54 fatal cases (Bloch *et al*., 2011).

All STEC strains had acquired the *stx* genes (*stx1* and/or *stx2*), coding for Shiga toxins, by lysogenization with lambdoid bacteriophages, called Shiga toxin-converting bacteriophages (Stx phages), and which occur in these bacteria as prophages (Allison, 2007; Łoś *et al*., 2011). Their lifecycle and genome organization are similar to those of phage λ, which is considered as a model organism in microbiology and molecular biology. A 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. It is worth to mention that the decision whether to propagate lytically or to lysogenize *E. coli* depends on the environmental factors (temperature, nutrients' availability and multiplicity of infection) and the physiology of bacteria (Ptashne, 2004; Węgrzyn & Węgrzyn, 2005; Węgrzyn *et al*., 2012). During lysogenic development, which is one of the replication strategies, phage DNA is incorporated into the host chromosome, forming a prophage. At this stage, the viral genome is replicated together with the bacterial DNA, the majority of phage genes are silenced and no new virions are formed. At the molecular level, this is due to repression of the early lytic promoters, p_R and p_L, by the cI protein (Węgrzyn *et al*., 2012). However, the lysogenic stage is not permanent because when the host cell is endangered by stress conditions the phage developmental switch to the lytic cycle is observed. In this process, virus DNA is excised from the bacterial chromosome and is replicated separately. This leads to synthesis of phage-encoded regulatory and structural proteins, and as a consequence, an assembly of virions is achieved. The lytic development implies the

death of bacterial cells which allows phage progeny output (Węgrzyn *et al*., 2012).

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 the RecA-dependent S.O.S. regulon. In many cases, one common signal that induces lambdoid prophages is bacterial DNA damage, generated by many different factors, such as low pH, the iron ions, UV irradiation, antibiotics and hydrogen peroxide. Under such conditions, the RecA protein recognizes bacterial single-stranded DNA fragments and is activated to stimulate the self-cleavage of the cI repressor. This leads to initiation of phage lytic cycle through transcription from the early p_L and p_R promoters (Łoś *et al*., 2009; Węgrzyn *et al*., 2012; Szych *et al*., 2013; Licznerska *et al*., 2016a).

As the *stx* genes are located in the "late" region of phage genome, downstream of the $p_{R'}$ promoter, their expression occurs only after prophage induction and during the lytic development. Effective expression of the *stx* genes leads to synthesis of a large amount of Shiga toxins that are released to the human intestine, where they attack eukaryotic cells and block protein synthesis, leading to cell death (Law, 2000; Herold *et al*., 2005).

The primary symptom of infections of humans by STEC is hemorrhagic colitis (HC). In some cases, especially in children and elderly persons, it may result in various complications, including the hemolytic-uremic syndrome (HUS) with the most common symptoms such as the renal failure, anemia and thrombocytopenia. The mortality rate among patients with HUS is about 10%, but can be even higher, especially without treatment (Razzaq, 2006; Gyles, 2007).

For clinicians, one of the most frustrating aspects of managing STEC infection has been a lack of known effective treatment strategies that diminish the risk of HUS progression. Treatment of infection caused by STEC bacteria is also difficult because many drugs are prophage inducers which enhance expression of the toxin genes and thus enhance severity of the disease symptoms (Kimmitt *et al*., 2000; Gamage *et al*. 2004; Seran & Boedeker, 2008). *In vitro* analyses show that some antibacterial agents, such as quinolones and trimethoprim, dramatically increase both, the bacteriophage burst size and Shiga toxin production in certain STEC strains, and these observations have been confirmed in a murine model (Zhang *et al*., 2000). Moreover, clinical studies have indicated that antibiotics are not effective in reducing the duration of STEC infection or bloody diarrhea. Therefore, the role of antibiotics for the management of STEC infections still remains undecided after decades of debate and conflicting viewpoints in the literature. Due to serious doubts of using antibiotics, there is an urgent need to develop new drugs for treatment of STEC-infected patients. Proteins crucial for phage development seem to be good molecular targets for such a drug's action.

In the light of the facts described above, it is obvious that detailed understanding of phage gene expression regulation during development of lambdoid viruses is crucial for both, the basic knowledge about the pathogenicity of STEC bacteria and putative further work on treatment of infections caused by these pathogens.

