UNIVERSITY IN NOVI SAD

FACULTY OF SCIENCES

DEPARTMENT OF BIOLOGY AND ECOLOGY

# FILAMENTOUS BACTERIOPHAGES OF SPECIES *PSEUDOMONAS AERUGINOSA*

PhD Thesis

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Novi Sad, 2023

**УНИВЕРЗИТЕТ У НОВОМ САДУ**
**Природно-математички факултет**

**КЉУЧНА ДОКУМЕНТАЦИЈСКА ИНФОРМАЦИЈА**[1]

| | |
|---|---|
| Врста рада: | Докторска дисертација |
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| Наслов рада: | Филаментозни бактериофаги врсте *Pseudomonas aeruginosa* |
| Језик публикације (писмо): | Енглески (латиница) |
| Физички опис рада: | Унети број:<br>Страница 177<br>Поглавља 10<br>Референци 378<br>Табела 13<br>Слика 23<br>Графикона 17<br>Прилога 1 |
| Научна област: | Биологија |
| Ужа научна област (научна дисциплина): | Микробиологија |
| Кључне речи / предметна одредница: | *Pseudomonas aeruginosa*, Бактериофаги, *Inoviridae* |
| Резиме на језику рада: | Филаментозни бактериофаги су широко распрострањени у бактерији *Pseudomonas aeruginosa* и њиховом инфекцијом долази до значајних промена у фенотипу ове бактеријске врсте. Ова група фага је означена као Pf и припадају фамилији *Inoviridae*. Први корак у испитивању ове групе |

[1] Аутор докторске дисертације потписао је и приложио следеће Обрасце:
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5г – Изјава о коришћењу.
Ове Изјаве се чувају на факултету у штампаном и електронском облику и не кориче се са тезом.

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| | фага је био добијање суспензије са високим титром вируса. С обзиром на то да не постоји описана метода за умножавање интегративних филаментозних фага, на примеру Pf филаментозних фага дизајниране су, описане и упоређене две методе за њихово умножавање. У ту сврху, искоришћена су три референтна соја бактерије *P. aeruginosa* LESB58, PAO1 і UCBPP-PA14 (PA14), која су природно инфицирана са филаментозним фагима PfLES58, Pf4 и Pf5. Метода 1 је подразумевала умножавања фага у 6 L бактеријске културе 48 сати, док је методом 2 искоришћено 600 mL културе и инкубација је трајала шест дана са додавањем свежег медијума и инокулума на свака два дана. Суспензија фага из метода 1 је пречишћена од бактерија центрифугирањем па потом филтрацијом (0,45 и 0,22 μm) док је суспензија фага из методе 2 пречишћена искључиво центрифугирањем у више наврата, односно док талог бактерије није више био видљив. У оба случаја, пречишћени фаги су потом ултрацентрифугирани и дијализирани. Метода 2 се по свим провереним параметрима показала као далеко супериорнија у односу на методу 1. Значај методе 2 је и у томе што се може примењивати и за умножавање филаментозних фага других бактеријских врста. Изолација виралне ДНК из умножених Pf фага и третман различитим ензимима указао је на присуство једноланчане ДНК код PfLES58, Pf4 і Pf5 фага. Такође, код сва три фага утврђено је присуство главног протеина капсида (CoaB) SDS анализом. CoaB протеин је карактеристичан за филаментозне фаге из фамилије *Inoviridae*. Од свих испитаних бактериофага, најшири литички спектар има PfLES58 (90.6%), а потом Pf5 (62.5%), док је најужи имао Pf4 (46.1%). Субинхибиторне концентрације ципрофлоксацина и митомицина Ц могу да повећавају продукцију PfLES58, Pf4 и Pf5 бактериофага. Сличан ефекат у продукцији је забележен и код мутаната који се јављају након инфекције облигатно литичким фагом. Успешно је утврђено да ослобођени вириони Pf фага могу инфицирати друге *P. aeruginosa* сојеве и утицати на промене у њиховом фенотипу. Разлике између Pf инфицираних и неинфицираних сојева су биле очигледне. Код инфицираних сојеве је забележено смањење у аутоагрегацији ћелија и у производњи пиовердина и пиоцијанина. С друге стране, значајно је повећана покретљивост флагелама и производња биофилма код инифицираних сојева. Осим тога, код неких сојева је повећана појава варијанти малих колонија (енг. *small colony variants*). Овакви ефекти доводе до појаве сојева који су склонији успостављају хроничних инфекција. Pf бактериофаги су повећавали осетљивост инфицираних сојева на одређене антибиотике, као што су тетрациклин, цефтазидим и стрептомицин, отварајући ново поље у терапији фагима где би се филаментозни фаги користили у борби против бактеријских инфекција. |
| Датум прихватања теме од стране надлежног већа: | 28.10.2021. |

| | |
|---|---|
| Датум одбране:<br><br>(Попуњава одговарајућа служба) | |
| Чланови комисије:<br><br>(титула, име, презиме, звање, институција) | Председник: др Верица Алексић Сабо, доцент, Природно-математички факултет, Универзитет у Новом Саду<br><br>Члан: др Петар Кнежевић, редовни професор, Природно-математички факултет, Универзитет у Новом Саду<br><br>Члан: др Малгоржата Лобоцка, редовни професор, Институт за биохемију и биофизику Пољске академије наука, Варшава |
| Напомена: | |

**UNIVERSITY OF NOVI SAD**
**FACULTY OF SCIENCES**

**KEY WORD DOCUMENTATION[2]**

| | |
|---|---|
| Document type: | Doctoral dissertation |
| Author: | Damir Gavrić |
| Supervisor (title, first name, last name, position, institution) | Dr. Petar Knežević, full professor, Faculty of Sciences, University of Novi Sad |
| Thesis title: | Filamentous bacteriophages of species *Pseudomonas aeruginosa* |
| Language of text (script): | English language (latin) |
| Physical description: | Number of:<br>Pages 177<br>Chapters 10<br>References 378<br>Tables 13<br>Illustrations 23<br>Graphs 17<br>Appendices 1 |
| Scientific field: | Biology |
| Scientific subfield (scientific discipline): | Microbiology |
| Subject, Key words: | *Pseudomonas aeruginosa*, Bacteriophages, *Inoviridae* |
| Abstract in English language: | Filamentous bacteriophages are widely distributed in the bacterium *Pseudomonas aeruginosa* and their infection leads to significant changes in the phenotype of this bacterial species. This group of phages is designated Pf and belongs to the *Inoviridae* family. The first step in analyzing this group of phages was to obtain a suspension with a high virus titer. Given that there is no described method for multiplying integrative filamentous phages, two methods for their multiplication were designed, described and compared |

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| | using the example of Pf filamentous. For this purpose, three reference strains of *P. aeruginosa* LESB58, PAO1 and UCBPP-PA14 (PA14), which are naturally infected with filamentous phages PfLES58, Pf4 and Pf5, were used. Method 1 involved propagation of phages in 6 L of bacterial culture for 48 hours, while method 2 used 600 mL of culture and incubation lasted six days with the addition of fresh medium and inoculum every two days. The phage suspension from method 1 was purified from bacteria by centrifugation and then by filtration (0.45 and 0.22 μm), while the phage suspension from method 2 was purified only by centrifugation on several occasions, that is, until the bacterial sediment was no longer visible. In both cases, the purified phages were then ultracentrifuged and dialyzed. Method 2 proved to be far superior to method 1 according to all tested parameters. The significance of method 2 is that it can also be used for the multiplication of filamentous phages of other bacterial species. Isolation of viral DNA from multiplied Pf phages and treatment with different enzymes indicated the presence of single-stranded DNA in PfLES58, Pf4 and Pf5 phages. Also, in all three phages, the presence of the main capsid protein (CoaB) was determined by SDS PAGE analysis. The CoaB protein is characteristic of filamentous phages from the *Inoviridae* family. Of all tested bacteriophages, PfLES58 (90.6%) had the widest lytic spectrum, followed by Pf5 (62.5%), while Pf4 (46.1%) had the narrowest. Subinhibitory concentrations of ciprofloxacin and mitomycin C can increase the production of PfLES58, Pf4 and Pf5 bacteriophages. A similar effect in production was also recorded in mutants that appear after infection with an obligatory lytic phage. It was successfully established that the released Pf phage virions can infect other *P. aeruginosa* strains and affect changes in their phenotype. Differences between Pf infected and uninfected strains were apparent. In infected strains, a decrease in cell autoaggregation and pyoverdine and pyocyanin production was recorded. On the other hand, motility by flagella and biofilm production were significantly increased in infected strains. In addition, the occurrence of small colony variants has increased in some strains. Such effects lead to the appearance of strains that are more prone to establishing chronic infections. Pf bacteriophages increased the sensitivity of infected strains to certain antibiotics, such as tetracycline, ceftazidime and streptomycin, opening a new field in phage therapy where filamentous phages would be used in the fight against bacterial infections. |
| Accepted on Scientific Board on: | 28.10.2021 |
| Defended:<br><br>(Filled by the faculty service) | |

| | |
|---|---|
| Thesis Defend Board:<br><br>(title, first name, last name, position, institution) | President: Dr. Verica Aleksić Sabo, assistant professor, Faculty of Sciences, University of Novi Sad<br><br>Member: Dr. Petar Knežević, full professor, Faculty of Sciences, University of Novi Sad<br><br>Member: Dr. Małgorzata Łobocka, full professor, Institute of Biochemistry and Biophysics of the Polish Academy of Sciences, Warsaw |
| Note: | |

*I would like to take this opportunity to express my gratitude to all the employees of the Department of Biology and Ecology and the Faculty of Science and Mathematics who made this doctoral dissertation possible. I would like to express my heartfelt gratitude to the following individuals:*

*Professor Dr. Petar Knežević, my mentor, distinguished filamentous phage scientist, Chair of the Tubulavirales Working Group of the International Committee on Taxonomy of Viruses and head of PK Lab. Your guidance and unwavering support since the very beginning have made all of this possible. Your understanding, as well as your selfless sharing of knowledge, have been invaluable. Your life advice and constant presence for me and my family are deeply appreciated.*

*To the members of the commission, Dr. Malgorzata Lobocka and Dr. Verica Aleksić Sabo, for all the advice and suggestions during the preparation of the doctoral dissertation.*

*To the members of* Petar Knezevic's Lab *– PK Lab: Verica Aleksić Sabo, Darija Vuković, Sonja Gostimirović, Jelena Narančić, Jovana Pejić, Isidora Nikolić and Jelena Cvetanović, who were always ready to help. I express my gratitude to Verica for her valuable support throughout my academic journey. I extend my appreciation to Darija, who has been by my side since the beginning of my doctoral studies, and for consistently being a dependable source of support.*

*I am deeply indebted to my professors, Dr. Dragan Radnović, Dr. Jelica Simeunović, and Dr. Maja Karaman. I also extend my gratitude to the rest of the Department of Microbiology for their wholehearted support. I would like to express my special appreciation to Ana Volarić and Petar Davidović, friends whose presence and shared experiences made my doctoral journey enjoyable and memorable.*

*Furthermore, I extend my gratitude to Dr. Aleksandra Petrović Fabijan for her help and support during the initial stages of my laboratory work.*

*My sincere appreciation goes to Dr. Rok Kostanjšek of the Biotechnical University in Ljubljana for his invaluable help in obtaining electron micrographs.*

*I am also very grateful to Dr. Elvira Vukašinović from the Chair of Biochemistry and Molecular Biology for advice and help with SDS-PAGE analysis of bacteriophage proteins.*

*Special thanks to Dina Tenji from LECOTOX laboratory, and the colleagues from the Chair of Biochemistry, Molecular Biology for their insights and guidance during the qRT-PCR analysis and the colleagues from the Chair of Biochemistry, Molecular Biology, and Genetics for their coordination in sequencing*.

