The *ea22* gene of lambdoid phages: preserved prolysogenic function despite of high sequence diversity

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Abstract
The *exo-xis* region of lambdoid phages contains open reading frames and genes that appear to be evolutionarily important. However, this region has received little attention up to now. In this study, we provided evidence that *ea22*, the largest gene of this region, favors the lysogenic pathway over the lytic pathway in contrast to other characterized *exo-xis* region genes including *ea8.5, orf61, orf60a*, and *orf63*. Our assays also suggest some functional analogies between Ea22 and the phage integrase protein (Int). While it is unsurprising that Ea22 operates similarly in both *λ* and Stx phages, we have observed some distinctions that may arise from considerable sequence dissimilarity at the carboxy termini of each protein.

Keywords *Exo-xis* region · Lambdoid phages development · Shiga toxin-producing *Escherichia coli* (STEC)

Introduction
The recognition of Shiga toxin-producing *Escherichia coli* (STEC) bacteria as a public health problem took place in 1982 during an outbreak of hemorrhagic colitis (HC) tied to the consumption of contaminated hamburgers [1]. In 2011, the Shiga toxin-producing *E. coli* serotype O104:H4 was responsible for a serious epidemic in twelve European countries [2, 3].

The main virulence factors of STEC are Shiga toxins (Stx toxins), encoded by genes *stx1* and *stx2* located in genomes of bacteriophages that infect these bacteria [4]. Shiga toxin-converting bacteriophages (Stx phages) belong to the lambdoid family of phages, of which bacteriophage *λ* is the best investigated member [4–9]. All lambdoid phages indicate high similarities in the life cycle and organization of the genome. Depending on the intracellular conditions, lambdoid phages follow either a lysogenic or lytic developmental pathway leading to dormancy or the production of new viral particles, respectively [10–12]. The effective production of Shiga toxins and their release in human intestine occurs only
upon prophage induction and its further lytic development [13–15] suggesting that a therapeutic route may be found by studying the lysogenic-lytic decision further.

Despite over sixty years of research, not all genes in bacteriophage λ have been explored [16]. In other model systems, sequence comparisons are usually enough to fill in any knowledge gaps; however, in bacteriophage λ, this approach suffers due to a lack of similarity to reference proteins [17, 18]. Shiga toxin-converting bacteriophage Φ24B has rather small genome size (approximately 60 kb) carry 55% of sequences predicted to encode hypothetical proteins. Surprisingly, in its host, Escherichia coli bacterium (with genome of about 4600 kb), such sequences represent only 24% and the difference is even more remarkable when sizes of the both genomes are taken into consideration [19]. An imbalance between the amount of functional genomics studies conducted on Stx phages and their host bacteria, makes the functional characterization of Stx phage genomes challenging.

Among all lambdoid bacteriophages, the exo-xis gene region remained unknown for long time, not only in terms of the hypothetical proteins encoded within it but also their functional attributes. The early pL promoter, one of two major promoters repressed by cI protein during lysogeny, is also responsible for expression of exo-xis region genes [8, 16, 18]. In λ phage, the exo-xis region consists of three already tested open reading frames (ORFs) designated as orf60a, orf63, and orf61 and two recognized genes called ea22 and ea8.5 (Fig. 1). Except ea8.5, all of them as well as some other uncharacterized ORFs are found in Stx phages [20].

Genes within the exo-xis region were previously observed to repress transcription from λ cII-dependent promoters: pE, pL and pAQ that are known to promote lysogenic pathway [21]. Using a multicopy plasmid approach, Bloch and collaborators later revealed new features of exo-xis region including impaired lysogenization and more effective induction of prophages, both spontaneous and stimulated by various agents [22]. Furthermore, Licznerska and co-workers demonstrated that in the absence of the exo-xis region, the expression of critical Stx phage genes directing lytic development was decreased after prophage induction [23]. Together, these observations demonstrate that lambdoid phage development may be more complex than it was thought previously and that the exo-xis region serves a role in this process.

The analysis of individual genes of the exo-xis region has been instructive. For example, the ea8.5 gene contributed to significant repression of pAQ, a minor effect on pL, and negligible effect on the pE promoter [21]. These results suggested that ea8.5 affects the pL and pAQ promoters, though its influence is relevantly weaker than the whole exo-xis region. It seems that the ea8.5 product may be partially responsible for the negative regulation of pL and pAQ. However, the activity of these promoters also depends on at least one other factor located between exo and xis [21]. The structure of Ea8.5 is a hybrid of two common folds, a homeodomain and a zinc finger, each with a potential nucleic acid binding and protein–protein interaction function [24]. Apart from Ea8.5, the only other exo-xis region gene product that has been analyzed biochemically is Orf63, a small oligomeric protein consisting of two alpha helices. Functionally, orf63 represses expression of the major phage genes responsible for prophage induction thereby delaying the time and decreasing the efficiency of this process [25]. While gene products of orf60a and orf61 remain essentially uncharacterized from a biochemical viewpoint, they do serve important roles in the control of phage lytic development since their deletions significantly delay the induction of Stx prophage treated with hydrogen peroxide and reduce the efficiency of such process. Furthermore, deletion of either orf60a or orf61 enhances post-infection bacterial survival and promotes the formation of lysogens. As was observed for orf63, the effects of orf60a or orf61 deletions were more pronounced in the Stx phage Φ24B than in λ [26].