Region of lambdoid phage DNA between the *exo* and *xis* genes, which is located in the central part of the genome (called the *exo-xis* region), is transcribed from the an early p_L promoter, active during the lytic cycle of lambdoid phages and switched off by cI protein during prophage maintenance. In case of phage λ, the *exo-xis* region consists of two recognized genes: *ea22* and *ea8.5*, open reading frame *55* (ORF) and four highly conserved ORFs, named: *orf60a*, *orf63*, *orf61* and *orf73* (Figure 1), whose functions are not yet clear. Comparatively, the *exo-xis* region of phage $\Phi24_B$, a representative of the Stx phages, contains additional ORFs, but there is no homolog of the *ea8*.5 gene which encodes the λ Ea8.5 protein (Fig. 1).

First speculations that the *exo-xis* region is involved in regulation at the stage of the phage decision whether to lysogenize the host cell or to enter into the lytic development, appeared in 2002. Sergueev and co-workers demonstrated that expression of some genes and ORFs, located between *exo* and *xis* on a defective prophage, causes inhibition of host DNA replication and help the

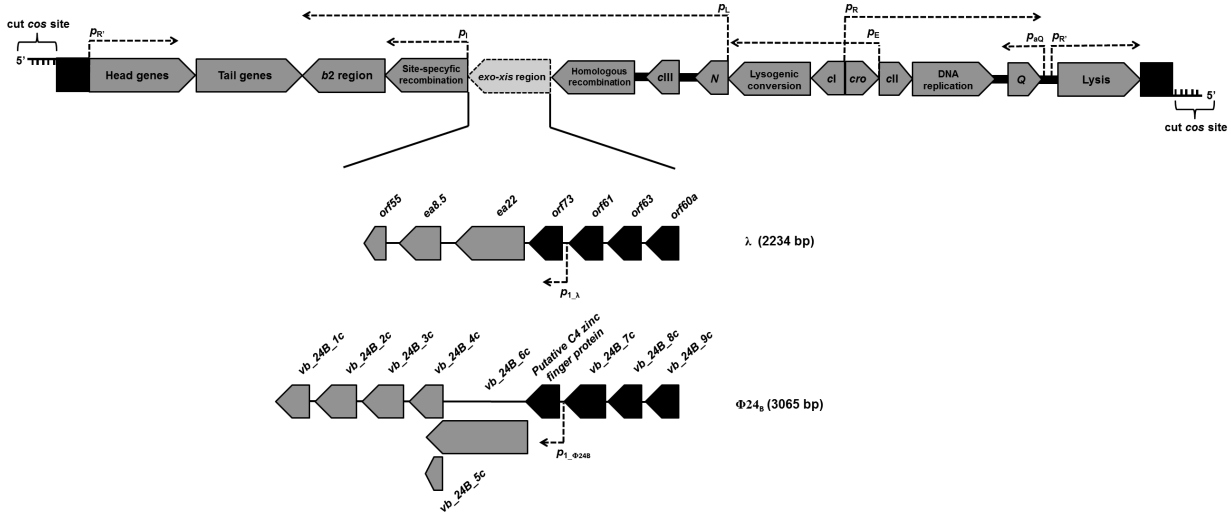

Figure 1. Location and composition of the *exo-xis* region of the lambdoid viruses: λ and $\Phi24_B$.
Black arrows represent four open reading frames: *orf60a*, *orf63*, *orf61* and *orf73* that are highly conserved in the genomes of the tested viruses (≥97% nucleotide sequence identity). Genes and ORFs with lower nucleotide identity or additional ORFs that occur in the *exo-xis* region of phage $\Phi24_B$ are marked by gray arrows. Directionality of transcription from promoters is indicated as thick, punctuated, dashed arrows. Localization of promoters predicted with BPROM: p_{1_λ} and $p_{1\_\Phi24B}$ is exactly the same in the case of λ and $\Phi24_B$ bacteriophages (Bloch *et al*., 2014).

virus to maximize its DNA replication during lytic development (Sergueev *et al*., 2002). However, no experimental data supporting such a hypothesis were published until 2008. In that year, the first results presenting evidence for the relation between the *exo-xis* region and phage development were shown (Łoś *et al*., 2008a). These data indicated that overexpression of the *exo-xis* region impairs the lysogenization process and contributes to a decreased transcription from the cII-stimulated promoters p_I, pa_Q and p_E, which are responsible for promotion of the lysogenic cycle (Łoś *et al*., 2008a). Moreover, a few years later it was demonstrated that more efficient induction of λ and $\Phi24_B$ prophages, induced by mitomycin C and hydrogen peroxide, occurred in cultures of host cells bearing a plasmid with the *exo-xis* region (Bloch *et al*., 2013). Following prophage induction, an increase in the 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 (Bloch *et al*., 2013 and Bloch *et al*., 2014). These observations were supported by a finding that deletion of the phage *exo-xis* region resulted in a dramatic decrease in the level of phage gene expression that are crucial for the lytic development of lambdoid viruses (Licznerska *et al*., 2016b).