*I extend heartfelt gratitude to my brother, Darko Gavrić, for his love and support. My profound thanks go to our parents, Angelina and Božo Gavrić, for their selfless dedication in providing us with the means and opportunities to achieve our aspirations. I hope they take pride in our accomplishments.*

*I am also grateful for the support provided by family of my wife Ana: Saša Đorđević, Borka Đorđević, Ivana Pašić, and Vojislav Đorđević.*

*My deepest appreciation goes to my family. To my wife, my dearest friend, Ana, I am truly thankful for your constant presence through thick and thin, and your unyielding commitment to our journey. Your contributions to our family are immeasurable. Lastly, I am thankful to my son, Uroš, for every joyful smile and warm hug. This doctoral dissertation is dedicated to both of them.*

*MSc Damir Gavrić*

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**Резиме.** Филаментозни бактериофаги су широко распрострањени у бактерији *Pseudomonas aeruginosa* и њиховом инфекцијом долази до значајних промена у фенотипу ове бактеријске врсте. Ова група фага је означена као Pf и припадају фамилији *Inoviridae*. Први корак у испитивању ове групе фага је био добијање суспензије са високим титром вируса. С обзиром на то да не постоји описана метода за умножавање интегративних филаментозних фага, на примеру Pf филаментозних фага дизајниране су, описане и упоређене две методе за њихово умножавање. У ту сврху, искоришћена су три референтна соја бактерије *P. aeruginosa* LESB58, PAO1 i UCBPP-PA14 (PA14), која су природно инфицирана са филаментозним фагима PfLES58, Pf4 и Pf5. Метода 1 је подразумевала умножавања фага у 6 L бактеријске културе 48 сати, док је методом 2 искоришћено 600 mL културе и инкубација је трајала шест дана са додавањем свежег медијума и инокулума на свака два дана. Суспензија фага из метода 1 је пречишћена од бактерија центрифугирањем па потом филтрацијом (0,45 и 0,22 μm) док је суспензија фага из методе 2 пречишћена искључиво центрифугирањем у више наврата, односно док талог бактерије није више био видљив. У оба случаја, пречишћени фаги су потом ултрацентрифугирани и дијализирани. Метода 2 се по свим провереним параметрима показала као далеко супериорнија у односу на методу 1. Значај методе 2 је и у томе што се може примењивати и за умножавање филаментозних фага других бактеријских врста. Изолација виралне ДНК из умножених Pf фага и третман различитим ензимима указао је на присуство једноланчане ДНК код PfLES58, Pf4 i Pf5 фага. Такође, код сва три фага утврђено је присуство главног протеина капсида (CoaB) SDS анализом. CoaB протеин је карактеристичан за филаментозне фаге из фамилије *Inoviridae*. Од свих испитаних бактериофага, најшири литички спектар има PfLES58 (90.6%), а потом Pf5 (62.5%), док је најужи имао Pf4 (46.1%). Субинхибиторне концентрације ципрофлоксацина и митомицина Ц могу да повећавају продукцију PfLES58, Pf4 и Pf5 бактериофага. Сличан ефекат у продукцији је забележен и код мутаната који се јављају након инфекције облигатно литичким фагом. Успешно је утврђено да ослобођени вириони Pf фага могу инфицирати друге *P. aeruginosa* сојеве и утицати на промене у њиховом фенотипу. Разлике између Pf инфицираних и неинфицираних сојева су биле очигледне. Код инфицираних сојеве је забележено смањење у аутоагрегацији ћелија и у производњи

пиовердина и пиоцијанина. С друге стране, значајно је повећана покретљивост флагелама и производња биофилма код инифицираних сојева. Осим тога, код неких сојева је повећана појава варијанти малих колонија (енг. *small colony variants*). Овакви ефекти доводе до појаве сојева који су склонији успостављају хроничних инфекција. Pf бактериофаги су повећавали осетљивост инфицираних сојева на одређене антибиотике, као што су тетрациклин, цефтазидим и стрептомицин, отварајући ново поље у терапији фагима где би се филаментозни фаги користили у борби против бактеријских инфекција.

**Abstract.** Filamentous bacteriophages are widely distributed in the bacterium *Pseudomonas aeruginosa* and their infection leads to significant changes in the phenotype of this bacterial species. This group of phages is designated Pf and belongs to the *Inoviridae* family. The first step in analyzing this group of phages was to obtain a suspension with a high virus titer. Given that there is no described method for multiplying integrative filamentous phages, two methods for their multiplication were designed, described and compared using the example of Pf filamentous. For this purpose, three reference strains of *P. aeruginosa* LESB58, PAO1 and UCBPP-PA14 (PA14), which are naturally infected with filamentous phages PfLES58, Pf4 and Pf5, were used. Method 1 involved propagation of phages in 6 L of bacterial culture for 48 hours, while method 2 used 600 mL of culture and incubation lasted six days with the addition of fresh medium and inoculum every two days. The phage suspension from method 1 was purified from bacteria by centrifugation and then by filtration (0.45 and 0.22 μm), while the phage suspension from method 2 was purified only by centrifugation on several occasions, that is, until the bacterial sediment was no longer visible. In both cases, the purified phages were then ultracentrifuged and dialyzed. Method 2 proved to be far superior to method 1 according to all tested parameters. The significance of method 2 is that it can also be used for the multiplication of filamentous phages of other bacterial species. Isolation of viral DNA from multiplied Pf phages and treatment with different enzymes indicated the presence of single-stranded DNA in PfLES58, Pf4 and Pf5 phages. Also, in all three phages, the presence of the main capsid protein (CoaB) was determined by SDS PAGE analysis. The CoaB protein is characteristic of filamentous phages from the *Inoviridae* family. Of all tested bacteriophages, PfLES58 (90.6%) had the widest lytic spectrum, followed by Pf5 (62.5%), while Pf4 (46.1%) had the narrowest. Subinhibitory concentrations of ciprofloxacin and mitomycin C can increase the production of PfLES58, Pf4 and Pf5 bacteriophages. A similar effect in production was also recorded in mutants that appear after infection with an obligatory lytic phage. It was successfully established that the released Pf phage virions can infect other *P. aeruginosa* strains and affect changes in their phenotype. Differences between Pf infected and uninfected strains were apparent. In infected strains, a decrease in cell autoaggregation and pyoverdine and pyocyanin production was recorded. On the other hand, motility by flagella and biofilm production were significantly increased in infected strains. In

addition, the occurrence of small colony variants has increased in some strains. Such effects lead to the appearance of strains that are more prone to establishing chronic infections. Pf bacteriophages increased the sensitivity of infected strains to certain antibiotics, such as tetracycline, ceftazidime and streptomycin, opening a new field in phage therapy where filamentous phages would be used in the fight against bacterial infections.

# 1. Introduction

*Pseudomonas aeruginosa* is a ubiquitous Gram negative bacterium and is able to survive in a wide range of environments (Silby et al., 2011). It is known for its coding capability, which enables great metabolic versatility and high adaptability to environmental changes (Klockgether et al., 2011; Stover et al., 2000). *P. aeruginosa* has been recognized as an opportunistic pathogen, most commonly associated with nosocomial infections and ventilator-associated pneumonia (Barbier et al., 2013). *P. aeruginosa* is susceptible to infection by different bacteriophages, including filamentous phages from the *Inoviridae* family (Knezevic et al., 2015). Unlike other phages, filamentous bacteriophages have a unique life cycle where they continuously exit the host cell without lysing the bacterial cell (Knezevic et al., 2021). Pf phages are filamentous bacteriophages found in *P. aeruginosa* and are known to affect the fitness of their hosts. Pf1 and Pf4 are the most extensively studied phages in this group (Rice et al., 2009; Secor et al., 2017). Here, detailed analyzes of their biology and influence on the phenotype of different strains of *P. aeruginosa* will be conducted in order to determine their role in the virulence of this bacterial species.

## *1.1. Pseudomonas aeruginosa*

### 1.1.1. Characteristics of *Pseudomonas aeruginosa* species

Taxonomic position of the species *P. aeruginosa*, which is the type representative of the genus *Pseudomonas*, is as follows (Brenner et al., 2005):

Regnum (Empire): *Procaryotae*

Phylum (Division): *Proteobacteria*

Classis (Class): *Gammaproteobacteria*

Ordo (Order): *Pseudomonadales*

Familia (Family): *Pseudomonadaceae*

*P. aeruginosa* was likely first reported in human infections by Luke in 1862, who observed rod-shaped particles in blue-green pus of some infections. Similar coloration had been previously observed by Sedillot in 1850 on surgical dressings. The microorganism was first isolated from infections by Gessard in 1882, who called it *Bacillus pyocyaneus*. At the end of the 19th century,

the genus *Pseudomonas* was first described (Migula 1894), and the description was based on phenotype (Cohn 1872). However, at the beginning of the next century, differences in morphology, Gram stain, type of flagellation and metabolism with respect to the oxygen were used to differentiate species within the genus *Pseudomonas* (Orla-Jensen 1909; Bergey et al., 1923). Later, techniques based on DNA enabled the division of pseudomonads into five rRNA subgroups and this classification was reported in Bergey's Manual of Systematic Bacteriology published in 1984 (Palleroni 1984). Sequencing and analysis of 16S ribosomal RNA (16S rRNA) have placed *Pseudomonas* in the class of *Gammaproteobacteria* (Woese et al., 1984). More detailed analyzes of the 16S rRNA gene sequences of 128 *Pseudomonas* species indicated that many species do not strictly belong to the *Pseudomonas* cluster. Individual members of this genus were eventually divided into more than 25 genera that belonged to the classes *Alpha-*, *Beta-* and *Gammaprotebacteria* (Anzai et al., 2000; Peix et al., 2009, García-Valdés and Lalucat, 2016). Since then, a large number of new species have been discovered from different samples within the genus *Pseudomonas*.

*P. aeruginosa* is a Gram-negative, aerobic, oxidase positive rod, 1.5 – 3.0 µm long and 0.5 – 0.8 µm wide, without dormant forms (Fig. 1). This bacterium is an opportunistic pathogen under various circumstances. Due to its ability to colonize various environmental niches and to utilize many environmental compounds as energy sources, *P. aeruginosa* has ubiquitous occurrence (Green et al., 1974; Williams et al., 1976; Glazebrook et al., 1978). The bacterium is motile by one polar flagellum. In addition to flagella, *P. aeruginosa* has pili, which are important virulence factors, being responsible for adhesion and twitching movements (Brenner et al., 2005).