Our preliminary research on ea22 showed that deletion of this gene in both λ and Φ24B genomes did not affect phage titer, but promoted a rapid induction of mutant prophages after stimulation by UV irradiation. For other inducing agents, however, the effects were slight or absent [23]. Thus, we hypothesize that ea22 may play a different function during phage life cycle than other already studied exo-xis region genes.
Materials and methods

Bioinformatics

The translated amino acid sequences of ea22 from λ (NC_001416) and from three Stx phages: Φ24B
(HM208303), 933W (NC_000924), and P27 (KU238067) were submitted to the QUICK2D utility for secondary structural prediction, the CLUSTAL utility for alignment, and the MARCOIL/PCOILS utilities for coiled-coil prediction, all hosted on the HHPred server at https://toolkit.tuebingen.mpg.de [27]. The hairpin RNA structures were predicted using Mfold web server available at https://unafold.rna.albany.edu/?q=mfold. The RNA–RNA interaction was identified using IntaRNA software available at https://rna.informatik.uni-freiburg.de/IntaRNA.

Bacterial strains and bacteriophages

The bacterial strains and bacteriophages used in this work are presented in Table 1. Strains with deletion of gene ea22 or vb_24B_6c were constructed by using the λRed homologous recombination system following the procedure described previously [23]. Each deletion was confirmed by DNA sequencing (Genomed) and showed that there was no sequence changes in the other open reading frames from that region. Lysates of lambdoid bacteriophages were stored in TM buffer (10 mM Tris–HCl, 10 mM MgSO₄, pH 7.2) at 4 °C.

Growth conditions and bacteriological media

All experiments were performed in the Luria–Bertani (LB; Lab Empire) medium at 30 °C with agitation. The same LB medium, supplemented with 1.5% bacteriological agar (BTL), was used as a bottom agar. The adsorption of phage particles onto the surface of the host cells was conducted by the addition of 10 mM MgSO₄ (phage λ; Chempur) or 10 mM MgSO₄ and 10 mM CaCl₂ (Stx phages; Chempur) to the liquid LB medium. Titration of phage λ and its derivative was carried out by a standard double-layer agar assay with the top agar consisting of LB medium with 0.7% (w/v) bacteriological agar (BTL). The visualization of the plaques of Stx phages was analogous to the standard phage titration procedure, but additionally the bottom agar was supplemented with 2.5 µg/mL of chloramphenicol (Sigma-Aldrich) [28]. Plates were incubated at 37 °C overnight.

Table 1  Escherichia coli strains and bacteriophages

| Bacterial strains and bacteriophages | Genotype or relevant characteristics | References |
|-------------------------------------|-------------------------------------|------------|
| **E. coli strains**                |                                     |            |
| MG1655                             | F− λ− ilvG rfb-50 rph-1             | [43]       |
| MG1655 (λ)                         | MG1655 bearing λ prophage           | [22]       |
| MG1655 (λΔea22)                    | MG1655 bearing λΔea22 prophage      | [23]       |
| MG1655 (Φ24B)                      | MG1655 bearing Φ24BΔstx2::cat prophage | [22]   |
| MG1655 (Φ24BΔea22)                 | MG1655 bearing Φ24BΔsrx2::cat Δvb_24B_6c prophage | [23] |
| MG1655 (933W)                      | MG1655 bearing 933WΔsrx2::catGFP prophage | This work |
| MG1655 (P22)                       | MG1655 bearing P22Δsrx2::catGFP prophage | This work |
| MG1655 (P27)                       | MG1655 bearing P27Δsrx2::catGFP prophage | This work |
| MG1655 (P32)                       | MG1655 bearing P32Δsrx2::catGFP prophage | This work |
| **Bacteriophages**                 |                                     |            |
| λ                                   | Carries a frameshift mutation relative to Ur-lambda | [44] |
| λΔea22                             | λ phage with deletion of ea22 gene  | [23]       |
| Φ24B                               | Φ24BΔsrx2::cat in which stx genes were replaced with a chloramphenicol resistance gene (cat) | [45] |
| Φ24BΔea22                          | Φ24B phage with deletion of vb_24B_6c, the homolog of λ ea22 gene | [23] |
| 933W                               | 933WΔsrx2::catGFP in which stx genes were replaced with a chloramphenicol resistance gene (cat) and gene of green fluorescent protein (GFP) | [46] |
| P22                                | 22Δsrx2::catGFP in which stx genes were replaced with a chloramphenicol resistance gene (cat) and gene of green fluorescent protein (GFP) | [46] |
| P27                                | 27Δsrx2::catGFP in which stx genes were replaced with a chloramphenicol resistance gene (cat) and gene of green fluorescent protein (GFP) | [46] |
| P32                                | 32Δsrx2::catGFP in which stx genes were replaced with a chloramphenicol resistance gene (cat) and gene of green fluorescent protein (GFP) | [46] |
Transcriptional analysis of tested genes by quantitative real-time reverse transcription PCR (qRT-PCR) after bacterial infection with lambdoid phages