In order to explain the role of individual genes or ORFs from the *exo-*xis region involved in the regulation of the lysis-*vs*.-lysogenization decision, Kwan and others (Kwan *et al*., 2013) reported that Ea8.5 contains a fused homeodomain/zinc finger fold, which suggests a regulatory role of this protein. Interestingly, overexpression of the *ea8.5* gene was responsible for the fuzzy plaque phenotype of the λ *cI*b2 phage, rapid virus development after prophage induction and repression of the cII-dependent promoters (p_I, p_{aQ}, p_E) (Łoś *et al*., 2008a; Bloch *et al*., 2014). On the other hand, expression of *orf60a*, *orf63* and *orf61* promotes the lytic pathway of lambdoid phage development (Dydecka *et al*., 2017 and Dydecka *et al*., 2018). Mutant phages with deletions of *orf60a* and *orf61* influenced the lysis-*vs*.-lysogenization decision, and impaired prophage induction provoked by different agents, such as mitomycin C and hydrogen peroxide. Moreover, during the induction process, the efficiency of lytic development of the tested mutants was lower relative to the control variants. What is important, the effects of the *orf60a* and *orf61* deletions were more spectacular for phage $\Phi24_B$ than for phage λ (Dydecka *et al*., 2018). Similar results were obtained for lambdoid phages with deletion of *orf63*. Dydecka and others (Dydecka *et al*., 2017) demonstrated that Orf63 is a small-size, functional protein (63 aa.) with two alpha helices, likely intertwined to form an oligomer. Functionally, the Orf63 protein probably participates in regulation of expression of crucial phage genes and ORFs from the *exo-xis* region during prophage induction. Moreover, lack of sequences of *orf63* in the genomes of λ and $\Phi24_B$ resulted in the delay in phage development after prophage induction and increased survival of the host cells during lytic cycle (Dydecka *et al*., 2017).

In this study, we have concentrated on the physiological role of *orf73* in regulation of the lambdoid phage development.

MATERIALS AND METHODS

Bacteria, bacteriophages and growth conditions. All *Escherichia coli* strains and lambdoid viruses used in this study are presented in Table 1. Bacterial cells were routinely cultured in the Luria-Bertani (LB) liquid medium at 30°C under aerobic conditions. The same broth, supplemented with 0.7% or 1.5% bacteriological agar, was used as a top or bottom agar, respectively. Bacteriophage suspensions were stored in TM buffer (10 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.2) at 4°C. The phage titration procedure was performed by using the standard double overlay method with some modifications. Briefly, 1 ml of the indicator *E. coli* MG1655 strain was mixed with 2 ml of top agar supplemented with $MgSO_4$ (λ and λΔ*orf73* phages) or $MgSO_4$ and $CaCl_2$ ($\Phi24_B$ and $\Phi24_B$Δ*orf73* phages), to a final concentration of 10 mM of each. The mixtures were poured onto Petri plates filled with bottom agar or bottom agar with sublethal concentration of chloramphenicol (2.5 μg/ml), according to a procedure described by Łoś and others (Łoś *et al*., 2008b). Supplementation of top agar with appropriate antibiotic was used to obtain visible plaques formed on a bacterial lawn by Stx phages. Afterwards, 2.5 μl of each serial dilution of phage lysate was spotted onto double-agar plate and incubated at 37°C for 16 h. Next day, plaques were counted and the PFU/ml value was calculated.

