„Characterization of selected bacteriophages isolated from urban sewages – molecular studies and potential biotechnological applications"
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An increased interest in the process of formation and control of bacterial biofilms has been observed in the recent years. This applies to both biofilms produced by pathogenic and non-pathogenic bacteria. Biofilm is a community of microorganisms, either associated with a surface or adherent to one another and immersed in an extracellular polymeric exopolysaccharide matrix (EPSs) **[article no. 1]**. Principal components of EPS are proteins, lipids, extracellular DNA (e-DNA), RNA and polysaccharides [1,2]. Bacterial biofilm formation is a complex process divided into five stages: (1) attachment, (2) monolayer formation and matrix production, (3) formation of micro-colonies, (4) biofilm maturation, and (5) biofilm detachment and dispersion **[article no. 1]**. Importantly, one can identify positive and negative aspects of biofilm formation for human life. On the one hand, bacterial biofilms are crucial for various fermentation processes, wastewater treatment or in microbial fuel cells technology. On the other hand, biofilm formation has become a significant problem in the medical sector since pathogenic and multidrug resistant bacteria included in biofilms can cover surfaces of various materials used in medicine, as well as surfaces of patients' tissues. Moreover, the formation of biofilms creates problems in food industry and industrial water systems [3–7].

As estimated, 99% of all bacteria in natural environments are found in the form of biofilm [8]. It is known that many bacterial pathogens can easily attach to surfaces and form stable biofilms which may be composed of only a single species or, more often, many species of microorganisms. Importantly, bacteria within biofilms reveal 10–1000 times higher antibiotic resistance in comparison with planktonic cells [9,10]. Biofilm bacteria are resistant to antibiotics due to the number of factors: (i) interaction of antimicrobials with biofilm matrix components that delays and weakens their action, (ii) slower growth rates of bacteria in the biofilm (iii), hiding of binding sites for antibiotics due to genetic changes of target cells, (iv) action of the modifying enzymes, (v) generation of persister cells which are insensitive to antibiotics, (vi) changes of the chemical microenvironment, (vii) the age of biofilm, and (viii) formation of multispecies bacterial biofilms **[article no. 1].** The presence of more than one bacterial species in a biofilm is favorable as it can facilitate the biofilm's attachment to a surface, and enhances the resistance to antibacterial agents [5]. The difficulties in combating polymicrobial biofilm communities arise mainly from the diverse

resistance mechanisms occurring in bacteria, as well as the limitation of antibiofilm agent migration, caused by the presence of a large diversity of EPSs produced by heterogeneously distributed microorganisms. Undoubtedly, antibiotic resistance of multispecies bacterial biofilms is a serious problem. Various attempts are made to fight this type of biofilms. They are discussed in detail in **article no. 1** of this doctoral dissertation, with particular emphasis on the use of bacteriophages and the enzymes they encode (e.g. depolymerases) in both individual and combined (with other antibacterial agents) therapies.

Bacteriophages (phages) are viruses that infect and replicate only in bacterial cells. They can be either virulent or temperate. Virulent phages propagate due to lytic mode, where rapid viral replication ends in progeny release and the bacterial death through cell lysis. Temperate phages can follow either a lytic or a lysogenic cycle in which viral genetic material is integrated into the host genome and is maintained as a prophage. Nevertheless, phage-based therapies focus on lytic phages. These strategies are based on single phages or phage cocktails, phage-derived enzymes, genetically modified phages or phages in combination with antibiotics (**article no 1**). Advantages of using bacterial viruses against biofilms result from their properties which can be summarized as follows: (i) lytic activity against bacteria with reduced (although not completely inhibited) metabolism, (ii) specificity (usually a phage is able to infect a single bacterial species or even specific strains), (iii) auto-control ability (phages propagate only if their host bacteria are available), (iv) safety (they are inactive against eukaryotic cells), (v) easy sourcing and accessibility, and (vi) ability to disseminate within the biofilm and replicate there, at least to some extent [11].

Over the past years, the number of articles relating to bacteriophages investigated as tools to control the biofilm formation has increased significantly. In the light of the above facts, the aim of this study was to isolate and characterize previously unknown lytic phages with a potential application in the fight against biofilms.

Abundance of bacteriophages in urban sewage demonstrated that this might be a rich source of such viruses. Sewage are a breeding ground for many bacterial species, what in turn affects the variety of phages in this environment. Therefore, both phages presented in this study, were isolated from sewage water samples collected at Gdansk Wastewater Treatment Plant. They were obtained according to protocol described previously [12]. In the first step of this approach, a raw urban

sewage sample was mixed with a culture of a particular bacterial strain to obtain lysates of bacteriophages able to propagate in the host cells. I my studies, I have focused on two bacterial species *Escherichia coli* and *Enterococcus faecalis* - commonly found in polymicrobial biofilms.