Bacterial host *E. coli* MG1655 was grown with shaking to OD of 0.3 (A600 nm) at 30 °C and from it, a 12 mL of sample was collected and centrifuged at 2000×*g* for 10 min at 4 °C. The pellet was washed once with 0.85% NaCl (Chempur) and suspended in 3 mL of LB supplemented with 10 mM MgSO₄ and 10 mM CaCl₂. After cooling the mixture on ice, phage particles were added to the bacterial sample at an m.o.i. of 1 and incubated for 30 min at 4 °C. Following this treatment, infected bacteria were transferred to a shaking incubator at 30 °C. After an appropriate period, 10⁹ bacterial cells were harvested and treated with 10 mM NaN₃ to inhibit the growth of the host. Total RNA was isolated using the High Pure RNA Isolation Kit (Roche Applied Science) following treatment with 2 U of TURBO DNase (Life Technologies) for 60 min at 37 °C as recommended by the manufacturer. The quantity of the total RNA sample was determined by UV absorbance using a Nanodrop spectrophotometer (Eppendorf) and the quality was determined electrophoretically. A cDNA preparation was obtained following the vendor’s protocol using 1.25 µg of RNA template. Transcriptor reverse transcriptase and random hexamer primers (Roche Applied Science). The cDNA preparation was then diluted tenfold and analyzed by qRT-PCR using a LightCycler 480 Real-Time PCR System [29]. Amplification was performed using following program: 95 °C for 5 min; 55 cycles of 95 °C for 10 s; 60 °C for 15 s; and 72 °C for 15 s. All oligonucleotide primers, listed in Table 2, were created by Primer3web version 4.0.0. and synthesized by Sigma-Aldrich or Genomed. The isocitrate dehydrogenase *icdA* was selected as a housekeeping gene [30]. The relative changes in gene expression were analyzed with LinRegPCR using the E-Method with efficiency correction [29].

**One-step growth curve**

One-step growth experiment was carried out as described previously with only a few modifications [29]. Briefly, bacteria were cultured in LB until early log-phase (2 × 10⁸ CFU/mL). Following that, a 10 mL sample was centrifuged (2000×*g*, 10 min, temp. 4 °C) and the pellet was suspended in 1 mL of LB supplemented with 3 mM NaN₃ (Sigma-Aldrich). Phage particles were added to *E. coli* MG1655 host to a multiplicity of infection (m.o.i.) of 0.05 and allowed to be adsorbed for 10 min at 30 °C. After incubation, the mixture was diluted tenfold in LB with 3 mM NaN₃ and centrifuged (2000×*g*, 10 min, 4 °C) to remove unadsorbed phages. This procedure was repeated three times. A 25 µL aliquot of this suspension was added to 25 mL of LB and incubated with shaking at 30 °C. At a series of time points, samples were withdrawn and titrated under permissive conditions. A free bacteriophage count was determined by using a double-layer agar plate method [28]. The burst size of lambdoid bacteriophages (PFU/cell) was calculated as a ratio of phage titer (PFU/mL) and the number of infection centers.

### Table 2: Primers used for qRT-PCR of *exo-xis* region and associated genes

| Primer name | Sequence (5′ → 3′) |
|-------------|-------------------|
| pF_λ_Stx_exo | TGGCGTACTGCACTAAACC |
| pR_λ_Stx_exo | TCATATCGGACGAAATAGTC |
| pF_λ_ea22   | GCAGTTCGACGATACTGGA |
| pR_λ_ea22   | AATGATGTCGAGACTGGG |
| pF_Φ24ba_ea22 | TCAGCAACATGGGCACTC |
| pR_Φ24ba_ea22 | GTGGTGAAAGCTGAGTTG |
| pF_P27_ea22 | ACATCGTGGCGGCACTGG |
| pR_P27_ea22 | CGGTTGTCCTTCTGATTT |
| pF_933W_P22_P32_ea22 | TCAGCAACATGGGCACTC |
| pR_933W_P22_P32_ea22 | AAGCTTGCCGTTGAGCTT |
| pF_λ_Stx_orf61 | TTACGCTGACCGCCATG |
| pR_λ_Stx_orf61 | CGGACATGGCGATCTTTCA |
| pF_λ_orf63 | ACCGTGTGTCCTCTAGCTG |
| pR_λ_orf63 | GTTACGGCGATCCCTTTC |
| pF_Stx_orf63 | GGGTCTTCTCTGTGCTT |
| pR_Stx_orf63 | TACGCCACCTCTTTACCAA |
| pF_λ_orf60a | GCATAACCGGCTCTGTTT |
| pR_λ_orf60a | CGGAAATCCACTGAAGCAC |
| pF_Stx_orf60a | CATAACCGGCTGCTTT |
| pR_Stx_orf60a | CGGAAATCCACTGAAGCAC |
| pF_λ_N | AAGCGCAAATCTCCCAGT |
| pR_λ_N | AGCGGTTGCTGAGATCTC |
| pF_Stx_N | TACGCCACCTCTTTACCAA |
| pR_Stx_N | AGCGGTTGCTGAGATCTC |
| pF_λ_cl | CACCTAGCAGGAAAGAAC |
| pR_λ_cl | TACGCCACCTCTTTACCAA |
| pF_Stx_cl | TACGCCACCTCTTTACCAA |
| pR_Stx_cl | TACGCCACCTCTTTACCAA |
| pF_λ_cro | ATGCGGAAAGAGTCAAGCCC |
| pR_λ_cro | TGGAAATGTAAGGCGGG |
| pF_Stx_cro | CGAACGTTCTGTGAGATGAG |
| pR_Stx_cro | GGTGGGAGTGGGTAGT |
| pF_λ_Q | ATCGACGTGTCGAGAAAGA |
| pR_λ_Q | ATCGACGTGTCGAGAAAGA |
| pF_Stx_Q | ATCGACGTGTCGAGAAAGA |
| pR_Stx_Q | ATCGACGTGTCGAGAAAGA |
| pF_λ_R | GGTTGGATGTGAAAGGCTT |
| pR_λ_R | GGTTGGATGTGAAAGGCTT |
| pF_Stx_R | GGTTGGATGTGAAAGGCTT |
| pR_Stx_R | GGTTGGATGTGAAAGGCTT |
| pF_E.coli_icdA | GTTACGGTTTTCGGGAGGCC |
| pR_E.coli_icdA | GTTACGGTTTTCGGGAGGCC |
Survival of E. coli bacteria after infection with lambdoid bacteriophages