Bioinformatics analysis. Multiple sequence alignment of the nucleotide and amino acid sequences of *orf73* from λ phage (NC_001416) and Stx phages: $\Phi24_B$ phage (HM208303), 933W phage (NC_000924), VT2 Sakai phage (AP000422), Stx1 converting phage (NC_004913) and Stx2 converting phage II (NC_004914), was per-

Table 1. ***Escherichia coli*** **strains and bacteriophages used in this work.**

Bacterial strains or bacteriophages	Relevant genotype or description	References
E. coli strains		
MG1655	F– λ– *ilvG rfb-50 rph-1*	Jensen, 1993
MG1655 (λ)	MG1655 bearing λ prophage	Bloch *et al*., 2013
MG1655 (λΔ*orf73*)	MG1655 bearing λ prophage with deletion of *orf73*	Licznerska *et al*., 2016b
MG1655 ($\Phi24_B$)	MG1655 bearing $\Phi24_B$ prophage	Bloch *et al*., 2013
MG1655 ($\Phi24_B$Δ*orf73*)	MG1655 bearing $\Phi24_B$ prophage with deletion of *orf73*	Licznerska *et al*., 2016b
Bacteriophages		
λ	carries a frameshift mutation relative to Ur-lambda	Hendrix and Duda, 1992
λΔ*orf73*	λ phage with deletion of *orf73*	Licznerska *et al*., 2016b
$\Phi24_B$	$\Phi24_B$Δ*stx2*::*cat*	Allison, 2003
$\Phi24_B$Δ*orf73*	$\Phi24_B$ phage with deletion of *orf73*	Licznerska *et al*., 2016b

formed by using the ClustalW algorithm (https://www.genome.jp/tools-bin/clustalw).

Monitoring of phage lytic development during one round of infection of the *E. coli* host cells. Lytic cycle of the tested bacteriophages was studied in one-step growth experiments, according to the procedure described by Bloch and others (Bloch *et al.,* 2013). Host bacteria were grown in the LB medium at 30°C to A_{600}=0.2. Samples (10 ml) were centrifuged (2000×*g* for 10 min at 4°C), the supernatant was discarded and the bacterial pellet was suspended in 1/10 of the initial volume of LB medium supplemented with appropriate ions and 3 mM sodium azide. After 5-min incubation of the sample at 30°C, the phage particles were added to bacteria to m.o.i.=0.05. Phage adsorption was carried out at 30°C for 10 min. Then, the mixture was diluted tenfold in LB medium with 3 mM sodium azide and centrifuged (2000×*g* for 10 min at 4°C). To remove unadsorbed virions, the centrifugation procedure was repeated three times. In the next step, the suspension was diluted 1000-fold in LB medium pre-warmed to 30°C, supplemented with 3 mM sodium azide and aerated with shaking at the same temperature. The number of infective centers was estimated in the interval of 0–15 min after dilution by plating under permissive conditions. Samples taken at later times were cleared by vigorously shaking for 1 min with chloroform. Following the centrifugation step (2000×*g* for 5 min at 4°C), the number of phage progeny was estimated by plating on indicator *E. coli* MG1655 strain. Plates were incubated at 37°C overnight and then the burst size was calculated as a ratio of PFU/ml of the tested samples to the PFU/ml of infection centers.

Survival test of host bacteria after phage infection. To determine the survival rate of *E. coli* bacteria after phage infection, a previously published method was used (Dydecka *et al*., 2017). Bacterial cells were grown in LB liquid medium at 30°C to A_{600}=0.2. Samples (4 ml) were withdrawn and centrifuged (2000×*g* for 10 min at 4°C). The obtained pellets were suspended in 1.2 ml of LB medium supplemented with appropriate ions. In the next step, the phage lysate was added to the samples to m.o.i. of 1, 5, or 10, and following incubation at 30°C, serial dilutions in 0.85% sodium chloride were prepared. Afterwards, 40 µl of each dilution were plated onto bottom agar and incubated at 37°C overnight. The fraction of surviving bacteria was calculated in relation to the control variants treated with TM buffer (10 mM Tris-HCl, 10 mM $MgSO_4$, pH 7.2) instead of the phage lysate.

Lysogenization test of bacterial cells after phage infection. To estimate the percentage of lysogenic bacteria after bacteriophage infection, a procedure presented previously was used (Dydecka *et al*., 2017). Host bacteria were cultivated in LB liquid medium at 30°C to A_{600}=0.2. Aliquots of these cultures were centrifuged (2000×*g* for 10 min at 4°C) and the pellets were washed twice with the TCM buffer (10 mM Tris-HCl pH 7.2, 10 mM $MgSO_4$, 10 mM $CaCl_2$). In the next step, phage suspensions were added to bacterial samples to m.o.i.=1, 5 or 10. Following incubation of the mixtures at 30°C, serial dilutions were prepared and 20 µl of each was spread on LB agar plates. After overnight incubation at 37°C, the obtained bacterial colonies were passaged in each well of a 96-well plate filled with LB medium. The putative lysogens were shaken at 37°C to A_{600}=0.1. Then, the bacterial cultures were treated with ultraviolet radiation at 50 J/m² for 20 s and incubated for 2 h at 37°C. After the induction process, the putative lysogens were mixed with chloroform and centrifuged (2000×*g* for 10 min at 4°C). The water phase was spotted onto a double-layer LB agar. After overnight incubation at 37°C, the percentage of lysogens among survivors was determined and presented as a ratio of number of lysogens to all tested bacterial colonies.