E. coli and *E. faecalis* belong to the group of a biofilm-forming pathogens that have an enormous impact on global economy and medicine. *E. coli* is one of the most prevalent microorganisms able to form bacterial biofilms. Importantly, such microbial community can be formed by both non-pathogenic and pathogenic bacteria such as enterohemorrhagic *E. coli* (EHEC), enteropathogenic *E. coli* (EPEC) or enteroaggregative *E. coli* (EAggEC). *E. coli* biofilm is one of major contributors to the occurrence of various medical device-associated infections, and it is also responsible for food spoilage and contaminations in food industry [13,14]. *E. coli* can attach to various surfaces such as stainless steel, polymer materials, glass, polystyrene, PVC or teflon, causing corrosion and damage [5]. *E. faecalis* are the most important enterococcal species which accounts for 80-90% of all clinical isolates [15]. These microorganisms cause serious infections, including urinary tract infections (UTIs), endocarditis, bacteremia and infections associated with root canal treatment [16,17]. In most cases, the infections are associated with biofilm formation (biofilm-associated infections) and exhibit highly antibacterial resistance, especially in the nosocomial environment. Moreover, 75% of all UTIs are associated with urinary catheterization [18]. Recent reports indicated that *E. faecalis* strains are responsible for about 20% of all cases of UTIs, including infections derived from catheters (CA-UTIs), due to their colonization and formation of biofilm [19]. This results from the ability of microorganisms to successfully adhere to many polymers used for urological catheter production such as propylene, polystyrene, silicone, polyvinyl chloride or silicone coated latex [20]. Furthermore, biofilm of *E. faecalis* is also responsible for contaminations in the food-processing industry [21].

At the beginning of my research, I characterized and analyzed morphological and biological properties of the newly isolated phage vB_EcoS-95 **[article no. 2].** The lytic activity of bacteriophage vB_EcoS-95 was determined using the spot test by observing the formation of growth inhibition zones. The obtained results revealed bacteriolytic activity against some *E. coli* strains, including some clinical isolates. Transmission electron microscopy showed that vB_EcoS-95 belongs to the family *Siphoviridae*. As demonstrated by one-step growth experiments, the latent period at optimal conditions was as short as 4 min, and the burst size was about 115 plaque-forming

units (PFU) per cell. Therefore, vB_EcoS-95 can produce enough virions within a short time to lyse the host bacteria efficiently, which makes it an attractive agent for applications in treatment of infections or protection of various materials against bacterial colonization. The complete nucleotide sequence of the phage genome has been determined, annotated and compared with other phages. Interestingly, such genome analysis indicated that this phage does not encode its own DNA polymerase, while coding for an untypical lytic protein which might potentially contribute to rapid lysis of the host cell. In the **article no. 2**, I also demonstrated results of experiments on a biofilm-forming strain. Biofilm degrading efficacy of this phage was assessed by using various methods, including crystal violet and resazurin assays, as well as holographic 3D microscopy. The obtained results indicated that this phage is able to destroy bacterial biofilm. The most effective reduction of bacterial cell count in biofilm was observed using bacteriophage treatment at an m.o.i. of 10, while lower m.o.i. (7, 5, 4 and 2) resulted in a gradual reduction of the biofilm. The results presented in this paper indicated that vB_EcoS-95 is a newly discovered *E. coli* phage that may be potentially used to control the formation of biofilms in food protection and/or medicine. Furthermore, an extremely short latent period of this phage suggested that it can be used in further studies on development of novel biotechnological tools applicable in genetic engineering **[article no. 2]**.

E. faecalis is responsible for a large proportion of nosocomial infections, and it developed resistance to many antibiotics. This bacterium is also able to form biofilm on various surfaces. The **article no. 3** presents isolation and characterization of a newly discovered virulent phage vB_EfaS-271, infecting a clinical strain of *E. faecalis* 271. Firstly, I determined host range and microbiological properties of this phage. The newly isolated phage showed lytic activity against a few tested clinical *E. faecalis* strains. Host range analysis revealed that although the phage exhibited lytic spectrum against some of the tested *E. faecalis* strains, it could not lyse strains from other species. Electron microscopic studies and phylogenetic analysis revealed that phage vB_EfaS-271 belongs to the *Siphoviridae* family. Analysis of lytic development indicated a relatively rapid development (with a latent period of 8 min) and burst size of approximately 70 plaque-forming units per infected cell. Furthermore, average nucleotide identity analysis indicated its similarity to some other phages infecting enterococci. Interestingly, whole genome analysis demonstrated that vB_EfaS-271 contains genes coding for putative DNA polymerase B-like protein, bifunctional DNA primase/polymerase, two endonucleases, helicase and primase. This