The number of host bacteria survived after phage infection was estimated according to the procedure described previously with a few minor modifications [31]. E. coli MG1655 was cultured to A600 = 0.2 in LB at 30 °C. For each experimental repeat, a 1 mL sample was centrifuged (2000xg, 10 min, temp. 4 °C) and the pellet was washed twice with 1 mL of freshly prepared TCM buffer (10 mM Tris–HCl, 10 mM MgSO4, 10 mM CaCl2, pH 7.2). The bacterial pellet was then suspended in 1 mL of TCM buffer and phage lysate was added to a m.o.i. of 1.5, and 10. After an incubation at 30 °C, serial dilutions of each mixture were prepared in TCM buffer and 40 µL was spread onto plates containing LB supplemented with 1.5% agar. Plates were incubated at 37 °C overnight. The number of viable cells was calculated on the basis of counted colonies. The percentage of surviving bacteria after infection with phage mutants were calculated in relation to control experiment in which bacteria were infected with wild-type λ or Φ24B phages (assumed as 100%).

Measurement of the number of bacterial lysogens after bacteriophage infection

This assay and an estimate of the efficiency of prophage formation have previously been described [25]. In brief, 96 colonies of bacteria that survived phage infection were cultivated in a multi-well plate containing 200 µL of LB. Bacterial growth proceeded at 37 °C with shaking until an A600 of 0.2 was observed. Cultures were then treated with UV light at 50 J/m² for 20 s to induce prophages and incubated with shaking at 37 °C for 2 h. After the UV induction process, putative bacterial lysogens were mixed with 1% (v/v) chloroform and centrifuged at 2000xg for 10 min. A plate containing double-layer LB agar was spotted with 2.5 µL of each supernatant. Lysogens were indicated by the appearance of turbid lysis spots after overnight incubation at 37 °C [28]. In the first step, the efficiency of lysogenization was determined as a percentage of lysogens among all tested 96 bacterial colonies (Table 4). Each experiment was repeated three times. Next, the obtained results were used to calculate a percentage of lysogens per infected cell (Fig. 5). The number of cells in the bacterial population infected with one or more phages was determined by the Poisson distribution.

Results

Sequence characteristics

Since ea22 is the largest gene in the exo-xis region, its gene product presents the opportunity for a comprehensive sequence analysis as shown in Fig. 2a. Among the lambdoid phages selected for this study, Ea22 from the Stx phages P32 and P22 Stx are nearly identical to Ea22 from the Stx phage 933W and demonstrate 99% of similarity (Fig. 2). From a multiple sequence alignment shown in Fig. 2a, the amino-terminal portion is highly conserved among Stx phages but appears to be truncated in λ, leaving only one predicted α-helix, while the remaining Stx Ea22 proteins have three additional predicted β-strands. Given that the amino-terminal portion represents an autonomously folded domain, as shown schematically by a circle in Fig. 2c, λ ea22 could possibly be lacking a critical function that is retained in Stx phages. A coiled-coil region follows supported by the prediction of one or more long α-helices with a characteristic repeating heptad motif of hydrophobic and hydrophilic amino acids at key positions. Among the phages selected for comparison, there is considerable similarity in the first third of the coiled-coil region followed by some variabilities in the middle (one sequence for 933W/Φ24B or another for P27/h) and more divergent final third that is truncated in the λ Ea22 sequence. While all four Ea22 sequences are predicted to have an internal coiled-coil sequence, it remains unknown what oligomeric state the coiled-coil confers to each expressed protein. The C-terminal portion of each Ea22 sequence appears to be unique to each protein not only in terms of length but also in terms of predicted secondary structures suggesting that each Ea22 protein in this set presents a different domain with possibly different functional consequences. Despite the considerable functional divergence in the C-terminal portion of the sequence, all Ea22 proteins end with a common tripeptide consisting of a basic lysine or arginine followed by a glycine and glutamic acid (+GE) (Fig. 2). The significance of the +GE motif is unknown.