The rate of adsorption of virions on the host cells. The efficiency of phage adsorption process was measured according to the procedure described previously, with some modifications (Bloch *et al.,* 2013). Bacteria were grown in LB liquid medium at 30°C to A_{600}=0.1. Samples (1 ml) were centrifuged (2000×*g* for 10 min at 4°C) and the pellets were washed twice with 0.85% sodium chloride. Finally, the pellets were suspended in 0.15 ml of LB medium supplemented with appropriate ions. Tested bacteriophages were added to the bacterial samples to m.o.i.=0.1. During incubation at 30°C, the phage titers were determined at specified times by using the double-layer LB agar method. Plates were incubated at 37°C for 16 h. Samples withdrawn immediately after addition of phage particles to the host cells (time 0) were considered as 100% of non-adsorbed viruses and other values were calculated relative to them.

Statistical methods. Each experiment was prepared in three independent, biological replicates. The variation among replicates was presented as error bars indicating standard deviation (S.D.). The significance of differences between mean values of two measured parameters was assessed by using *t*-test. Differences were considered significant when $p<0.05$ or $p<0.01$, and are marked on the figures by one or two asterisks, respectively.

RESULTS

The nucleotide sequences of *orf73* and amino acid sequences of its putative products are conserved among the family of lambdoid viruses

The *orf73* locus is placed between *orf61* and the *ea22* gene in the genome of λ and $\Phi24_B$ viruses. According to available data, this ORF remains under control of the early p_L and BPROM-predicted p_1 promoters (Fig. 1) (Bloch *et al*., 2014). As we demonstrated in Table 2, the nucleotide sequence of *orf73* is highly conserved among the group of lambdoid viruses, with the scores of similarities ≥97%. Moreover, we have also tested the identity

Table 2. Scores of pairwise alignments of the nucleotide sequences of *orf73* from the λ phage (NC_001416) and Stx phages: $\Phi24_B$ phage (HM208303), 933W phage (NC_000924), VT2 Sakai phage (AP000422), Stx1 converting phage (NC_004913) and Stx2 converting phage II (NC_004914).

The multiple sequence alignment was performed by using the ClustalW algorithm. Pairwise scores represent the percentage identity between two sequences, taking into account length of the alignment.

	λ	$\Phi24_B$	933W	VT2 Sakai	Stx1	Stx2_II
λ		97	97	97	97	97
$\Phi24_B$			99	99	99	99
933W				100	100	100
VT2 Sakai					100	100
Stx1						100
Stx2_II						

Table 3. Scores of pairwise alignments of the predicted amino acid sequences of Orf73 from the λ phage (NC_001416) and Stx phages: $\Phi24_B$ phage (HM208303), 933W phage (NC_000924), VT2 Sakai phage (AP000422), Stx1 converting phage (NC_004913) and Stx2 converting phage II (NC_004914).
The multiple sequence alignment was performed by using the ClustalW algorithm. Pairwise scores represent the percentage identity between two sequences, taking into account length of the alignment.

	λ	$\Phi24_B$	933W	VT2 Sakai	Stx1	Stx2_II
λ		97	97	97	97	97
$\Phi24_B$			100	100	100	100
933W				100	100	100
VT2 Sakai					100	100
Stx1						100
Stx2_II						

of the predicted amino acids sequences of the putative products of *orf73*. As we present in Table 3, the high level of similarity is kept at the protein level of Orf73 for all tested λ and Stx phages (≥97%). Taking into account the results of these comparisons, we suppose that *orf73* can be a true gene that encodes a real protein product.