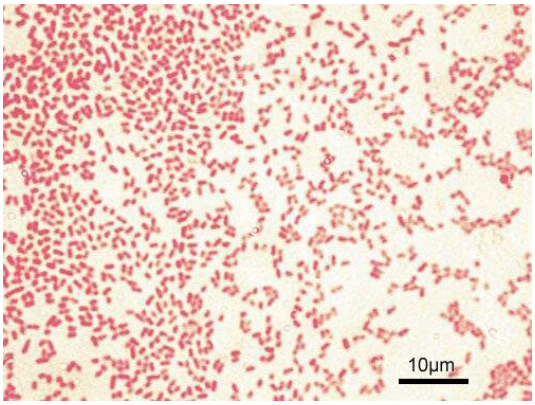


**Figure 1.** Microscopic image of Gram-negative rods of *P. aeruginosa* (Wilson and Pandey, 2022).

*P. aeruginosa* grows on most bacteriological media in broad temperature range from 4 to 42 °C, and its optimum growth temperature is between 37 and 41 °C. Most other pseudomonads cannot grow at 41 °C. Given that it is an obligate aerobe, oxygen is necessary, but growth is present even under anaerobic conditions in the presence of nitrates. High salt concentrations are well tolerated, but heat or desiccation are not. *P. aeruginosa* is catalase and oxidase positive. Bacteria of this species uptake sugars and break them down oxidatively, without being able to ferment them. Also, they reduce nitrates to nitrites and adopt arginine from which they produce ammonia. They liquefy gelatin, break down lipids, but do not hydrolyze starch. They do not utilize maltose and xylose, like many other sugars. *P. aeruginosa* is capable of synthesizing growth factors and vitamins necessary for its own growth (Stratton 1983; Cullimore, 2000; Brenner et al., 2005; Pitt and Simpson, 2006).

Colonies on nutrient agar are large, smooth, bulging in the center or small, convex and rough (Lam et al., 1980). Other types of colonies such as mucoid ones are formed less often. The first type of colony is mostly isolates from pathological material, others are isolated from natural environments, while mucoid ones are isolated from clinical material in cases of urinary and respiratory infections. Also, *P. aeruginosa* can adopt an alternative phenotype termed as small colony variants (SCVs) in standing conditions (Besse et al., 2023). SCVs are characterized by a smaller colony size compared to wild type (WT) colonies. Also, SCVs have pronounced piliation and bind more strongly to abiotic surfaces. *P. aeruginosa* cultures have a characteristic smell due to the production of aminoacetophenone from tryptophan and the smell is most reminiscent of violets or linden.

One of the specificities of *P. aeruginosa* is the production of blue-green phenazine pigment pyocyanin and yellow-green fluorescent pigment pyoverdine. In addition to these pigments, the production of pyorubin (dark red) and pyomelanin (brown) is also possible. To confirm the production of pyocyanin, King A medium is used, containing potassium and magnesium that suppress the production of fluorescein (King et al., 1954). On the other hand, the production of pyoverdine is determined on the King B medium, which has a much lower concentration of the mentioned ions. After incubation, the culture is exposed to UV rays, and only pyoverdine show fluorescence. Among the nonselective media, Mueller-Hinton agar is

particularly suitable for cultivation, as it stimulates the production of the characteristic pigment. The most commonly used differential media for isolation and subcultivation are McConkey, Columbia blood or Endo agar. On blood agar, *P. aeruginosa* usually gives a zone of β-hemolysis. Cetrimide or acetamide are often added to selective media in combination with nalidixic acid. Nitrofurantoin, as well as many other substances such as benzalkonium chloride, irgazan and malachite green, are also added to selective media. Other most often used antibiotics are penicillin G and novobiocin, and the antifungal agent cycloheximide (Shooter et al., 1966; Sands and Rovira, 1970; Thom et al., 1971; Geuenich and Miller, 1982, Atlas, 1995). Substrates containing trimethoprim and lauroyl sarcosine are used less often, such as S1 substrate, which contains casamino acids and S2, rich in L-asparagine (Gould et al., 1985).

Characterization of isolated strains is currently based on serotyping and analysis of sequenced bacterial genomes, and less often on pyocin typing or phagotyping (Pitt and Simpson, 2006; Cunningham et al., 2022).

*P. aeruginosa* can grow both planktonically and in a form of a biofilm, being attached to a surface. The key steps in the biofilm life cycle are reversible or irreversible adhesion, formation and maturation of the biofilm and its dispersion (Fig. 2) (Rumbaugh and Sauer, 2020). Reversible attachment is achieved through electrostatic and hydrophobic interactions. These interactions are unstable and the cells often return to the liquid phase. The main role in attaching cells to the surface is played by flagella, but it has been proven that pili (type I and IV), curli fibers and antigen 43 also help attachment (Kostakioti et al., 2013). After binding to the surface, the cells begin to form microcolonies. At the same time, they produce the extracellular matrix. The extracellular matrix is important for biofilm adhesion and protection, and is composed of exopolysaccharides, proteins, and extracellular DNA (eDNA) (Costerton et al., 1999; Wei and Ma, 2012). In addition to the production of biofilm matrix components, this sessile way of growth also implies loss of expression of flagellum genes, induction of antibiotic resistance mechanisms and increased production of virulence determinants (Williamson et al., 2012; Liao et al., 2013; Heacock-Kang et al., 2017). Mature biofilms can have different structures (Rumbaugh and Sauer, 2020). The existence of unstructured and flat biofilms, as well as structures reminiscent of mushrooms, was confirmed. There are also pillar-like structures, often designated as micro- or macrocolonies, that

are interspersed with fluid-filled channels. Biofilm dispersion can be divided into active and passive dispersion (Wille and Coenye, 2020; Wille et al., 2020). Active dispersion is regulated by the intracellular second messenger cyclic di-GMP (Corrigan and Gründling, 2013). An increase in cyclic di-GMP leads to the formation of a biofilm, while a decrease in it leads to the dispersal of biofilm components. Cells leave the biofilm most likely through small holes in the matrix structure, where upon exiting they leave large transparent cavities or hollow structures composed of non-motile cells (Sauer et al., 2002; Purevdorj-Gage et al., 2005; Davies and Marques, 2009). This dispersion of cells can also occur due to increased production of rhamnolipids which cause detachment in biofilm via central hollowing pattern (Wang et al., 2013; Wood et al., 2018). Additional inducers of dispersion are *cis*-2-decenoic acid (Marques et al., 2015), oxygen (Thorman et al., 2006; Petrova et al., 2012), pyruvate (Eschblach et al., 2004; Schreiber et al., 2006) as well as cell death (Thomas and Hancock, 2009). However, these mechanisms that lead to cell dispersion are still not fully understood.

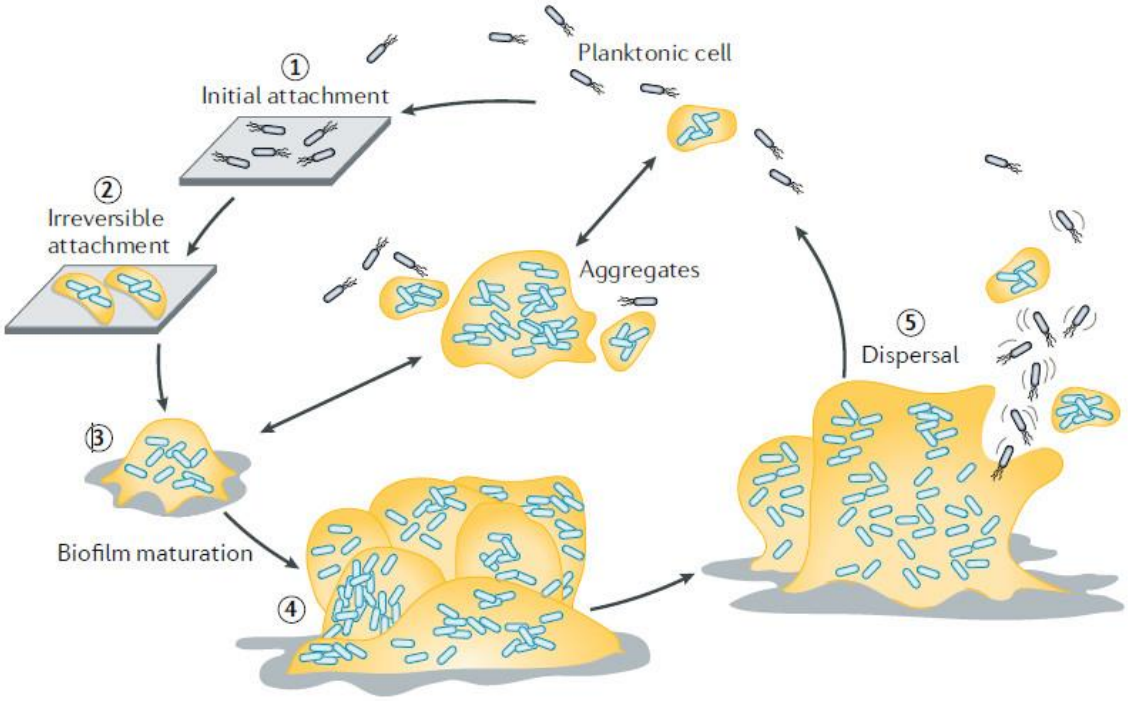


**Figure 2.** Biofilm formation and dispersion (Rumbaugh and Sauer, 2020).

During biofilm maturation, bacteria undergo physiological changes and become more resistant to environmental stresses. *P. aeruginosa* biofilms are thought to be the cause of many chronic and recurrent infectious diseases that complicate the treatment of bacterial infections. Antibiotics have been found to be ineffective in bacteria growing in biofilms (Burns et al., 1993; Hoiby, 1993; Costerton et al., 1999; Singh et al., 2000; Yuan et al., 2019). It has been proven on several occasions that bacteria in biofilm are 10 – 1000 times more tolerant to antibiotics than planktonic cells (Rumbaugh and Sauer, 2020; Ju et al., 2020; Wu et al., 2021). In general, this effect is attained as the biofilm acts as a physical obstacle that hinders the entry of antibiotics into bacterial cells (Drenkard, 2003). Moreover, the presence of multidrug-tolerant persister cells within biofilms contributes to the prolonged and recurrent infections observed in cystic fibrosis patients (Mulcahy et al., 2010).

In the case of *P. aeruginosa*, the primary mechanisms of resistance in the biofilm include prevention of the antibiotic passage into the cells, antimicrobial neutralization by biofilm matrix, alteration in the biofilm cell growth rate, trigger of an adaptive stress response, and persister presence (Stewart, 2002). Multiple factors contribute to the regulation of *P. aeruginosa* biofilms, with a significant reliance on the quorum sensing system, two regulatory systems GacS/GacA and RetS/LadS, cdiGMP, and exopolysaccharides (Rasamiravaka et al., 2015). Another crucial component of any biofilm is the release of eDNA from lysed cells. eDNA facilitates cell adhesion and aggregation on surfaces, but also indirectly influences the expression of specific genes after recombinations, leading to increased resistance to antibiotics, such as aminoglycoside (Das et al., 2010; Wilton et al., 2016).