The ea22 gene expression during phage infection

Our analysis of ea22 began with a comparison of the expression levels between it and a set of genes that includes previously analyzed exo-xis region genes and other phage genes that are active during infection (Fig. 3). In accordance with our earlier studies [26, 29], all bacterial cultures were grown at 30 °C to optimize phage adsorption and DNA injection. Levels of mRNAs were measured by RT-qPCR 15 min after λ phage infection and 35 min after infection with Stx phages (Φ24B, 933W, P32, P27, P22). These post-infection times were chosen according to our prior knowledge of development of these phages in E. coli bacteria [26, 32]. As shown in Fig. 3, differences in the levels of phage mRNAs were observed between ea22 and exo-xis genes orf61, orf63, or orf60a. In the case of λ phage, the expression of ea22 is significantly lower than other tested genes from the exo-xis region. The opposite outcome, however, was observed for the
Stx phages, and notably, *ea22* expression level was the highest among all phage genes tested. The lower expression of both early (*N*, *cI*, *cro*) as well as middle or late phage genes (*Q*, *R*), than this observed in *ea22*, may suggest that *ea22* is active during the earliest stages of phage infection. This effect is particularly evident in phages P32, P27, and P22 in which the level of *ea22* expression significantly exceeds the low and surprisingly equal level of expression of other analyzed genes. Our previous report indicated that the tested phage genes, both early and late, revealed different levels of expression which increased during the time-course infection experiment [29]. In that infection assay, which differ in the applied m.o.i. = 5 [29], the expression of early genes *N* and *cro* reached the highest values in 10–20 min after infection with λ, or in 30–40 min after infection with Stx phage Φ24B. Importantly, Φ24B showed kinetics of development, following infection of *E. coli* cells, similar to those of 933W, P32, P27, and P22 [32]. In this light, the equal level of expression of the studied genes (except *ea22*), observed in this work, may indicate that their efficient expression have not started yet under these conditions.

**Effect of *ea22* deletion on phage development and host survival**

To be consistent with previous developmental studies, that we have performed on *exo-xis* region genes [25, 26], we also selected λ and Φ24B as representative Stx− (λ) or Stx+ lambdoid phages in this report. Importantly, these two phages show the highest Ea22 sequence divergency (Fig. 2) and among the Stx+ phages, Φ24B offers the practical benefits of stable titer of the phage lysate during storage, in addition to developing efficiently in *E. coli* bacteria at 30 °C. A growth temperature 30 °C was followed according to our previous studies [25, 26, 29] to slow down bacterial metabolism and the rate of phage propagation.

In a ‘one-step’ growth experiment, we observed that both λ and Φ24B phages lacking the *ea22* gene presents amino- and carboxy-terminal regions that may be functionally important individually or constitute independent protein domains. Pairwise scores shown as the number of identities between the two sequences, divided by the length of the alignment, and represented as a percentage. A schematic representation of the sequences to highlight the distinctiveness of the amino- and carboxy-terminal regions and a penultimate R/KGE sequence of unknown significance.
shorter latent period, however, produced more progeny per infected cell and thus caused a more rapid lysis of the bacterium than their wild-type counterparts. These distinctions were more pronounced for phage Φ24B, suggesting that Stx phages may possess additional factors that synergize the effects of ea22 (Table 3).

As shown in Fig. 4, host survival after infection was lower for ea22 mutant phages with the overall effect being greater for the Stx phage Φ24.

Considering the formation and maintenance of lysogens, the overall efficiency of this process was reduced over a tenfold range of m.o.i. in λ ea22 phage mutants (Table 4,

\[ \text{Expression patterns of selected genes measured by RT-qPCR from } \lambda \text{ and five Stx phages (Φ24B, 933W, P32, P27, P22) upon infection of } E. coli \text{ MG1655 host at m.o.i. of 1. Data represent mean values from three independent experiments with error bars indicating SD. The significance of differences between levels of mRNAs derived from ea22 and other genes of the } \text{exo-xis region are observed and marked by asterisks } P < 0.05 (\ast) \text{ or } P < 0.01 (\ast\ast) \text{ or } P < 0.001 (\ast\ast\ast). \]
Interestingly, a different situation was observed in the case of Φ24B phage. At m.o.i. 1 and 5, infection with Φ24B mutant phage resulted in similar level of lysogens that was respectively higher (1) and lower (5) in comparison to wild-type phage. The opposite effects observed at both m.o.i. values were unexpected, though almost constant level of lysogens generated by the deletion mutant was also surprising. Importantly, this level of lysogens was persisted at the highest m.o.i. 10. It seems that the deletion of ea22 gene alleviates the effect of increasing m.o.i. Again these effects were more pronounced for phage Φ24B.

Discussion

The focus of this investigation was ea22, a relatively large gene in the exo-xis region that is common to both λ and Φ24B, a phage that we have used in this and prior studies as a representative of the Stx phages. From developmental assays of ea22 mutant phages, we have observed that unlike other exo-xis genes such as orf60a, orf63, and orf61, expression of ea22 favors the lysogenic over the lytic pathway and acts early in development. As ea22 from both λ and Φ24B is considered to resist lytic development, Stx phages may possess additional genes that work in conjunction with ea22 to produce stronger effect than what we observed for λ bacteriophage. While two-hybrid studies of phage–host [33] and phage–phage [34] proteins failed to identify any interactions involving Ea22, they could have been impeded by low expression levels of one partner, weak interactions, the possibility that Ea22 acts in a multiprotein complex, or the possibility that Ea22 is a nucleic acid binding protein.

