“The morphological and molecular characterization of bacteriophage vB_Eco4M-7, virulent to the pathogenic *Escherichia coli* O157:H7”

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Escherichia coli is a Gram-negative bacteria that belongs to the *Enterobacteriaceae* family. The vast majority of *E. coli* strains exist as a commensals in the gut of humans and warm-blooded animals. These microorganisms play a crucial role in food digestion, provide resistance against pathogenic bacteria and take part in the production of vitamins from B and K groups [1]. However, some isolates among *E. coli* bacteria have the potential to cause severe diseases, which is probably associated with acquisition of the virulence determinants through the horizontal gene transfer (HGT) [2]. This process leads to creation of new groups of pathogenic bacteria causing various symptoms in humans. Among them, Shiga toxin-producing *E. coli* (STEC) strains, with its subgroup - enterohemorrhagic *E. coli* (EHEC), appear to be one of the most dangerous pathogens [3].

The main virulence factors of STEC are Shiga toxins. These proteins consist of a single A subunit and five B subunits. The B pentamer of this protein is responsible for recognizing the globotriaosylceramide (Gb3) receptor, located in the host cell membranes. Following endocytosis and retro-translocation from Golgi apparatus to endoplasmic reticulum, the enzymatically active A subunit is liberated from the B pentamer by a partial proteolysis, and delivered to cytoplasm where it cleaves an adenine nucleotide from 28S rRNA within the 60S ribosomal subunit. Such a modification leads finally to the inhibition of protein synthesis and cell death [4].

It is wort to mention, that Shiga toxins are encoded by *stx* genes, located in the genomes of Shiga toxin-converting bacteriophages (Stx phages). These temperate viruses occur in STEC cells in the form of prophages. During the lysogenic cycle, the expression of majority of phage genes, including *stx* genes, is silenced. However, various external factors, like UV irradiation, EDTA, high hydrostatic pressure, low pH, hydrogen peroxide or some DNA-binding antibiotics may provoke the induction of Stx prophages. The consequence of this process is excision of phage DNA from the chromosome of host bacteria and initiation of the lytic development. At this stage, besides expression of genes coding for phage regulatory and structural proteins, *stx* genes are also activated. This leads to production of a large amounts of Shiga toxins which are released to the intestine after phage-mediated lysis of the host cell. Then, Shiga toxins reach to the lumen of blood vessels and may be transported on, or within, blood cells in the circulation to target organs, especially the kidneys and the brain [4].

STEC infections may often cause profuse diarrhoea or haemorrhagic colitis (HC), manifested with bloody diarrhoea, abdominal cramps and an increased temperature of the body. Unfortunately, in some patients, HC progresses to hemolytic uremic syndrome (HUS). HUS is the disease characterised by the triad of symptoms: acute renal failure, haemolytic anaemia and thrombocytopenia. Importantly, HUS develops in 5-10% of individuals infected with STEC and may be accompanied by long-term

sequel, which causes a mortality rate of 3-17%. However, children less than 5 years old and elderly persons are the most susceptible groups endangered by severe complications, with mortality as high as 50% [5,6].

Unfortunately, nowadays, there are no protective measures or therapies against STEC. The treatment of STEC infection is very problematic because many of antibiotics and chemotherapeutics are potent inducers of Stx prophages. The administration of such kind medicaments to a STEC-infected patients may result in massive production of Shiga toxins and exacerbation of the disease symptoms. Additionally, the use of anti-diarrhoeal agents that inhibit intestinal peristalsis and delay clearance of the organisms are also not recommended, as they can increase the concentration of Shiga toxins in the intestinal lumen. Moreover, such an approach poses a risk factor for HUS progression. Currently, the safety treatment of STEC infection is based on the rehydration therapy and dialysis [7]. Therefore, new therapeutic approaches and novel, promising strategies for both effective treatment of STEC-infected patients and prevention of STEC infections should be developed.

Interestingly, according to the World Health Organization (WHO), STEC bacteria are classified as a major foodborne pathogens all-around the world. These microorganisms are transmitted to the humans by food products, like vegetables and fruits contaminated with animal feces, raw milk or undercooked ground meet. Sources of STEC infections have been also associated with water, organic fertilizers and equipment in slaughterhouses. This is because gastrointestinal tracts of cattle and other domestic animals are undoubtedly the main reservoir of STEC strains. Importantly, these animals are resistant to the Shiga toxins because the Gb3 receptors are not present on the surface of their vascular cells. It is worth to underline that food contamination with STEC bacteria contributes not only to the explosion of many local epidemic events but also leads to the large economic losses in food industry [5, 8]. Therefore, it is crucial to develop effective methods of food protection against STEC contamination.