Persister cells emerge as a transient phenotype resulting from the heterogeneous response of *P. aeruginosa* to environmental changes within a genetically identical bacterial population (Balaban et al., 2013). Comprising approximately 1% of biofilm cells, persister cells are metabolically inactive, exhibit slow growth, and display high tolerance to antibiotics (Lewis, 2010; Wood et al., 2013). Also, the cells within the biofilm together produce a large amount of antibiotic degrading enzymes. Due to these characteristics, these cells continue to proliferate once antibacterial treatment ceases, enabling the survival of *P. aeruginosa* and the establishment of chronic infections (Maisonneuve and Gerdes, 2014).

This type of resistance of *P. aeruginosa* to antibiotics represents adaptive resistance. Adaptive resistance is a phenomenon in which a bacterium enhances its ability to withstand antibiotic attack by undergoing temporary changes in gene and/or protein expression in response to environmental factors. These alterations enable the bacterium to survive the presence of antibiotics. Importantly, adaptive resistance is a reversible process that ceases when the environmental stimulus is no longer present (Sandoval-Motta and Aldana, 2016). However, in addition to adaptive resistance, other mechanisms employed by *P. aeruginosa* to counteract antibiotics can be categorized into intrinsic and acquired resistance.

Intrinsic resistance involves factors such as low permeability of the outer membrane, the expression of efflux pumps that actively remove antibiotics from the cell, and the production of enzymes that inactivate antibiotics. The majority of antibiotics need to traverse the cell membrane in order to reach their target and exert their effects (Lambert et al., 2002). Quinolones and β-lactams pass through the membrane via porin channels, while polymyxins and aminoglycosides facilitate their own uptake by interacting with bacterial lipopolysaccharides on the outer membrane of Gram-negative bacteria. However, the outer membrane permeability of *P. aeruginosa* is highly restricted, being approximately 12 to 100 times lower than that of *E. coli* (Bellido et al., 1992; Hancock and Brinkman, 2002). Bacterial efflux pumps have a significant role in eliminating harmful substances from the cell and can be categorized into five families: resistance-nodulation-division (RND) family, major facilitator superfamily (MFS), ATP-binding cassette (ABC) superfamily, small multidrug resistance (SMR) family, and multidrug and toxic compound extrusion (MATE) family (Sun et al., 2014).

*P. aeruginosa* also produces enzymes that can degrade or modify antibiotics. Many antibiotics contain chemical bonds, such as amides and esters, which are susceptible to hydrolysis by enzymes such as β-lactamases and aminoglycoside-modifying enzymes (Poole, 2005; Wright, 2005; Wolter and Lister, 2013). Certain *P. aeruginosa* isolates also produce extended-spectrum beta-lactamases (ESBLs), which confer resistance to most β-lactam antibiotics, including penicillins, cephalosporins, and aztreonam (Paterson and Bonomo, 2005; Rawat and Nair, 2010). The aminoglycoside-modifying enzymes includes aminoglycoside phosphotransferase (APH),

aminoglycoside acetyltransferase (AAC), and aminoglycoside nucleotidyltransferase (ANT), which play a dominant role in *P. aeruginosa* resistance to this group of antibiotics.

Acquired resistance can be achieved through horizontal gene transfer of antibiotic resistance genes or through mutations (Breidenstein et al., 2011). In conjunction with the significant inherent antibiotic resistance observed in *P. aeruginosa*, the acquisition of resistance mechanisms plays a crucial role in the emergence of multidrug-resistant strains, posing challenges in effectively eliminating this microorganism and contributing to the persistence of infections (Henrichfreise et al., 2007). Antibiotic resistance genes can be present on various mobile genetic elements such as plasmids, transposons, integrons, and prophages. Bacteria have the ability to acquire these genes through horizontal gene transfer from the same or different bacterial species (Breidenstein et al., 2011). Integrons, in particular, are genetic elements that facilitate the insertion of mobile gene cassettes into specific sites through site-specific recombination (Hall and Collis, 1995). Studies have demonstrated the crucial role of integrons in the dissemination of antibiotic resistance among *P. aeruginosa* strains (Chen et al., 2009; Nikokar et al., 2013; Odumosu et al., 2013; Khosravi et al., 2017; Urbanowicz et al., 2021). The acquisition of aminoglycoside and β-lactam resistance genes has been documented in *P. aeruginosa* (Bonomo and Szabo, 2006; Yan et al., 2006; Poole, 2011; Cavalcanti et al., 2015; Hong et al., 2015).

Mutations have the capability to induce diminished uptake of antibiotics, modifications in antibiotic targets, and increased expression of efflux pumps and antibiotic-inactivating enzymes, enabling bacteria to persist in the presence of antimicrobial agents (Munita and Arias, 2016). For instance, Mandsberg et al. (2009) conducted a study showing that the inactivation of the DNA oxidative repair system in *P. aeruginosa* results in higher mutation frequencies, leading to enhanced production of β-lactamase and upregulation of the MexCD-OprJ efflux pump. Furthermore, spontaneous mutations can affect the function or expression of porins, leading to changes in membrane permeability and increased resistance to antibiotics (Fernandez and Hancock, 2012). Additionally, mutations can alter the target sites for antibiotics, rendering the bacterium more resistant to the given antibiotic (Munita and Arias, 2016). Mutations in genes encoding DNA gyrase or topoisomerase IV can confer increased tolerance of *P. aeruginosa* to

quinolones, as these two enzymes are precisely the target sites for this group of antibiotics (Bruchmann et al., 2013; Aldred et al., 2014).

New antibiotics with novel modes of action have been investigated in recent years, along with new methods of administration and resistance modification against bacterial enzymes. Some of these new antibiotics have demonstrated excellent *in vitro* antibacterial activity against *P. aeruginosa* (El Solh and Alhajhusain, 2009; Walkty et al., 2014; Cigana et al., 2016). Furthermore, recent studies have highlighted several innovative non-antibiotic therapeutic approaches that exhibit high efficacy in eradicating antibiotic-resistant *P. aeruginosa*. These approaches include the inhibition of quorum sensing and bacterial lectins, iron chelation, vaccine strategies, nanoparticles, antimicrobial peptides, electrochemical scaffolds, and phage therapy (Chatterjee et al., 2016).

### 1.1.2. *P. aeruginosa* sensitivity to antimicrobial agents

*P. aeruginosa* is most often sensitive to semi-synthetic penicillins (ticarcillin), ureidopenicillins (azlocillin, mezlocillin and piperacillin), carboxypenicillins (carbenicillin), third-generation cephalosporins (cefotaxime, ceftriaxone, ceftazidime and cefpodoxime), fourth generation cephalosporins (cefepime and cefpirome), cephamycins (cefoxitin, cefotetan and cefmetazol), carbapenems (imipenem, meropenem), monobactams (aztreonam), aminoglycosides (gentamicin, tobramycin, netilmicin, amikacin), fluoroquinolones (ciprofloxacin, norfloxacin) and polymyxins (polymyxin B and colistin). Although the mentioned antibiotics are active against *P. aeruginosa*, this bacterium can acquire resistance to each of them. Because monotherapy rarely results in bacterial eradication, a combination of two and sometimes more antibiotics is most often used (Paul et al., 2004; Giamarellou and Antoniadou, 2001). The acquisition of resistance, together with innate resistance to antibiotics, contributes to the emergence of multidrug-resistant strains of *P. aeruginosa*, whose infections are difficult to treat. The World Health Organization (WHO) has included carbapenem-resistant *P. aeruginosa* on the list of three priority pathogens for which new antibiotics need to be developed (Tacconelli et al., 2017). Moreover, the excessive use of antibiotics in patient treatment accelerates the emergence

of new multidrug-resistant *P. aeruginosa* strains, rendering empirical antibiotic therapy ineffective against this microorganism (Hirsch and Tam, 2010).

### 1.1.3. The significance of *P. aeruginosa* species

*P. aeruginosa* is widely distributed in nature. It can be isolated from different types of freshwaters, including even distilled water, but not from sea water (Favero et al., 1971). It is present in various wet environments, such as bathrooms, washing machines, taps etc. In water distribution systems, it represents one of the most important microorganisms responsible for biofilm formation and persistance.

As an opportunistic pathogen, *P. aeruginosa* is known to cause various infections in humans. Some of these infections are mild, such as otitis externa, to life-threatening ones (Balasubramanian et al., 2013). It most often colonizes the lung epithelium and urinary tract (Balcht and Smith, 1994). Patients with burns, as well as immunocompromised patients with AIDS or cystic fibrosis (CF) are at high risk of developing serious *P. aeruginosa* infections, which account for high death rate in this population (Manfredi et al., 2000; Bouza et al., 2002; Valderrey et al., 2010). *P. aeruginosa* is a prevalent pathogen in CF lung infections. These infections are chronic and resistant to antibiotic treatment, leading to a decline in pulmonary functions and increased mortality in CF patients (Lyczak et al., 2002). Additionally, *P. aeruginosa* contributes to more than 5% of infectious exacerbations in individuals with chronic obstructive pulmonary disease, and its presence has been linked to higher mortality rates in these patients (Murphy, 2009).

*P. aeruginosa* is able to colonize medical devices while growing in biofilms. *P. aeruginosa* can be found as a microbiota of the intestinal tract in 10% of people, and sometimes in the saliva of 5% of people. However, the highest prevalence is among hospitalized patients with burns (Holder, 1977; Mousa, 1997; Estahbanati et al., 2002; Church et al., 2006). There are no major differences between strains isolated from natural environments and from pathological material, but strains from the natural environment have the ability to absorb carbon from a wide range of organic compounds (Foght et al., 1996). Moreover, *P. aeruginosa* is able to interact and coexist

with other microorganisms in multispecies communities. Such infections are often detrimental to patients.

The emergence and spread of multidrug-resistant *P. aeruginosa* strains have recently become a public health concern (Horcajada et al., 2019). Beside many mechanisms of resistance, the bacterium is more tolerant to antibiotics in biofilms and more resistant to host responses, which leads to the chronic infections (Lebeaux et al., 2014; Maurice et al., 2018). For all these reasons, the bacterium is one of the most common nosocomial pathogens.

Animal models have multiple significance in understanding *P. aeruginosa* pathogenesis and for the development of new antibiotics or novel biologicals to control infections caused by this bacterium. Most of the studies related to this bacterium have been done on mouse or rat models (Wood et al., 2023). The first animal model used to investigate chronic pulmonary infection caused by *P. aeruginosa* was the rat model (Cash et al., 1979). The presence of *P. aeruginosa* can be proven even after 35 days from the beginning of the infection. Later, numerous other animal models such as rabbits, porcine, dogs and cats were used to study acute and chronic pneumonia caused by *P. aeruginosa* infection (Camolli et al., 1999, Van Heeckeren et al., 2002, Li Bassi et al., 2014, Luque 2014, Gras et al., 2019, Dear 2020, Nguyen et al., 2021).