We suppose, ea22 may act as key regulator of phage lysis vs. lysogeny decision in response to changing ratio of phage particles to bacterial cells. Although the presence of ea22 favored lysogeny in most cases, the only deviation was observed in Stx phage at the lowest applied m.o.i. Based on it, we speculate that there is a putative additional regulation at m.o.i value = 1 that is considered as low and favorable for the lytic development of phages. It appears that additional factor(s) may interact with ea22 under such conditions, abolish its action and even deepen the lytic response. Importantly, this additional regulation seems to be used only by selected phages. Taken together, ea22 serves a role in decision to choose lytic or lysogenic pathway with the observed effects on phage development and host survival being greater for Stx phage.

Table 3 Parameters of the intracellular development of wild-type phages or their ea22 deletion mutants in E. coli MG1655 bacteria

| Strain/phage       | Latent period | Burst sizea |
|--------------------|---------------|-------------|
| MG1655+λ           | 35 min        | 68 ± 15     |
| MG1655+λΔea22      | 30 min        | 96 ± 23     |
| MG1655+Φ24B        | 135 min       | 25 ± 7      |
| MG1655+Φ24BΔea22   | 85 min        | 110 ± 33    |

*The burst size of lambdoid bacteriophages (PFU/cell) was calculated as a ratio of phage titer (PFU/mL) and the number of infection centers. The presented values were estimated at 90 min for λ or at 180 min for Φ24B of phages development in E. coli bacteria.

Table 4 Percentage of lysogens among survived E. coli MG1655 bacteria infected with wild-type phages or their ea22 deletion mutants

| Strain/phage       | Efficiency of lysogenization (% of lysogens among survivors) |
|--------------------|---------------------------------------------------------------|
|                    | m.o.i. = 1 | m.o.i. = 5 | m.o.i. = 10 |
| MG1655+λ           | 48 ± 3    | 63 ± 3    | 90 ± 3     |
| MG1655+λΔea22      | 23 ± 7    | 68 ± 8    | 60 ± 5     |
| MG1655+Φ24B        | 10 ± 3    | 35 ± 6    | 59 ± 7     |
| MG1655+Φ24BΔea22   | 19 ± 5    | 25 ± 11   | 25 ± 7     |

Fig. 5). Interestingly, a different situation was observed in the case of Φ24B phage. At m.o.i. 1 and 5, infection with Φ24B mutant phage resulted in similar level of lysogens that was respectively higher (1) and lower (5) in comparison to wild-type phage. The opposite effects observed at both m.o.i. values were unexpected, though almost constant level of lysogens generated by the deletion mutant was also surprising. Importantly, this level of lysogens was persisted at the highest m.o.i. 10. It seems that the deletion of ea22 gene alleviates the effect of increasing m.o.i. Again these effects were more pronounced for phage Φ24B.
in the N-terminal part of all the analyzed Ea22 protein sequences. Interestingly, a large number of the identified phage integrases (including λ Int) have extended coiled-coil domains which on the one hand, facilitate association and stabilization of the initial complex of recombination, and on the other hand, prevent prophage excision in the absence of the main excision protein [35].

The sequence divergence also extends to the expression patterns of ea22 in λ and Stx phages during infection of E. coli host. With reference to λ phage, the level of mRNA for ea22 is significantly lower when compared with the number of transcripts for orf60a, orf63, and orf61, whereas in Φ24B and other Stx phages, we observe the opposite relationship. In fact, the level of ea22 gene expression in Stx phages (especially in P32, P27, and P22) was significantly higher than the expression of other tested genes (even the early genes like, N and cro). Surprisingly, in opposite to ea22, the other genes (both early and late) were expressed at almost equal level. As we know from previous report, Stx phage genes present different levels of expression which increase during time-course infection experiment [29]. Due to this, we suspect that the efficient expression of early genes, e.g., N and cro have probably not started yet in the tested phages P32, P27, and P22. Importantly, at the same time, the level of expression of ea22 was high, suggesting that despite belonging to the pl operon, the ea22 gene might be expressed earlier and regulated independently of the studied phage genes. Above that, the expression of two other phage genes ea8.5 and int have been analyzed by us previously in the frame of similar but not exactly the same infection experiment [29]. The obtained results indicate that the ea8.5 presents a level of expression similar to that observed for ea22 leading us to speculate that ea22 has a similar mechanism of regulation.