The literature data indicate that currently more than 100 serotypes (e.g. O26, O55, O104, O111, O121, O145, O165, O174, O183) of STEC bacteria have been identified, however, among them the endemic *E. coli* O157:H7 strain is predominant [8]. The significance of these bacteria in the public health was recognized in the United States of America (USA) about forty years ago, during an outbreak of hemorrhagic colitis related with the consumption of contaminated hamburgers [9]. Since then, this pathogen has been estimated to cause more than 60,000 foodborne illnesses, and over 60 deaths each year in USA, resulting in a significant economic impact on healthcare and food liabilities. *E. coli* O157:H7 infections are mainly associated with bloody diarrhoea, hospitalisations and HUS development [10]. High virulence of this serotype is determined by the synthesis of Shiga toxins, accompanied by products of the pathogenicity island and plasmid pO157 [11]. Numerous studies have found that *E. coli* O157:H7 can also form biofilms on both biotic (e.g. vegetables, meat) and abiotic (e.g. stainless steel, glass) surfaces [12]. A biofilm is generally defined as a community of microorganisms enclosed within the self-producing extracellular polysaccharide matrix. This type

of structure protects *E. coli* O157:H7 bacteria from a variety of external factors, such as antibiotics and disinfectants used in food industry [13,14]. Thus, the public health and economic incentives have become the driving force to look for effective, unconventional and safety strategies leading to the elimination of *E. coli* O157:H7 bacteria, the main representant of the STEC group.

A one of strategies with a potential to eliminate unwanted STEC bacteria involves employment of lytic bacteriophages (phages). These bacterial viruses are the most abundant entities in the nature and exist in all environments (including human body, water, and soil). They can infect only prokaryotic cells and efficiently replicate inside them. Importantly, vast majority of bacteriophages are specific to a single bacterial species, or even strain, thus they do not disrupt the natural microbiota. Moreover, they are not harm to human cells, indicated their safety [15]. Therefore, during the last century, bacteriophages have been screened and used for the purpose of the therapy for various diseases caused by human pathogens. It is worth emphasizing that these bacterial viruses are also able to penetrate the biofilm and degrade its matrix [16], thus they could be used as a tool for biocontrol of foodborne pathogenic bacteria, including *E. coli* O157:H7. Nowadays, many laboratories around the word are pursuing the development of strategies that are based on utilization of phages for reduction of bacterial contamination on many different surfaces and various foods. As a result, in recent years, several phage-based preparations against food pathogens (e.g. *E. coli*, *Listeria monocytogenes*, *Salmonella* ssp., *Xanthomonas campestris* pv. vesicatoria and *Pseudomonas syringae* pv. tomato) have been approved for safety food applications in the United States, China and Europe. Moreover, the commercial phage products effective against *E. coli* O157:H7 (e.g. EcoShield PX™, Ecolicide™, PhageGuard E™ and Finalyse®) have been developed and are used in the food industry [17].

In the light of the facts described above, it is obvious that unconventional strategies to prevent and treat STEC infections are urgently needed. Therefore, the aim of my work was to characterize a newly discovered STEC-specific bacteriophage, vB_Eco4M-7, as a potential tool in human therapy and food biocontrol.

The vB_Eco4M-7 phage was isolated from urban sewage collected at Gdańsk Wastewater Treatment Plant. The procedures of its isolation and propagation in the *E. coli* O157:H7 (ST2-8624) host have been described previously by the team of researchers I joined at the beginning of my PhD studies [18].

At the first step of my research, I decided to conduct basic characteristics of the vB_Eco4M-7 genome by using bioinformatics tools [**article no. 1**]. The analysis indicated that the genetic material of the tested phage is composed of double-stranded DNA. The vB_Eco4M-7 genome contains 96 open reading frames (ORFs), among which 35 ORFs were assigned putative functions based on amino acids homologies of their predicted products to other, already known proteins deposited in databases. Interestingly, the majority of them belong to four modules: phage morphogenesis, DNA packaging, DNA replication and host lysis. It is worth emphasizing that the genome of vB_Eco4M-7 does not include genes characteristic to temperate bacteriophages, like integrase, recombinase, repressors and

excisionase, thus the vB_Eco4M-7 phage should be classified as a lytic virus. Moreover, antimicrobial resistance genes and those coding for virulence factor homologs were also absent in the phage genome. These features indicated that the vB_Eco4M-7 phage should not affect eukaryotic cells, and that is potentially applicable for STEC therapy and/or food biocontrol as an antimicrobial agent.