Deletion of *orf73* influences phage infection and host survival

Previous studies indicated that either overexpression or deletion of the whole *exo-xis* region or particular genes and ORFs affect the lytic cycle of lambdoid bacteriophages after infection of *E. coli* bacteria under standard laboratory conditions (Łoś *et al.*, 2008a; Bloch *et al.*, 2013 and Bloch *et al.*, 2014; Licznerska *et al.*, 2016b; Dydecka *et al.*, 2017 and Dydecka *et al.*, 2018). Here, we demonstrate that in one-step growth experiment both lambdoid bacteriophages with the deletion of *orf73* present shorter latent period and more efficient intracellular lytic development relative to the wild-type viruses (Fig. 2). Interestingly, the latent period of wild-type λ phage was about 5 min longer relative to the λ deletion mutant (Fig. 2, panel A), while in the case of phage $\Phi24_B$ the first phage progeny appeared about 50 minutes later in comparison to $\Phi24_B$ with deletion of the *orf73* homolog (Fig. 2, panel B). Moreover, the average burst size values of λ (68±15 PFU/cell) and $\Phi24_B$ (25±7 PFU/cell) were several times lower relative to their deletion mutants, λΔ*orf73* (500 PFU/cell±60) and $\Phi24_B\Delta$*orf73* (159±38 PFU/cell) (Fig. 2).

We also found that the survival rates of bacterial cells in the population infected with λ and $\Phi24_B$ phages devoid of *orf73* were lower than those in experiments with wild-type lambdoid viruses under all tested m.o.i. conditions (Fig. 3). In accordance with the host survival experiments, we have also observed that efficiency of lysogenization was less effective in the absence of *orf73* at m.o.i.=5 and 10 (Fig. 4). Moreover, the differences between mutants and wild-type phages observed during the analyzed processes did not depend on adsorption of viruses on the surface of bacterial cells. We demonstrated that no significant effects of *orf73* deletion on this parameter could be found for the λ and Stx phages (Fig. 5).

All of these observations allowed us to propose that *orf73* can play an important role in phage development, particularly at the stage of the lysis-*vs.*-lysogenization decision.

DISCUSSION

Although principles of the mechanism of phage development regulation have been broadly described for cells lysogenized with bacteriophage λ (Ptashne 2004; Węgrzyn & Węgrzyn, 2005; Węgrzyn *et al.*, 2012), and despite recent reports providing information about this regulation in the Stx phages (Murphy *et al.*, 2008; Łoś *et al.*, 2012; Riley *et al.*, 2012), it appears that our knowledge about this process is still far from completeness. There is evidence that genes and ORFs from the *exo-xis* region of lambdoid viruses can be involved in the lysis-*vs.*-lysogenization decision (Łoś *et al.*, 2008a; Bloch *et al.*, 2013 and Bloch *et al.*, 2014; Licznerska *et al.*, 2016b, Dydecka *et al.*, 2017 and Dydecka *et al.*, 2018). Moreover, many bioinformatics and transcriptomic approaches (e.g. microarray or RNA-Seq analyses) have indicated a growing number of genes encoding small proteins (20–130 amino acids) that can play a variety of roles in the

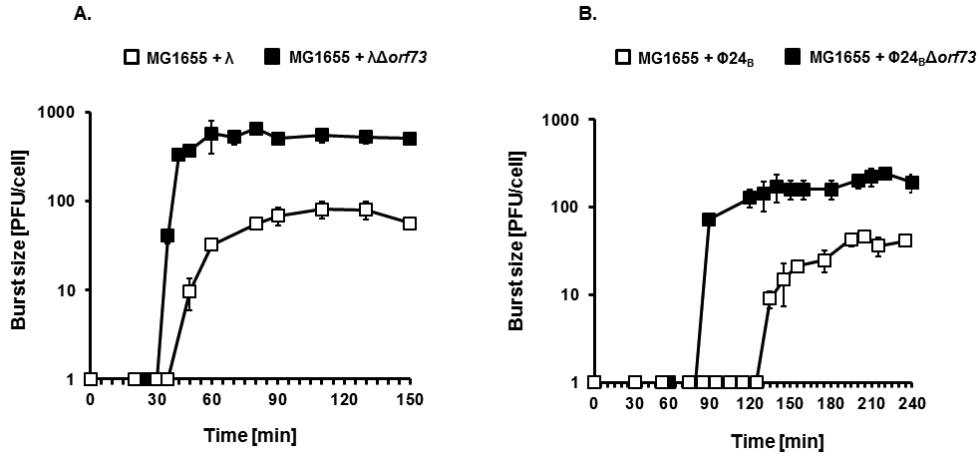

Figure 2. The intracellular development of λ (panel A, white squares), $\Phi24_B$ (panel B, white squares) and their recombinant mutants with deletion of *orf73* (panels A and B; black squares) following phage infection of the *E. coli* MG1655 host.
The presented results are mean values from three independent, biological experiments with error bars indicating S.D.