In addition to infecting vertebrates, *P. aeruginosa* is also pathogenic for invertebrates such as *Caernorhabditis elegans* (Mahajan-Miklos et al., 1999), *Drosophila melanogaster* (D'Argenio et al., 2001), *Galleria mellonella* (Jander et al., 2000 ; Miyata et al., 2003), *Bombyx morio* (Kaito et al., 2002) and *Plutella xylostella* (Jander et al., 2000) and many others. Plants are also prone to infection by *P. aeruginosa*. This bacterium most often causes leaf rot in *Arabidopsis thaliana*, *Lactuca sativa*, *Allium caepa*, *Solanum tuberosum*, *Nicotiana tabacum* and *Musa* sp. (Rahme et al., 1997; Plotnikova et al., 2000).

*P. aeruginosa* can also cause food spoilage. Members of the genus *Pseudomonas* cause spoilage of red meat, chicken meat, eggs and seafood. The presence of *Pseudomonas* in fresh meat leads to stickiness, rancidity and changes in the color of the meat. Eating such food can lead to serious and even fatal diseases (Baltch and Smith, 1994; Arnaut-Rollier et al., 1999; Franzetti and Scarpellini, 2007).

### 1.1.4. *P. aeruginosa* virulence factors

The wide spectrum of virulence factors possessed by *P. aeruginosa* can be divided into those related to the cell surface (pili, flagella, lipopolysaccharides and alginate) and those secreted from the cell (proteases, phospholipase C, chromophores, pyocins, exoenzymes and exotoxins). While cell wall and membrane-associated virulence factors are generally effective in colonization and establishment of chronic infection, extremely toxic extracellular factors are related to acute infection (Benie et al., 2017).

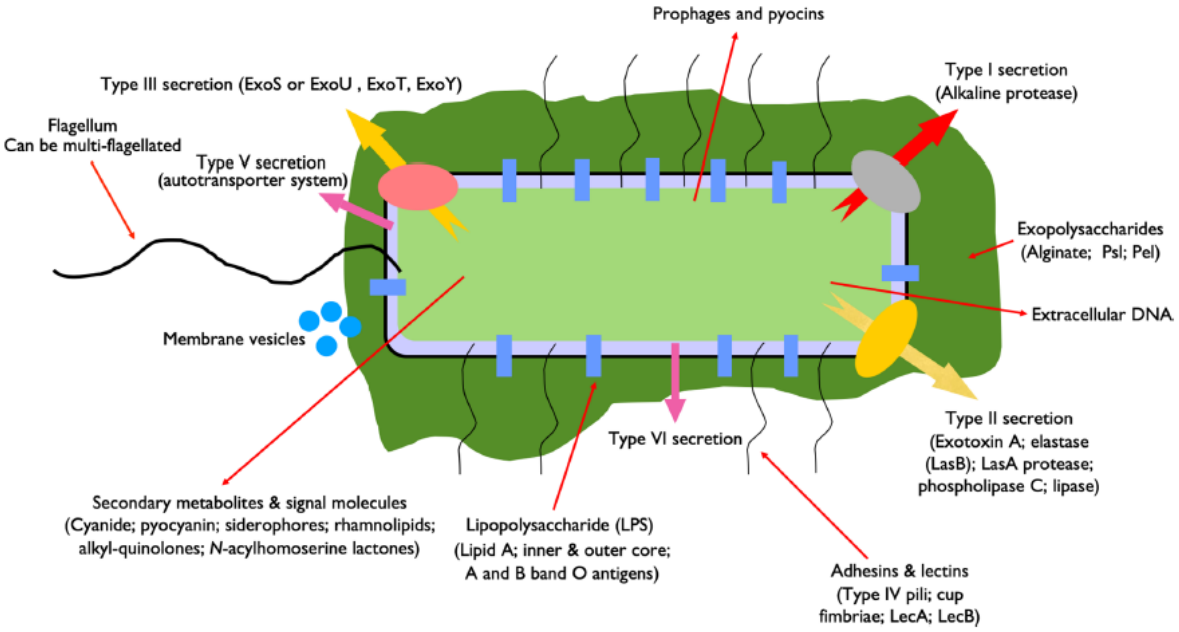


**Figure 3.** Schematic representation of the main virulent factors of the bacterium *P. aeruginosa* (Diggle and Whiteley, 2020).

Bacteria exhibit twitching motility using type IV pili (Fig. 3) (Bradley, 1980). This type of movement is important during the grouping of cells into microcolonies and the formation of biofilms (O'Toole and Kolter, 1998). Moreover, type IV pili are also important during the interaction of bacteria with mammalian cells. *P. aeruginosa* also has both swarming and swimming motility, using flagella (Fig. 3). With the help of flagella, the bacterium manages to overcome the repulsive forces of the surface on which it is located and thus establishes initial contact (Pratt and Kolter, 1999). Flagella also help in the acquisition of essential nutrients and are a great immunogen (Feldman et al., 1998).

Lipopolysaccharides (LPS) are important in the interaction of bacterial cells with the external environment (Fig. 2.) (Le Berre et al., 2011). LPS consist of a hydrophobic domain known as lipid A, which is a toxin, a non-repeating oligosaccharide, and an extrinsic polysaccharide (O-antigen). Modifications of lipid A can enable greater resistance to cationic antimicrobial peptides or activate inflammatory responses (Ernst et al., 1999; Moskowitz et al., 2004; Pamp et al., 2008; Gellatly et al., 2012). O-antigen is used in *P. aeruginosa* serotyping (Faure et al., 2003). The presence of lipopolysaccharide in the host activates innate and adaptive immune responses, but less than exotoxins. Most often, LPS as pyrogens lead to dysregulated inflammatory responses that contribute to morbidity and mortality (Rocha et al., 2019). In addition to LPS, *P. aeruginosa* possesses the exopolysaccharides, such as alginate (Fig. 3). Alginate has a mucoid structure made of mannuronic and guluronic acid polymers. Alginate production protects *P. aeruginosa* from dehydration, antibiotics, phagocytosis and acquired host response (Benie et al., 2017; Van Delden, 2004). Also, alginate is an integral part of the biofilm structure, but it is not crucial for its formation (Stapper et al., 2004).

Rhamnolipids belong to the group of biosurfactants consisting of a dimer of fatty acids alkanoic acids, composed of ten carbon chains that are attached to one or two molecules of L-rhamnose (Fig. 3) (Constantino-Teles et al., 2022). Rhamnolipid synthesis is under the control of various transcriptional and post-transcriptional regulators, but mostly under the control of the Rhl quorum sensing system (Ochsner et al., 1994; Rahim et al., 2001; Soberón-Chávez et al., 2021). Rhamnolipids play an important role in respiratory infections because they slow down the ciliary beat frequency and can affect mucociliary transport, which disrupts bacterial clearance (Read et al., 1992; Kanthakumar et al., 1996). Also, rhamnolipids inhibit phagocytosis by macrophages in the lungs and induce necrosis of neutrophils (McClure and Schiller, 1996; Jensen et al., 2007; Van Gennip et al., 2009). The presence of rhamnolipids also affects swarming motility, biofilm structure by maintaining channels that enable the diffusion of nutrients and oxygen, help in biofilm dispersion and increase the bioactivity of quinolone signals that control several virulence factors (Davey et al., 2003; Caiazza et al., 2005; Calfee et al., 2005; Tremblay et al., 2007; Nickzad and Déziel, 2014; Lin et al., 2018). Finally, rhamnolipids inhibit the growth of

other microorganisms in the lungs in the presence of *P. aeruginosa* such as *Staphylococcus aureus* (Radlinski et al., 2017; Gdaniec et al., 2021).

*P. aeruginosa* produces enzymes Elastase A (LasA) and B (LasB) (Fig. 3). LasA is a staphylolysin, while LasB is a pseudolysin and both represent important virulence factors. Elastases participate in the degradation of host tissues and components such as collagen, immunoglobulin G, surfactants and complementary proteins (Mariencheck et al., 2003; Matsumoto, 2004; Mun et al., 2009; Le Berre et al., 2011). In addition to elastases, proteases are also present. Alkaline protease is a metallo-endopeptidase (Fig. 3), known as aeruginolysin, and has multiple roles. For example, alkaline protease interferes with complement-mediated cell lysis (Laarman et al., 2012). This protease also inhibits bacterial clearance by cleaving the C2 component of the complement system, so the complement-mediated phagocytosis does not occur (Laarman et al., 2012). On the other hand, protease IV is a lysyl endopeptidase and specifically cuts carboxyl side substrates of lysine-containing peptides. Protease IV degrades fibrinogen, plasminogen, immunoglobulin G, surfactant proteins and certain complement components of the immune defense system (Van Delden, 2004; Hoge et al., 2010). *P. aeruginosa* secretes phospholipase C via the type II secretion system (Fig. 3). Phospholipase C is a cytotoxin responsible for β-hemolysis and affects signaling processes in various eukaryotic cells (Barker et al., 2004).

*P. aeruginosa* pigments are also important for virulence (Lau et al., 2004). Pyocyanin is a redox active phenazine pigment which produces reactive oxygen species due to the intracellular redox cycle (Fig. 3). Phenazines have numerous beneficial physiological roles because they participate in cellular signaling, in protection from oxidizing agents, supporting survival in the case of limited electron acceptor, getting nutrients (e.g. iron), in energy production, in protection from UV radiation and in the formation of biofilms (Laursen and Nielsen, 2004; Dietrich et al., 2006; Wang et al., 2010; Wang et al., 2011; Glasser et al., 2014). It is known that pyocyanin inhibits neutrophil superoxide formation and lymphocyte proliferation, increases the production of IL-8 by human airway epithelial cells, inhibits cell respiration, impairs ciliary function and causes oxidative damage to the lung epithelium. Also, it is assumed that it influences neutrophil

apoptosis, lowers intracellular cAMP levels as well as the production of reactive oxygen intermediates (Van Delden, 2004; Le Berre et al., 2011; Meirelles and Newman, 2018).

Pyoverdine is an iron-chelating molecule that is also produced by *P. aeruginosa*. In addition to its role as a siderophore that chelate iron, pyoverdine is a signaling molecule because it leads to the production of the extracellular virulence factor protease PrPL and exotoxin A (Visca et al., 2007; Cornelis and Dingemans, 2013).

Exotoxin A (ExoA) inhibits elongation factor 2 (EF-2) and thus inhibits protein synthesis in eukaryotic cells with its ADP-ribosyl transferase property, leading to cell death (Fig. 3). Also, it was found that this toxin damages host tissue and aids in bacterial invasion (Van Delded and Iglewski, 1998; Rocha et al., 2019).

However, in addition to all the described virulence factors, the most important one for *P. aeruginosa* is the biofilm formation capacity. Forming a biofilm, *P. aeruginosa* has a great advantage in establishing infections. As already said, biofilms are structured communities of sessile bacteria that are protected by an exopolysaccharide matrix and they provide bacteria with homeostasis and stability in unfavorable environmental conditions (Costerton et al., 1999; Mann and Wozniak, 2012). Because of its ability to protect bacteria from host defenses and antimicrobial agents, biofilm research has become a focus in order to develop alternative ways to control infections caused by *P. aeruginosa* (Costerton et al., 1999; Lebeaux et al., 2014; Lewis 2008; Maurice et al., 2018).