Electron microscopic studies and the phylogenetic analysis of the relationships between the vB_Eco4M-7 phage and other viruses revealed that the investigated phage belongs to the *Myoviridae* family [**article no. 1**]. Furthermore, the nucleotide sequence of the vB_Eco4M-7 genome shares significant similarity (coverage 94% and identity 92%) to the genome of the vB_ECML-117 virus that is an ingredient of the anti-*E.coli* O157:H7 preparation, named EcoShield PX™ [19]. Interestingly, both phages show differences in the sequence of the vB_Eco4M-7_27 gene encoding the tail fibre protein, which is possibly responsible for the host recognition. Bearing in mind that this incompatibility may have the influence on the host range of vB_Eco4M-7 and vB_ECML-117, in the next step of my work I decided to characterize and compare the morphological and biological properties of these closely related viruses [**article no. 1**]. Firstly, I analysed the ability of vB_Eco4M-7 and vB_ECML-117 to infect different bacterial strains by using a standard spot method. I observed that both bacterial viruses were able to form plaques on lawns of 16 Shiga toxin-producing *E. coli* O157:H7 isolates, 3 *E.coli* O157:H7 non-STEC isolates, 11 *E. coli* O157 STEC isolates, and 4 *E. coli* O157 non-STEC isolates. Interestingly, the vB_Eco4M-7 phage could not infect any of the *E. coli* O26 STEC isolates and 41 other non-O157 strains, including non-pathogenic bacteria. However, in contrast to the vB_Eco4M-7 virus, phage vB_ECML-117 exhibited lytic spectrum against 6 *E. coli* O25 strains. Moreover, all tested laboratory *E. coli* strains and bacteria from other species were resistant to the tested phages. Afterwards, I determined the plaque morphologies of tested viruses. The investigated phages form small, clear and uniform plaques on the *E. coli* O157:H7 (ST2-8624) lawn, which is characteristic for the group of lytic bacteriophages. Interestingly, my studies indicated that vB_Eco4M-7 and vB_ECML-117 phages are resistant to some laboratory disinfectants, like soap, dish soap, Line-Antibacterial 70 and Virusolve. In the **article no. 1**, I also presented the kinetics of the lytic development of tested phages in the host cells. As indicated by one-step growth experiments, the intracellular development of both vB_Eco4M-7 and vB_ECM-117 is rapid (lasting about 10 min) and efficient (the burst size of approximately 100 phages per cell) with short eclipse and latent periods. Interestingly, the vB_Eco4M-7 phage appeared more efficient in decreasing the host strain viability than vB_ECML-117 during cultivation of infected bacteria. Importantly, both investigated viruses did not cause the induction of the ST2-8624 prophage (belonging to Stx phages) that is present in the genome of the *E. coli* O157:H7 (ST2-8624) strain.

Taking these results into consideration, I concluded that there is a high probability that the vB_Eco4M-7 virus, because of its biological properties, could be a promising candidate for further studies on its use in phage therapy and/or food protection against Shiga-toxin producing *E. coli*. Moreover, in composition to vB_ECML-117 or other newly isolated phages, it might be used as a new

phage preparate in the fight against foodborne pathogens. It is worth to mention that the first step in this direction has been taken, thus research in this field resulted in granting a patent from the Polish Patent Office (patent no. 240170).

As I mentioned above, *E. coli* O157:H7 strains can occur in a wide variety on food-related environments due to their ability to attach, colonize and create biofilms on different surfaces. It is worth to underline that cells forming biofilms are often more resistant to several stresses than planktonic cells [20]. This phenomenon is related to a complex structure of biofilm where bacterial cells are submerged in a mixture of extracellular polymeric substances (like proteins, lipids, polysaccharides and nucleic acids) [14]. Several approaches are currently employed to eliminate *E. coli* O157:H7-mediated food contamination, ranging from simple washing to chemical or physical decontamination of foods [21,22]. However, some of them have considerable drawbacks, like large costs, potential damage of equipment or a deleterious impact on environment and organoleptic qualities of foods [23].