suggested important functions of these enzymes for the efficient replication of the phage genome. I have also demonstrated that this phage was able to destroy a biofilm formed by *E. faecalis* 271 **[article no. 3]**. To determine efficiency of this process, I analyzed the level of the biofilm density after treatment with the phage. I observed that phage vB_EfaS-271 caused a significant decrease in the biofilm density even if applied at a very low quantity of 10^2 plaque forming units. In addition, analysis of biofilm biomass with crystal violet staining and metabolic activity/viability estimated by the resazurin assay showed phage-mediated effect similar to those observed in the biofilm density assay. The presented results indicated that vB_EfaS-271 is potentially applicable for biological control of some *E. faecalis* strains, suggesting its potential usefulness as antibacterial agent. Although this phage is able to infect only a small group of *E. faecalis* strains, obtained results indicated that it might be potentially used in phage therapy or in genetic engineering. Importantly, it is effective in destroying the biofilm formed by *E. faecalis* 271 **[article no. 3]**.

In this light, I decided to carry out experiments aimed at to characterize vB_EfaS-271-host interactions in the light of potential applications of this virus in phage therapy **[article no. 4]**. I analyzed efficiency of phage vB_EfaS-271 in the prevention of formation of biofilms by *E. faecalis* on the catheter surface. For this purpose, I used Foley silicone catheter. I observed that vB_EfaS-271 is able to efficiently reduce number of viable bacterial cells on catheter surfaces. While the infection with vB_EfaS-271 at m.o.i. 10 caused rapid (within 3 h) and significant decrease in the number of viable *E. faecalis* cell, lower m.o.i. (0.0001 or 0.01) required a longer time (6 h) to cause such effects. Interestingly, efficiency of formation of phage-resistant bacteria after 24-h incubation was dependent on m.o.i., and it was higher when the virion-cell ratio was as high as 10. Nevertheless, obtained results indicated that vB_EfaS-271 may be considered as a candidate for its further use in phage therapy. In studies described in the **article no. 4**, I also tested toxicity of this phage particles to mammalian cells lines and its effects on *E. faecalis* co-cultured with these cells, using mouse embryonic fibroblasts (BALB/c3T3 clone 31). When analyzing effects of the phage lysate on mammalian cells lines, I observed similar number of viable cells in both phage-treated and control cells. The mammalian cells exhibited similar morphology before and after 24 h incubation with phages. These results indicated that phage vB_EfaS-271 is non-toxic to mammalian cells. Moreover, while addition of *E. faecalis* cells to cultures of BALB/c3T3 fibroblasts resulted in a significant decrease in viability of mammalian cell, simultaneous treatment with vB_EfaS-271 partially restored viability of fibroblasts when m.o.i. of 0.0001, but not m.o.i.

of 10, was used. These results indicated that the efficiency of protection of mouse fibroblasts against *E. faecalis* by phages was higher at lower m.o.i. In addition, the appearance of phage-resistant bacteria was more rapid at higher m.o.i which corroborated previously mentioned results. On this basis, it was concluded that selection of phage-resistant bacteria is more effective when a host bacterial cell was is infected by several phages. Furthermore, I suggest that a phage-resistant mutant, although viable, is usually deficient (to some extent) in one of physiological processes, relative to wild-type cell. Although the appearance of vB_EfaS-271-resistant mutants might suggest a limitation in the use of this virus in phage therapy, the impaired growth and lower competitiveness of such mutants implicates that combination of phage therapy with other anti-bacterial treatment(s) can still be effective **[article no. 4]**.

In conclusion, the results obtained during this study indicated a great potential of the newly discovered phages to destroy and to prevent formation of *E. coli* and *E. faecalis* biofilms. In both cases, significantly reduced bacterial cell counts were observed. Considering the available literature data, together with obtained results, I suggest that vB_EcoS-95 and vB_EfaS-271 might be potentially used in phage-based strategies against polymicrobial biofilms, which are especially problematic in medical and industrial sectors. Currently, high hopes for the development of effective antibiofilm strategies are placed in combination therapy (phages or their derivatives together with antibiotics). Although I did not analyze the effectiveness of this approach in my doctoral thesis, it is undoubtedly worth investigating in the future.

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Articles included in the doctoral dissertation:

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Article no.4: **Topka-Bielecka, G**.; Nejman-Faleńczyk, B.; Bloch, S.; Dydecka, A.; Necel, A.; Węgrzyn, A.; Węgrzyn, G. Phage–Bacteria Interactions in Potential Applications of Bacteriophage vB_EfaS-271 against *Enterococcus faecalis*. *Viruses* **2021**, *13*, 318.