#### 1.1.4.1. Lysogenic conversion in *P. aeruginosa* contributing virulence

Due to its marked genomic versatility and the production of numerous different virulence factors, *P. aeruginosa* is a widespread bacterial species (Breidenstein et al., 2011). The diversity and widespread nature of *P. aeruginosa* is also reflected in the distribution of its specific bacterial viruses, also known as bacteriophages. *P. aeruginosa* phages can be found in various environments, including rich organic soil, water samples, and even in human tissue in case of infection with this bacterium (Ceyssens et al., 2010 James et al., 2015). Bacteriophages represent obligate intracellular parasites incapable of multiplying outside the bacterial cell. They are

categorized into two main groups based on their interactions with bacterial hosts: obligatory lytic phages, which cause lysis of bacterial hosts after replication (Sharma 2013), and temperate phages, which either integrate their genetic material into bacterial genomes or exist episomally (Howard-Varona et al., 2017). Bacteriophages integrated into the bacterial chromosome are designated as prophages, and their bacterial host as a lysogen. Temperate bacteriophages are known to affect the phenotype of its bacterial hosts through a process called lysogenic conversion (De Smet et al., 2017). Furthermore, it has been confirmed that their presence leads to accelerated genome diversification and adaptive evolution in bacteria (Davies et al., 2016A).

Interestingly, by 2010 at least 60 different temperate bacteriophages have been characterized in *P. aeruginosa* and most strains possessed at least one prophage (Kung et al., 2010). Today, that number is much higher. *P. aeruginosa* specific temperate phages can affect the virulence of their hosts through the transfer and expression of virulence factors. Although the most famous case was recorded in *V. cholerae* with CTXφ phage encoding cholera toxin (Waldor and Mekalanos, 1996), there is a similar pore-forming toxin in *P. aeruginosa* encoded with φCTX prophage (Hayashi et al., 1990; Nakayama et al., 1999). *P. aeruginosa* strains infected with φCTX are significantly more virulent. Similarly, the *P. aeruginosa* prophage encodes a peptide inhibitor of α-polymerase protein (Iap), similar to the Wzz protein that regulates the chain length of the O-antigen side chain of LPS (Taylor et al., 2013). In this way, serotype conversion to *P. aeruginosa* occurs, whereby superinfections by LPS-binding phages are prevented, as well as increased adherence and evasion of the host immune response. Superinfection exclusion is a phenomenon when a temperate phage within its host blocks a potential infection by another phage (Abedon 1994; Brüssow et al., 2004; Hyman and Abedon, 2010; Chung et al., 2014). This phage-mediated physiological change significantly benefits *P. aeruginosa*.

Temperate bacteriophages also help *P. aeruginosa* in establishing chronic infections and protect the bacterium from various host defensive mechanisms. For example, the FIZ15 phage, isolated from an infected human wound, effectively enhanced the strain's resistance to phagocytosis by mouse peritoneal macrophages, improved adhesion to human buccal epithelial cells, and increased its resilience against killing by normal human serum (Vaca-Pacheco et al.,

1998). Also, a connection between temperate phages and the occurrence of mucoid phenotypes of *P. aeruginosa* in patients with cystic fibrosis was established (Miller and Rubero, 1984). Using endolysin from a cryptic prophage, *P. aeruginosa* can cause lysis of its cells and release of eDNA, where eDNA as an important component of every biofilm matrix contributes to strengthening the biofilm structure (Gloag et al., 2013; Turnbull et al., 2016). Lastly, the highly virulent *P. aeruginosa* strain, LESB58, possesses as many as four different prophages that increase its fitness in chronic lung infections (Davies et al., 2016B; Lemieux et al., 2016). However, the exact mechanisms of their action are not yet known.

Among the temperate phages discussed, the predominant focus has been on tailed phages, primarily due to their ease of isolation and propagation. Nevertheless, it is important to note that bacteria can also be infected by non-tailed phages, such as filamentous representatives. Interestingly, 60% of analyzed isolates of this bacterium possessed at least one genetic element of filamentous prophages (Knežević et al., 2015). Still, *P. aeruginosa* filamentous phages represent a relatively understudied group, with limited research conducted on their influence on bacterial host phenotypes, which has been primarily limited to one example of interaction between a filamentous phage and its natural host (Pf4 – PAO1). There remains a lack of comprehensive knowledge about other *P. aeruginosa* filamentous phages and their broader impact on other *P. aeruginosa* strains.

### 1.2. Filamentous bacteriophages

Filamentous phages of *P. aeruginosa* belong to the family *Inoviridae* (Fig. 4) and differ significantly from other phages in terms of morphology and life cycle. These filamentous bacteriophages are classified as follows:

Realm: *Monodnaviria*

Kingdom: *Loebvirae*

Phylum: *Hofneiviricota*

Class: *Faserviricetes*

Order: *Tubulavirales*

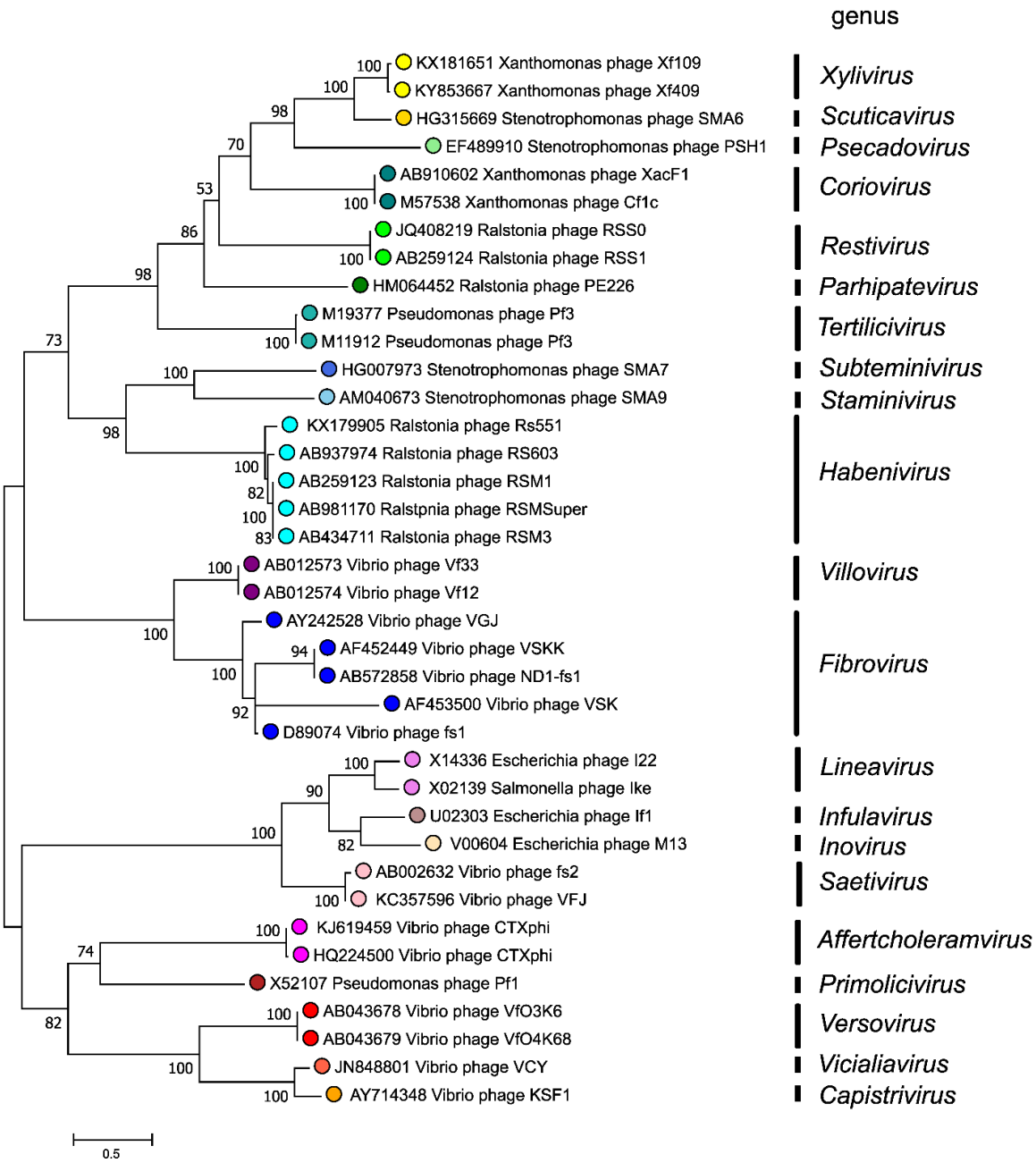


**Figure 4.** Phylogenetic relationships of recognized species of filamentous bacteriophages within the family *Inoviridae* (Knezevic et al., 2021).

The family *Inoviridae* is classified under the order *Tubulavirales*, together with the families *Plectroviridae* and *Paulinoviridae* (Knezevic et al., 2021). In contrast to *Inoviridae* phages, the members of *Plectroviridae* infect cell wall-less bacteria by attaching to the bacterial

surface. Representatives of the *Paulinoviridae* family infect *Thermus* and *Propionibacterium* species. The *Inoviridae* family comprises over 20 genera, with several of them having only one species (Fig. 4). Filamentous phages belonging to the same genus share high similarity in DNA sequence, but also >50% in amino-acid sequences of morphogenesis (p1) and major coat protein (p8) (Knezevic et al., 2021). Phages belonging to the same species share >95% in DNA sequence and have high similarity with the minor coat protein (p3) amino-acid sequence.

### 1.2.1. Morphology

Bacteriophages from the *Inoviridae* family exhibit helical symmetry, and the filaments are rigid or flexible, and contain single-stranded DNA (ssDNA) (Knezevic et al., 2021). The length of the virion is in a function of the size of the genome and changes may occur due to insertions, deletions or mutations that lead to changes in coat proteins (Fauquet et al., 2015). This group of phages can also be mobilized in alternative capsids and their physicochemical nature of DNA packaging can be changed and adapted (King et al., 2012). The diameter of most virions is similar for all members, measuring approximately 7 nm. The length varies from 700 nm (Pseudomonas phage Pf3) to 2000 nm (Pseudomonas phage Pf1). Electron micrographs reveal different structures at the virion ends of filamentous phages. For example, the composition of Escherichia phage M13 virions was analyzed, revealing the presence of approximately 2700 copies of the major coat protein (CoaB, p8), accompanied by the minor coat protein (CoaA, p3), as well as p6 at the rounded end (Fig. 5). Additionally, the blunt end was formed by five copies each of p7 and p9 proteins. Due to their distinctive morphology and simple genomes, filamentous bacteriophages find extensive utility in various biotechnological applications, including drug delivery nanocarriers and phage display techniques (Bradbury and Marks, 2004; Nam et al., 2008; Henry et al., 2015; Ju and Sun, 2017).

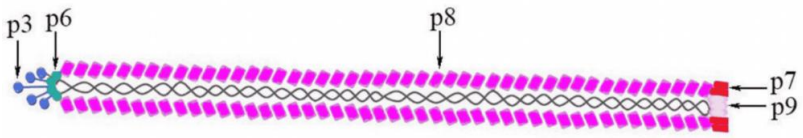


**Figure 5.** A schematic diagram of the virion structure of Escherichia phage M13 (species Escherichia virus M13), providing a visual representation of its key components (Knezevic et al., 2021).