Keeping in mind this information, during realization of my PhD thesis, I decided to answer the question whether the vB_Eco4M-7 bacteriophage may be used in food biocontrol. Therefore, I created the validation system for selection of bacteriophages against STEC contamination which employed the vB_Eco4M-7 bacteriophage, a model *E. coli* O157:H7 (ST2-8624) bacterial strain, and a model vegetable - cucumber [**article no. 2**]. Knowing that the peel of cucumber is a natural barrier, and it is significantly more difficult for bacteria to attach to, I made a decision to conduct this experiment by using a sliced cucumber. I noticed that the application of the vB_Eco4M-7 lysate onto the surface of cucumber slices significantly reduced the number of bacterial cells after 6 h incubation for each tested multiplicity of infection (MOI), ranking from 0.0001 to 10. However, longer period of incubation (24 h) resulted in a re-growth of pathogenic bacteria, though there were still significant differences in the titres between the control and STEC strains treated with the vB_Eco4M-7 bacteriophage. Interestingly, the highest lytic activity of vB_Eco4M-7 against STEC after 24 h was observed at the lowest MOI values (0.0001-0.01). Since the growth of the STEC strain depends on the number of phage particles applied to cucumber slices, I decided to check whether a similar phenomenon may occur in the liquid bacterial culture. A rapid decrease of the culture density after phage infection was observed during first 4 h at MOI of 0.1, 1 and 10, indicating effective killing of sensitive bacteria. As expected, under low MOI values (0.0001-0.01), culture density decreased slower, and it was not reduced as effectively as with higher MOIs. However, longer incubation (24 h) of susceptible bacteria with the vB_Eco4M-7 bacteriophage resulted in the appearance of phage-resistant mutants at all tested MOIs. Intriguingly, I noticed that the selection of mutants among survivors was more efficient at high MOI than at low MOI (like 0.001 and 0.0001). Thus, I can conclude that this phenomenon might arise from a strong selection pressure under high MOI values. In such conditions, every bacterial cell is probably infected by phages, thus only phage-resistant mutants may survive and propagate in the presence of the virus. Furthermore, I also suggested that under low MOI conditions, the selection pressure is weaker (the lower number of phage particles per one bacterial cell), thus the wild-type cells

can win the competition with bacterial mutants that are often deficient in some cellular functions. It is worth to underline that obtained results do not disqualify the use of the vB_Eco4M-7 phage as an antimicrobial agent in food biocontrol because such high selection pressure of mutants appears unlikely under environmental conditions. However, this speculation requires further investigation during real food storage [**article no. 2**]. Taking into account that the good anti-bacterial preparate should be safe for human health, in the next stage of my studies, I also tested toxicity of the vB_Eco4M-7 phage to mammalian cells (the Balb/3T3 line) [**article no. 2**]. Importantly, no decrease in viability of tested cells was found after 24 h incubation with the phage lysate. Moreover, Balb/3T3 cells indicated normal morphology as compared to the control variant. The obtained results confirmed my assumption that the vB_Eco4M-7 phage is non-toxic to mammalian cells and may be potentially used in food protection procedures [**article no. 2**]. Apart from that, I also indicated that this bacterial virus may propagate in and lyse *E. coli* O157:H7 cells at low temperatures (12 °C and 25 °C), resembling conditions occurring during vegetable storage [**article no. 2**]. The results presented in the **article no. 2** confirmed the applicable character of vB_Eco4M-7 in food protection against *E. coli* O157:H7, however, caution should be taken to the pressure of formation of phage-resistant bacteria.

Finally, **article no. 2** was selected as an Editor's Choice Article by the editors of the journal "*Toxins*". This selection means that it was recognized by editors as one of articles revealing the highest impact on the development of the field of studies on toxins. The selected articles are available at www.mdpi.com/journal/toxins/editors_choice.