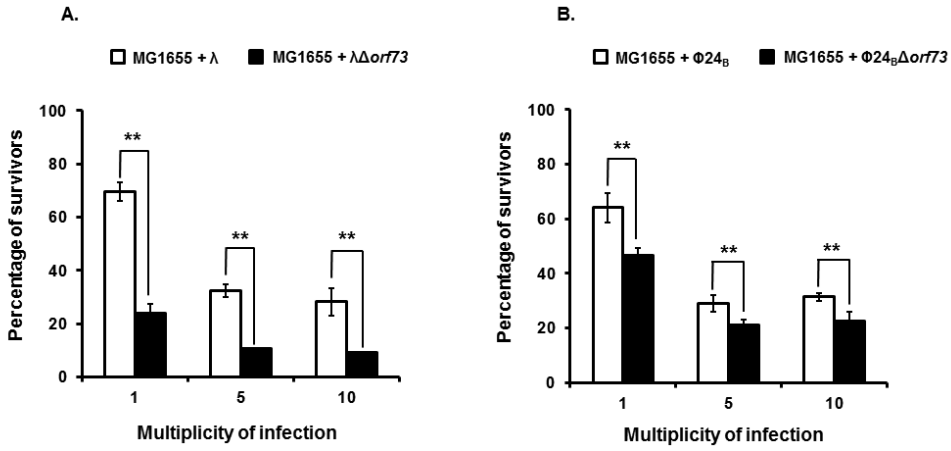

Figure 3. Effects of *orf73* deletion on survival of the host cells after infection with wild-type bacteriophages: λ (panel A, white columns) and $\Phi24_B$ (panel B, white columns) or their recombinant mutants (panels A and B; black columns).
Results are presented as mean values ± S.D. from three independent, biological experiments. A *t*-test was performed for results obtained at each m.o.i. The significance of differences between fractions of bacterial cells infected with wild-type phages and their deletion mutants is marked by asterisks, $p<0.01$ (**).

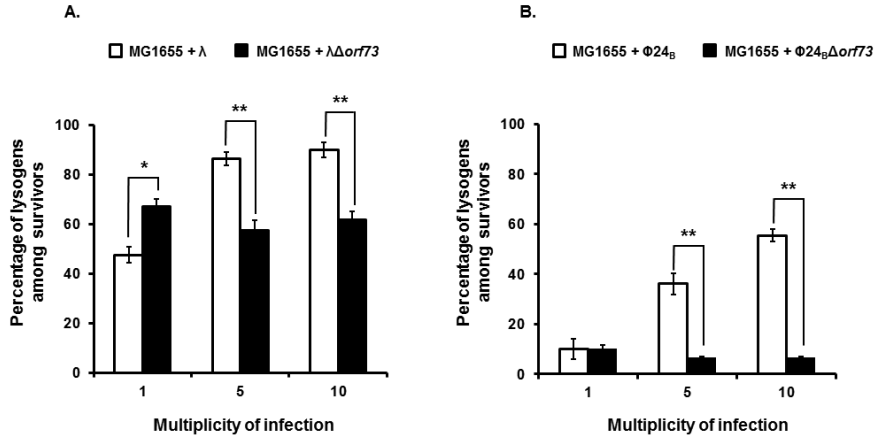

Figure 4. Effects of *orf73* deletion on lysogenization of *E. coli* cells with wild-type bacteriophages: λ (panel A, white columns) and $\Phi24_B$ (panel B, white columns) or their recombinant mutants (panels A and B; black columns).
Results are presented as mean values from three independent, biological experiments with S.D. indicated by error bars. A *t*-test was performed for results obtained at each m.o.i. Statistically significant differences between wild-type bacteriophages and their deletion mutants are marked by asterisks, $p<0.05$ (*) or $p<0.01$ (**).