### 1.2.2. Physicochemical characteristics

Members of the *Inoviridae* family are sensitive to chloroform, which is unusual for non-enveloped viruses, and resistant to heat and a wide range of pH. The flotation density of virions in CsCl is 1.29 ± 0.01 g/cm$^3$ and the DNA component is 6 – 14%. Mr virion is 12-34 x 10$^6$, while sedimentation coefficient (S20w) values are in a narrower range, 41 – 44 S. The rate of sedimentation is mostly determined by the ratio of virion mass and length (Fauquet et al. 2005).

### 1.2.3. Genome characteristics

Virions contain one molecule of infectious, circular, positive ssDNA (Fig. 1) (Knezevic et al., 2021).

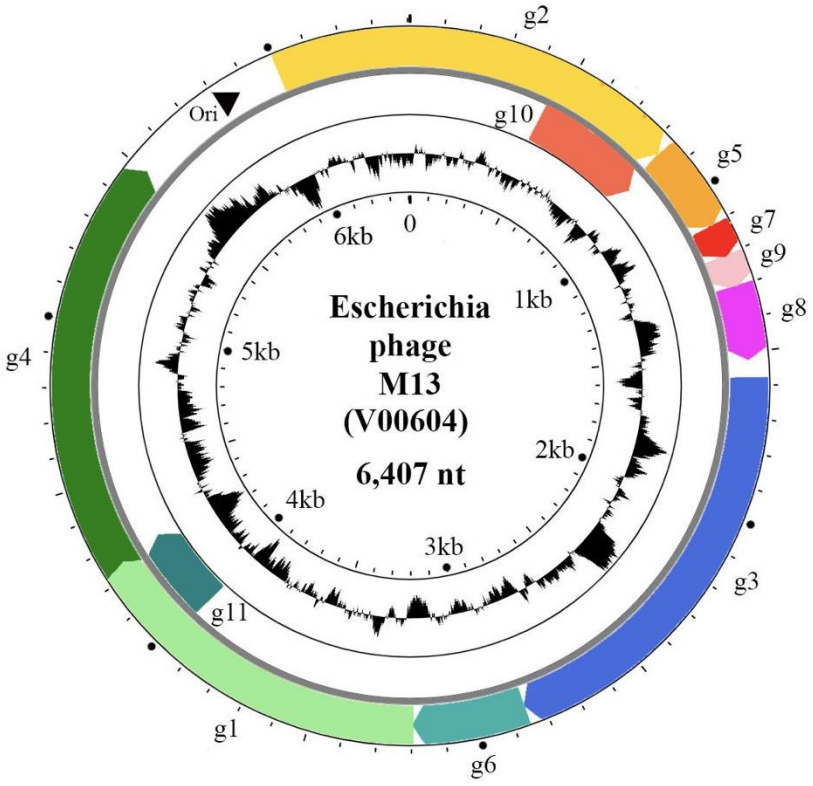


**Figure 6.** The genome of Escherichia phage M13 is presented in a circular format, highlighting its gene organization (Knezevic et al., 2021). The genes are depicted as colored arrows and can be classified into three modules: replication genes (*g2*, *g5*, and *g10*), structural genes (*g7*, *g9*, *g8*, *g3*, *g6*), and morphogenesis genes (*g1*, *g4*, and *g11*). The inner circle represents the GC content of the genome.

The size of the genome ranges from 4.5 to 12.4 kb and the genome encodes 4 – 17 proteins, depending on the species. The genes are closely positioned and often overlap. These bacteriophages possess three functional modules: a replication module, a structural module, and a morphogenesis (packaging/secretion) module. In the M13 phage, the replication module contains genes *g2*, *g5* and *g10* that encode proteins responsible for replication and ssDNA binding (Fig. 6). The structural module contains the genes for the structural proteins that build the viral particle (*g3*, *g6*, *g7*, *g8* and *g9*) (Fig. 6). The *g8* (*coaB*) encodes the major capsid protein (Table 1). Thousands of p8 subunits form a filament that wraps around the ssDNA. Genes *g7* and *g9* encode two small capsid proteins positioned at the end of the filament, while *g3* and *g6* encode two other proteins at the opposite end of the filament (Fig. 6). Protein p3 (CoaA) is the largest protein of filamentous phages and is crucial in their adsorption to the host cell. The morphogenesis module is responsible for morphogenesis and formation of viral particles (*g1*, *g4*, *g10* and *g11*) (Fig. 6).

Within the intergenic regions of filamentous phages, the origin of replication, packaging signals and promoters can be found (Knežević et al., 2021). In the case of integrative filamentous phages, the insertion sequences and proteins for latent stage regulation (repressors) are present, while some phages also encode integration proteins such as integrases or transposases.

In addition to the core genes, filamentous bacteriophages also possess accessory genes (Mai-Prochnow et al., 2015). For example, Pf4 bacteriophages encode several genes: reverse transcriptase (RT), ABC transporter ATPase, and toxin-antitoxin (TA) system (Rice et al., 2009). Genes for RT and ABC transporter are located at the 5' end of the genome, indicating a possible role in phage replication. RT in retroviruses (RNA viruses) is a multifunctional enzyme that is necessary for complementary DNA (cDNA) synthesis (Goff 1990). However, the role of this enzyme in filamentous phage, whose genome is composed of DNA and not RNA, is not clear. It is assumed that this polymerase could participate in the synthesis of the negative replication primer using the positive ssDNA of the filamentous phage as a template, a function that is most often performed by the host RNA polymerase (Higashitani et al., 1997; Mai-Prochnow et al., 2015). TA systems affect the viability and motility of bacteria, biofilm formation, quorum sensing and plasmid maintenance.

**Table 1.** Proteins of M13 bacteriophage and their function (Mai-Prochnow et al., 2015).

| Name of the functional module | The name of the protein (*gene*) | Function |
|---|---|---|
| **Replication** | **p2 (*g2*)**<br>**Endonuclease** | • Intercepts the replicative form and initiates replication.<br>• Binds the ends of newly formed DNA strands to form a circular ssDNA molecule after replication. |
| | **p5 (*g5*)**<br>**ssDNA binding protein** | • Prevents the conversion of newly synthesized ssDNA into dsDNA replicative form.<br>• It is replaced by protein p8 (CoaB) during virion formation on the cytoplasmic membrane of the host bacterium. |
| | **p10 (*g10*)** | • Translation product of *g2*, which is identical to the C-terminal domain of p2 protein, and which is synthesized from the internal start codon.<br>• It binds to dsDNA and prevents DNA hydrolysis by bacterial nucleases, and also prevents DNA replication with p5. |
| **Structural** | **p3 (*g3*)**<br>**CoaA protein** | • Mediator of phage adsorption for the primary and/or secondary receptor during the establishment of infection.<br>• Mediator in the release of membrane-bound virions via its C-terminal domain.<br>• Interacts with p6 and p8 proteins. |
| | **p6 (*g6*)** | • Forms a p3-p6 complex important for the termination of packaging of filamentous phages. |
| | **p7 (*g7*) + p9 (*g9*)** | • They initiate simultaneous packaging and extrusion of bacteriophages. |
| | **p8 (*g8*)**<br>**CoaB protein** | • Forms a helical filamentous capsid that envelops the viral ssDNA. |
| **Packaging and secretion** | **p1 (*g1*)** | • It integrates into the cytoplasmic membrane of the host and participates in phage packaging/extrusion by interacting with p4 and p11 proteins. |
| | **p4 (*g4*)** | • Facilitates extrusion by forming a channel through the outer membrane of the host with p1 and p11. |
| | **p11 (*g1*)** | • The product of the *g1* gene, which is formed by translation from the internal start codon.<br>• Prevents cleavage of p1 protein by bacterial proteases. |

### 1.2.4. Bacterial infection by filamentous bacteriophages

The range of potential hosts for members of order *Tubulavirales* is wide and includes Gram-negative bacteria: *Escherichia* (O'Callaghan et al., 1973), *Salmonella* (Khatoon et al., 1972), *Neisseria* (Meyer et al., 2016), *Pseudomonas* (Takeya and Amako, 1966; Mooij et al., 2007; Rice et al., 2008), *Vibrio* (Waldor and Mekalanos, 1996; Campos et al., 2003), *Ralstonia* (Askora et al., 2009; Murugaiyan et al., 2011; Addy et al., 2012), *Xanthomonas* (Tseng et al., 1990; Lin et al., 1994), *Erwinia* (Akremi et al., 2020), and *Thermus* (Pederson et al., 2001; Nagayoshi et al., 2016). There are only three published reports of filamentous phage that infect Gram-positive organisms (Kim and Blaschek, 1991; Chopin et al., 2002; Burckhardt et al., 2023).

A significant characteristic of filamentous phages, which distinguishes them from all other bacteriophages, is their ability to continuously produce new virions, without causing the death of the host. Based on differences in the life cycle, there are two types of filamentous phages: integrative, which become part of the host's genome, and non-integrative, which replicate exclusively extrachromosomally, i.e. episomally (Knezevic et al., 2021). The episomally replicating filamentous phages produce a large number of new virions, often reaching up to $10^{13}$ per mL of cultures. On the other hand, most chromosomally integrated phages produce a much lower number of new virions, on average 1 phage for every 10 – 100 cells under inducing conditions (Davis et al., 2002). This is not the rule for all chromosomally integrated phages, as is the case with Xanthomonas phage Cfltv, which reaches up to $10^{11}$ virions per milliter (Kuo et al., 1994).

Filamentous phages initiate infection by attaching to pili on the surface of the bacterial cell (Fig. 7) (Bradley 1973). There are three types of pili that can serve as primary receptors for these phages, namely conjugative F, N (or I) or type IV pili (Endemann et al., 1992; Waldor et al., 1997; Jouravleva et al., 1998; Deng et al., 1999; Campos et al., 2003b; Yang et al., 2004; Holland et al., 2006). Diverse phages such as *Inovirus M13* and *Affertcholeramvirus CTXϕ* also use a secondary receptor, which is the highly conserved TolQRA complex of inner membrane proteins (Click and Webster, 1997; Heilpern and Waldor, 2000). This complex belongs to the Tol-Pal complex, which is involved in cell division and maintenance of cell envelope integrity (Cascales et

al., 2007; Gerding et al., 2007). Using their CoaA protein, filamentous phages bind to receptors on bacteria, which leads to the retraction of the pili through the outer membrane, thus bringing the virion closer to the cell's periplasm. Two N-terminal domains of the protein bind to primary and secondary receptors, and the C-terminal domain serves for virion uncoating and DNA entry into the host cell cytoplasm (Fig. 7) (Reichmann and Holliger, 1997; Deng and Perham, 2002; Bennet and Rakonjac , 2006).

Depending on the lifestyle, the DNA of filamentous phages will begin replication upon entering the cell or will be integrated into the bacterial chromosome. Replication processes are assisted by RNA polymerase for initiation of negative strand DNA synthesis, while XerC recombinase is used for integration. In both situations, locally folded ssDNA forms quasi-double-stranded binding sites for the appropriate proteins: -35 and -10 boxes for RNA polymerase (Higashitani et al., 1997) and *dif* sites for the site-specific XerCD recombinase (Fig. 7) (Val et al., 2005).