Various studies have shown a clinical potential of the use of combinations of lytic phages and antibiotics to treat biofilms formed by STEC strains [24]. However, the major problem is to choose an appropriate antimicrobial agent that will not cause the induction of Stx prophages [25]. Thus, I decided to test the effects of the vB_Eco4M-7 phage alone or in a phage cocktail with vB_ECML-117, and/or in combination with different antibiotics on biofilm formed by the *E. coli* O157:H7 (ST2-8624) strain [**article no. 3**]. In my experiments, I employed two antibiotics with different mechanisms of action, ciprofloxacin and rifampicin [26,27], at supra-MIC concentrations (MIC - minimum inhibitory concentration). The biofilm was treated for 6 h with either antimicrobial agent individually, sequentially, or simultaneously. To evaluate the antibiofilm properties of vB_Eco4M-7 or the phage cocktail, I used standard methods of biofilm testing, like measurement of OD_{600}, estimation of the number of viable cells, quantification of the biofilm biomass through densitometric measurement, and crystal violet (CV) staining. In the first step of my analyses, I indicated that the application of the vB_Eco4M-7 phage onto bacterial biofilm layer to an MOI of 100 decreased the optical density of bacterial culture and the number of viable bacterial cells relative to the control variant. Interestingly, the phage cocktail treatment was even more pronounced, however the fraction of survivors was still significant. Therefore, in the next step of my studies, I tested the efficiency of mixtures of bacteriophages and antibiotics in eradication of bacterial biofilm. I observed that the combination of the vB_Eco4M-7 bacteriophage or the phage cocktail with tested antibiotics

was effective in the lowering of bacterial density, the number of survivors and the thickens of the biofilm layer. However, the best results in biofilm eradication were obtained when phage infection preceded antibiotic treatment. I conclude that such a synergistic effect might be related with the relaxation of the biofilm structure by investigated phages, thus the penetration of antibiotic molecules into the deeper layers of the biofilm was more effective and enhanced their influence on biofilm cells. Moreover, the sequential treatment of bacterial biofilm with tested agents also generated the lowest number of phage- and antibiotic-resistant mutants. Finally, I tested if analysed antimicrobial agents (individually or in combination) cause the induction of the ST2-8624 prophage in the liquid culture of *E. coli* O157:H7 (ST2-8624). As a positive control, I employed mitomycin C which is known as a strong inducer of temperate prophages [28]. I observed that ciprofloxacin was almost as efficient in provoking the ST2-8624 prophage induction as mitomycin C, while no virus particles could be detected in the host cultures grown in the presence of rifampicin. Interestingly, the treatment of STEC with the vB_Eco4M-7 bacteriophage or the phage cocktail, alone or in combination with rifampicin, has completely inhibited the spontaneous induction of the ST2-8624 prophage. Keeping in mind the results presented in the **article no. 3**, I conclude that the vB_Eco4M-7 bacteriophage alone, and with the phage cocktail, is able to destroy biofilm formed by STEC strains. Moreover, it is worth to underline that rifampicin or similarly acting compounds might be effective and safe candidates for drugs when used together with the vB_Eco4M-7 bacteriophage to combat STEC-mediated infection.

The results presented in this dissertation indicated that the newly isolated vB_Eco4M-7 bacteriophage presents many features that make it a potential candidate for phage therapy and food protection against Shiga-toxin producing *E. coli*. The morphology, host specificity, stability of phage particles, a lack of toxicity against eukaryotic cells, as well as its efficiency against *E. coli* O157:H7 cells were experimentally confirmed. It is worth to underline that this kind of research may result in improved understanding not only phage biology, but also genetics, immunology, and pharmacology. In fact, such studies indicate that bacterial viruses can be considered as one of the most promising tools in combating bacterial infections. However, the use of phages in medicine and food industry creates many challenges and problems that should be solved to avoid the limitations of this antimicrobial strategy.

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Articles included in the doctoral dissertation:

Article no. 1: **Necel A**, Bloch S, Nejman-Faleńczyk B, *et al*. Characterization of a bacteriophage, vB_Eco4M-7, that effectively infects many *Escherichia coli* O157 strains. *Sci Rep.* **2020**, *10(1)*, 3743; doi:10.1038/s41598-020-60568-4.

Article no. 2: **Necel A**, Bloch S, Nejman-Faleńczyk B, *et al*. A validation system for selection of bacteriophages against Shiga toxin-producing *Escherichia coli* contamination. *Toxins (Basel)* **2021**, *13(9)*, 644; doi:10.3390/toxins13090644.

Article no. 3: **Necel A**, Bloch S, Topka-Bielecka G, *et al*. Synergistic effects of bacteriophage vB_Eco4-M7 and selected antibiotics on the biofilm formed by Shiga toxin-producing *Escherichia coli*. *Antibiotics* **2022**, *11*, 712; doi:10.3390/antibiotics11060712.