On the other hand, expression of int was decreased compared to most of the analyzed genes from pl operon [29]. This was possible due to the occurrence of double control of the int expression during phage infection. As shown previously, product of the int gene is responsible for an integrative recombination of phage and bacterial DNA during lysogenic pathway and works in conjunction with Xis to achieve excision of prophage DNA after induction [36]. The int gene is located near the ea22 gene and is transcribed from both its own promoter, pl, and the leftward major pl promoter [37]. The pl promoter is positively regulated by cII protein which operates at the early stage of lysogenic infection. Shortly after making the decision to transition into a lytic cycle, when pl is inactive either due to the absence of cII [38], or by other exo-xis region proteins including Ea8.5 [21], int expression may still occur via pl [39]. Looking for convergence in the regulation of the int and ea22 genes expression, we came across the data from a comprehensive ribosomal profiling study of phage genes during early lytic infection. These data allowed us to identify int as a gene with a similar to ea22 profile and level of expression in the first twenty minutes of temperature-dependent prophage induction [39]—Fig. 1. In addition, upstream of the λ and Φ24B ea22 genes, there was a predicted but never investigated promoter named p1 [29]. Interestingly, the p1 promoter, like the well-established pl promoter, also contains binding sites for host RNA polymerase sigma factor 70 (RpoD17) and two host arginine-sensitive regulators ArgR and ArgR2, as found by BPROM software [29]. Undoubtedly, at this stage of knowledge, we cannot exclude that similar to that observed in int, the double control of ea22 expression also exists. However, further research are needed to confirm the activity of p1 promoter and to explain the mechanism of this regulation.

![Graph showing percentage of lysogens per infected cell](image-url)
The last aspect of this discussion focused on the physiological significance of ea22 expression. We showed that in contrast to other exo-xis genes (orf60a, orf63 and orf61), expression of ea22 favors the lysogenic over the lytic pathway. Wild-type λ and Φ24'th phages revealed higher efficiency of lysogenization of bacterial cells and lower efficiency of progeny phage production during the lytic cycle, when compared with Δea22 mutants. Interestingly, differences between wild-type and mutant phages were more pronounced in Φ24'th than in λ. In the context of ea22 gene expression, its disparate function relative to other exo-xis region genes, and some similarities in its mechanism of regulation to phage integrase, there is a possibility that Ea22 navigates the lysogenic-lytic decision by working in concert with the phage integrase and is dependent on the physiological state of the bacterial cell. It is worth noting that the function of ea22 that we have proposed is different from earlier assumptions. In one of previous reports, authors concluded that Ea22 from λ is similar to Ehly 2 protein that is associated with an enterohemolysin 2 activity and encoded by phage C3208 in E. coli O26:H11 [40, 41]. This finding was based on the sequence similarity that was estimated at level of 35% [40]. According to the work of Rost and collaborators, such a level of identity is considered as result of twilight zone that is difficult to interpret [42]. In addition, the location of the ea22 gene in the early region of the phage genome does not indicate on its enterohemolysin activity. Importantly, there were no other premises that indicated on similarity of Ea22 to Ehly2.

Conclusion

We have presented a series of investigation that appear to show that, Ea22 has a different function than previously assumed. Unlike other analyzed genes of the exo-xis region [30, 31], ea22 is a functional gene whose product acts to favor the lysogenic state over lytic infection, possibly by the kind of host protein partnerships it makes. The regulation of ea22 seems to be distinct from other exo-xis region genes, and thus more similar to the int gene. Using ea22 deletion mutants in λ and several Stx phages including Φ24'th, we have observed developmental differences that may arise due to a divergent carboxy-terminal sequence in each protein. Our work to date suggests that ea22 represents an important new gene to study aspects of lysogenic-lytic decision. Based on this, Ea22 could serve as a new target for potential therapy against STEC infections. Its prolysogenic effect could be used in the future to inhibit the lytic development of Stx phages and thereby to limit the production of dangerous to human health Shiga toxins.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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References