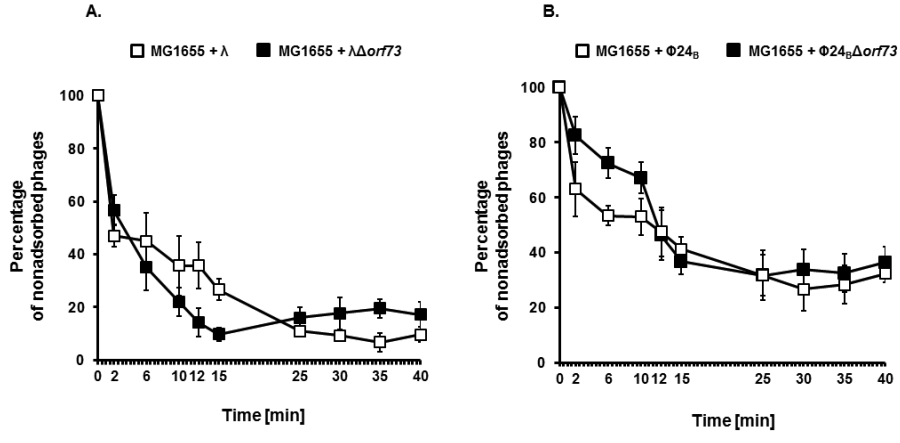

Figure 5. Rate of adsorption of lambdoid bacteriophages: λ (panel A, white squares) and $\Phi24_B$ (panel B, white squares) or their recombinant mutants (panels A and B; black squares) to the *E. coli* MG1655 host at m.o.i. of 0.1.
The presented results are mean values from three independent, biological experiments with error bars indicating S.D.

world of microorganisms (Hemm *et al.,* 2008; Ibrahim *et al.,* 2007; Hobbs *et al.,* 2011). Keeping these reports in mind, we would like to pay attention to the physiological role of *orf73* during infection process of *E. coli* with the λ and $\Phi24_B$ phages.

The sequence of *orf73* is located between *orf61* and *ea22* in the λ genome and it is also highly conserved among the Stx phages: $\Phi24_B$, 933W, VT2 Sakai, Stx1 and Stx2_II (Fig. 1 and Table 2). Our results from RT-qPCR analyses, as well as expression patterns of λ genome from ribosome profiling, show increased expression of *orf73* during phage λ development. Presence of the *orf73*-derived transcript was also confirmed by RT-qPCR during lifecycle of phage $\Phi24_B$ (Liu *et al.*, 2013; Bloch *et al.*, 2014). It is important to note that the level of *orf73* expression during infection or prophage induction in *E. coli* was comparable to that of the *ea22* gene. Moreover, *orf73* and *ea22* were expressed at significantly higher levels than the rest of the *exo-xis* region during $\Phi24_B$ prophage induction in cells treated with hydrogen peroxide. Interestingly, *in silico* analysis also predicted the existence of a newly detected, strong $p_{1\_\lambda/\Phi24B}$ promoter between *orf61* and *orf73*. This p_1 promoter, together with early p_L promoter, might control and enhance the expression of *ea22* and *orf73* (Fig. 1) (Bloch *et al.*, 2014). Therefore, it seems that the mechanisms of action and regulation of *orf73* and *ea22* can be similar.

Such observations allow us to suppose that *orf73* might be translated into an active polypeptide product and plays an important role in regulation of the lambdoid bacteriophage development, especially at the stage of the lysis-*vs.*-lysogenization decision. This hypothesis can be corroborated by the fact that the potential product of *orf73* contains a zinc finger fold. Such a structure, partially resembling those occurring in eukaryotic proteins involved in regulation of the tissue and organ development, may potentially interact with both, the nucleic acids and other proteins (Berg, 1990). Importantly, this domain is also characteristic for the λ Ea8.5 regulatory protein that probably interacts with bacterial proteins, including DnaA, DiaA or Had, and can also regulate transcription at the cII-dependent promoters which are involved in the lysis-*vs.*-lysogenization decision (Łoś *et al.*, 2008a; Kwan *et al.*, 2013).

In the light of results presented in this paper, we conclude that expression of *orf73* promotes the lysogenic pathway of the lambdoid bacteriophages' development. We observed that the mutant phages with deletions of *orf73* revealed lower efficiency of lysogenization of the host cells and higher level of burst size during the lytic cycle. Survival rates of *E. coli* bacteria in the population infected with deletion mutants were lower relative to the wild-type lambdoid phages. Moreover, the decision to propagate lytically did not depend on environmental factors, such as nutrients availability and multiplicity of infection.

Taking the above summarized results into consideration, we suggest that *orf73* is important, but not essential, in regulation of the lambdoid bacteriophage development. In contrast to *orf60a*, *orf63* and *orf61*, expression of *orf73* favors not the lytic, but the lysogenic cycle of the λ and Stx phages. However, the molecular mechanism of action of *orf73* still remains unclear. In addition, it seems that the products encoded in the *exo-xis* region strongly cooperate at the stage of the lysis-*vs.*-lysogenization decision.

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