1. Riley LW, Remis RS, Helgerson SD et al (1983) Hemorrhagic colitis associated with a rare Escherichia coli serotype: N Engl J Med 308:681–685. https://doi.org/10.1056/NEJM198309233081203
2. Muniesa M, Hammerl JA, Hertwig S et al (2012) Shiga toxin-producing Escherichia coli O104:H4: a new challenge for microbiology. Appl Environ Microbiol 78:4065–4073. https://doi.org/10.1128/AEM.00217-12
3. Bloch SK, Felczykowska A, Nejman-Faleńczyk B (2012) Escherichia coli O104:H4 outbreak– have we learnt a lesson from it? Acta Biochim Pol 59(4), 483–488. https://doi.org/10.18388/abp.2012_2081
4. Nataro JP, Kaper JB (1998) Diarrheagenic Escherichia coli. Clin Microbiol Rev 11:142–201
5. Sandvig K, van Deurs B (1996) Endocytosis, intracellular transport, and cytotoxic action of Shiga toxin and ricin. Physiol Rev 76:949–966. https://doi.org/10.1152/physrev.1996.76.4.949
6. Karmali MA, Steele BT, Petric M et al (1983) Sporadic cases of haemolytic-uraemic syndrome associated with faecal cytotoxin and cytotoxin-producing E. coli in stools. Lancet 1:619–620. https://doi.org/10.1016/s0140-6736(83)91795-6
7. Mizutani S, Nakazono N, Sugino Y (1999) The so-called chromosomal verotoxin genes are actually carried by defective prophages. DNA Res 6:141–143. https://doi.org/10.1093/dnares/6.2.141
8. Besser RE, Griffin PM, Slutsker L (1999) Escherichia coli O157:H7 gastroenteritis and the hemolytic uremic syndrome: an
emerging infectious disease. Annu Rev Med 50:355–367. https://doi.org/10.1146/annurev.med.50.1.355
9. Schmidt H (2001) Shiga-toxin-converting bacteriophages. Res Microbiol 152:687–695. https://doi.org/10.1016/S0923-2508(01)01429-9
10. Węgrzyn G, Węgrzyn A (2005) Genetic switches during bacteriophage λ development. Prog Nucleic Acid Res Mol Biol. 79:1–48. https://doi.org/10.1016/S0079-6603(04)79001-7
11. Piashne MA (2004) Genetic switch: phage lambda revisited, 2nd edn. Laurel Hollow, NY, Cold Spring Harbor Laboratory Press
12. Kędzierska B, Szambowska A, Herman-Antosiewicz A et al (2007) The C-terminal domain of the Escherichia coli RNA polymerase subunit plays a role in the CI-dependent activation of the bacteriophage pM promoter. Nucleic Acids Res 35:2311–2320. https://doi.org/10.1093/nar/gkm123
13. Wagner MK, Neely MN, Zhang X et al (2001) Role for a phage promoter in Shiga toxin 2 expression from pathogenic Escherichia coli strain. J Bacteriol 183:2081–2085. https://doi.org/10.1128/JB.183.6.2081-2085.2001
14. Wagner PL, Livny J, Neely MN et al (2002) Bacteriophage control of Shiga toxin 1 production and release by E. coli. Mol Microbiol 44:957–970. https://doi.org/10.1046/j.1365-2958.2002.02950.x
15. Waldor MK, Friedman DI (2005) Phage regulatory circuits and virulence gene expression. Curr Opin Microbiol 8:459–465. https://doi.org/10.1016/j.mib.2005.06.001
16. Węgrzyn G, Licznerska K, Węgrzyn A (2012) Phage λ—new insights into regulatory circuits. Adv Virus Res 82:155–178. https://doi.org/10.1016/B978-0-12-394621-8.00016-9
17. Hatfull GF (2008) Bacteriophage genomics. Curr Opin Microbiol 11(5):447–453. https://doi.org/10.1016/j.mib.2008.09.004
18. Hatfull GF (2015) Dark matter of the biosphere: the amazing world of bacteriophage. Divers J Virol 89:12745–12755. https://doi.org/10.1128/JVI.02495-13
19. Smith DL, Rooks DJ, Fogg PC et al (2012) Comparative genomics of Shiga toxin-converting bacteriophages. BMC Genomics 16:13–311. https://doi.org/10.1186/1471-2164-13-311
20. Sergueev K, Court D, Reaves L et al (2002) E. coli cell-cycle regulation by bacteriophage λ. J Mol Biol 324:297–307. https://doi.org/10.1006/jmbi.2001.6311. https://doi.org/10.1078/1438-4221-00176
21. Łoś JM, Łoś M, Wegrzyn A et al (2008) Role of the bacterio-merase subunit in λ development. Proc Natl Acad Sci USA 79:238–242. https://doi.org/10.1073/pnas.79.2.238
22. Rajagopala SV, Casjens S, Uetz P (2011) The protein interaction network of bacteriophage lambda with its host, Escherichia coli. J Virol 87:12745–12755. https://doi.org/10.1128/JVI.02495-13
23. Gupta K, Sharp R, Yuan JB et al (2017) Coiled-coil interactions mediate serine integrase directionality. Nucleic Acids Res 45:7339–7353. https://doi.org/10.1093/nar/gkx474
24. Campbell AM (1992) Chromosomal insertion sites for phages and plasmids. J Bacteriol 174:7495–7499. https://doi.org/10.1128/JB.174.23.7495-7499.1992
25. Guaneros G, Montañez C, Hernandez T et al (1982) Posttranscriptional control of bacteriophage λ int gene expression from a site distal to the gene. Proc Natl Acad Sci USA 79:238–242. https://doi.org/10.1073/pnas.79.2.238
26. Schindler D, Echols H (1981) Retroregulation of the int gene of bacteriophage lambda: control of translation completion. Proc Natl Acad Sci USA 78:4475–4479. https://doi.org/10.1073/pnas.78.7.4475
27. Liu X, Jiang H, Gu Z et al (2013) High-resolution view of bacteriophage lambda gene expression by ribosome profiling. Proc Natl Acad Sci USA 110:11928–11933. https://doi.org/10.1073/pnas.1309739110
28. Plunkett G, Rose DJ, Durfee TJ et al (1999) Sequence of Shiga toxin-converting bacteriophage Ф24B. Science 285:1 is a prototype phage for dissemination of the Shiga toxin-forming bacteriophage λ. Proc Natl Acad Sci USA 78:4475–4479. https://doi.org/10.1073/pnas.78.7.4475
29. Bloch S, Neiman-Faleńczyk B, Dydecka A et al (2014) Different roles of the bacterio-merase subunit in λ development. Proc Natl Acad Sci USA 79:238–242. https://doi.org/10.1073/pnas.79.2.238
30. Allison HE, Sergeant MJ, James CE et al (2003) Immunity profiles of wild-type and recombinant Shiga-like
toxin-encoding bacteriophages and characterization of novel double lysogens. Infect Immun 71:3409–3418. https://doi.org/10.1128/iai.71.6.3409-3418.2003

46. Gamage SD, Patton AK, Hanson JF et al (2004) Diversity and host range of Shiga toxin-encoding phage. Infect Immun 72:7131–7139. https://doi.org/10.1128/IAI.72.12.7131-7139.2004

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