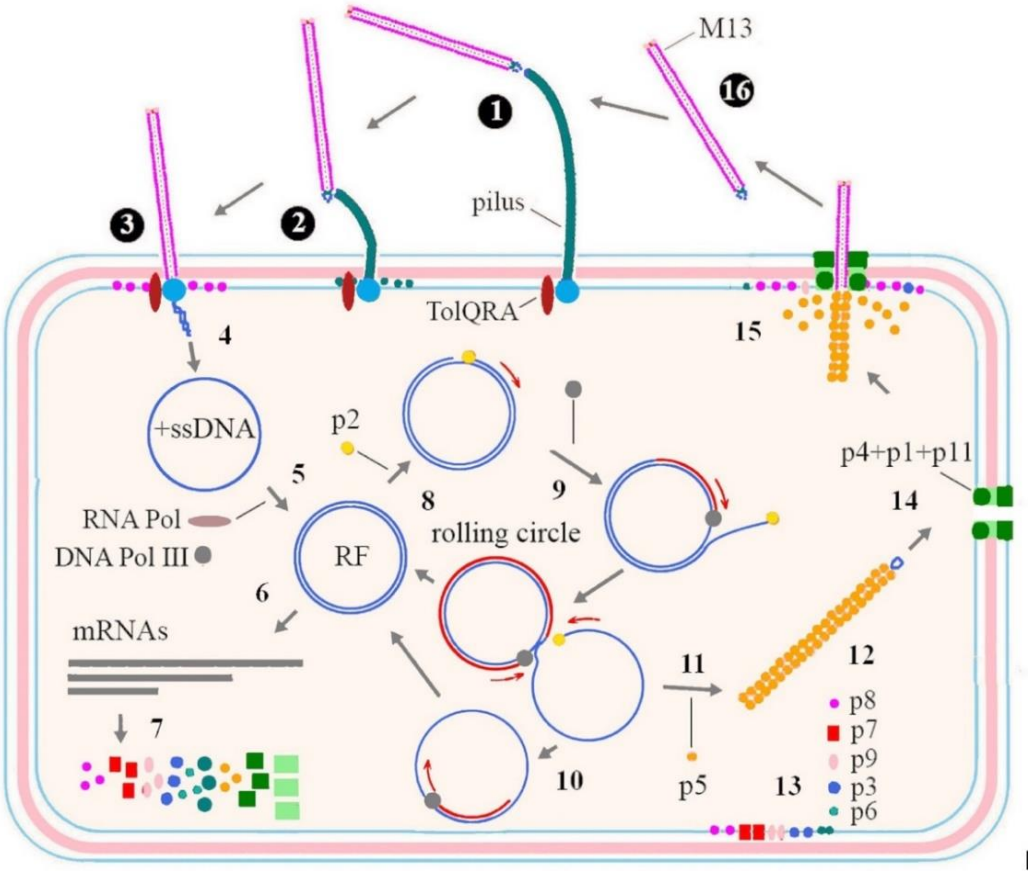


**Figure 7.** The life cycle of a representative member of the *Inoviridae* family (Knezevic et al., 2021).

*Inovirus M13* is one of the filamentous phages that multiply episomally. It has nine genes within its genome, and produces 11 proteins, due to internal translational starts within two genes, *g2* and *g1*. As already mentioned, within its genome, it does not have sequences for host integration and regulation of gene expression (Russel and Model, 2006; Endemann and Model, 1995; Marciano et al., 1999; Rapoza and Webster, 1995). After entering the cytoplasm of the cell, the ssDNA genome of the Escherichia phage M13 represents a positive strand that serves as a template to synthesize the negative strand. This process is initiated by bacterial RNA polymerase, not phage proteins. RNA polymerase successfully synthesizes an RNA primer that will be used by bacterial DNA polymerase III to synthesize the negative strand, resulting in double-stranded DNA. This double-stranded form is most often called the replicative form (RF) (Fig. 7).

Escherichia phage M13 is replicated by a rolling-circle mechanism, one strand at a time, without forming a concatemer. When the negative strand is synthesized, the old positive strand is replaced by a new one that has yet to be synthesized (Meyer and Geider, 1982; Asano et al., 1999). The newly synthesized positive strands are then used again to synthesize negative strands (Fig. 7). The number of dsDNA copies reaches up to 50 per cell, which leads to the synthesis of phage proteins. The structural proteins possess signal sequence and integrate into cytoplasmic membrane together with p1, p11 and p4 proteins that form the extrusion complex (Feng et al., 1999). Within this complex, p1/p11 proteins form the inner membrane ATPase/channel while p4 forms the outer membrane channel (Feng et al., 1999; Marciano et al., 1999). In the final stages of infection, when the appropriate protein concentration is reached, the positive strands are coated by the ssDNA-binding protein p5, with the exception of a hairpin loop that serves as a packaging signal (Fig. 7) (Michel and Zinder, 1989). The formed complex, ssDNA-p5, is directed to the p1/p11/p4 extrusion complex for export via the packaging signal. This packaging signal also helps minor proteins p7 and p9 to identify ssDNA genomes so that they could be packaged into virions and exported (Russel and Model, 1989). During extrusion, the p5 protein is replaced by p8, the major coat protein that was previously integrated into the cytoplasmic membrane (Rakonjac et al., 1999; Rakonjac and Model, 1998). The first proteins that leave the bacterial cell are p7 and p9, followed by numerous copies of the p8 protein. The process of extrusion is

completed by the addition of p3 and p6 proteins to the ends of the virions, during which they are released into the natural environment.

Vibrio phage CTXφ, which also initiates infection by binding to specific receptors on the surface of the bacterial host, belongs to the group of integrative bacteriophages. TcpA, the major subunit of the toxin-coregulated pilus (TCP), not only contributes to the pathogenicity of *V. cholerae* but also functions as a receptor for CTXφ phages (Waldor and Mekalanos, 1996; Herrington et al., 1998; Tacket et al., 1998). Additionally, the TolA protein, a component of the TolQRA complex, may have a role in infection as the secondary receptor for the CTXφ phage. After successful receptor binding, CTXφ inserts its ssDNA into the host cell and uses the host polymerase to synthesize the complementary strand, resulting in dsDNA (RF) (Quinones et al., 2005; Kamruzzaman et al., 2014). The process of phage integration depends on the host site-specific *dif*-site binding recombinase, XerCD, whose function is to resolve dimers of chromosomes (Huber and Waldor, 2002). The phage genome contains two inverted repeats of the *dif* site that form a forked stem-loop structure, thus reconstituting a double-stranded XerCD binding sequence in the ssDNA (Val et al., 2005). The action of XerCD recombinase on this secondary structure results in site-specific recombination and insertion of the phage genome into the *dif* site of the chromosome. In case when *V. cholerae* strain does not possess a CTXφ integration site, such DNA remains in the form of a plasmid (Fan and Kan, 2015). Beside the CTXϕ, many other phages, including Vibrio phage VGJϕ and Xanthomonas page XacF1 use the host-encoded recombinase XerC/D (Huber and Waldor, 2002; Das et al., 2011; Ahmad et al., 2014).

However, other filamentous bacteriophages can use integrases for their integration into the bacterial chromosome (Kawasaki et al. 2007). The presence of integrase was first determined in Ralstonia phage φRSM1, and then in other phages that infect *Ralstonia solanacearum, Ralstonia pickettii*, *Burkholderia pseudomallei* and *P. aeruginosa* bacteria (Webb et al., 2004; Mooij et al., 2007; Askora et al., 2009). Most phages with integrase use the host's tRNA gene as an integration site (*attB* site) (Campbell, 1962). Within the integrase there is a sequence (*attP*) that is identical to the *attB* sequence at the point where crossing over occurs. Interestingly, these are not the only ways filamentous bacteriophages can be integrated. Filamentous prophages of *Neisseria* bacterial species are integrated by a completely different strategy (Kawai et al., 2005).

In this case, each copy of the phage genome is flanked by a duplication of 5'-CT and possesses an ORF for transposase coding. Bille et al. (2005) successfully proved that *Neisseria* filamentous phages encode their own transposases.

After integration, the CTXφ phage does not replicate or its genes are transcribed because they are suppressed by a regulatory circuit composed of host, phage and satellite-encoded regulatory proteins (Davis et al., 2002; Waldor and Friedman, 2005; Kimsey and Waldor, 2009). However, the stresses from the environment that induce the lytic cycle in other temperate phages (e.g. tailed phages) actually induce the replication of integrated filamentous phages. In most cases, this type of induction is initiated via a regulatory circuit that involves the induction of SOS respond and the degradation of the LexA repressor protein (Quinones et al., 2005). In the case of dsDNA phage, prophage excision occurs via integrase and additional recombinase activity normally conferred by a phage-encoded recombination directionality factor (RDF) or excisionase (Bertani and Bertani, 1971; Gottesman, 1974; Couturier, 1976).

In the chromosome of *V. cholerae* there is a repeat sequence (type RS1) adjacent to the CTXφ genome. Within the intergenic region one (Ig-1) is the origin of replication for the CTXφ phage, where RstA causes breakage and creates a single-stranded nick that results in the occurrence of the 3' end of DNA (Moyer et al., 2001; Waldor et al., 1997). The host DNA polymerase binds to that 3’ end of DNA and begins synthesis of viral DNA. When the enzyme reaches the next nick at the origin of replication, DNA synthesis stops and the newly formed ssDNA is packaged into a virion. Assembly and packaging of temperate filamentous phages take place in fairly similar steps as in the case of episomal filamentous phages. Zot protein of CTXφ phage, which is homologous to PI protein of Escherichia phage M13, participates in the assembly and secretion of CTXφ virions (Koonin, 1992; Waldor and Mekalanos, 1996). This protein has an ATPase domain that probably provides energy for the mentioned processes. The release of the CTXφ phage from the cell occurs thanks to the chromosome-encoded secretin, EpsD, which is a component of the extracellular protein secretion (Eps) type II secretion system (Davis et al., 2000). Interestingly, cholera toxin, protease and chitinase are also released through this system (Sandkvist et al., 1993; Sandkvist et al., 1997). It is assumed that the release of newly formed

virions and the mentioned factors takes place without interruption, because *V. cholerae* produces extremely low titers of CTXφ phage.

### 1.2.5. Contribution of filamentous phage infection to bacterial phenotype

Infection by bacteriophages can affect the fitness of their bacterial hosts. In most cases, a phage infection enhances the host's ability to combat various unfavorable abiotic and biotic factors, to invade a new habitat, and to take part in the development of microbial communities. However, at times a phage infection lowers the fitness of the host bacterium, which also can be beneficial for bacteriophages if the bacterial host happens to be a pathogen (Oh et al., 2019; Wendling et al., 2021). Interestingly, bacteriophages from the *Inoviridae* family can carry additional, bacterial genes and can alter their adsorption specificity for new hosts (Knezevic et al., 2015). Moreover, it is known that filamentous bacteriophages can play a significant role in altering their hosts.

Filamentous bacteriophages can affect the growth rate of their bacterial hosts (Brown and Dowell, 1968; Kuo et al., 1991; Askora et al., 2009; Yu et al., 2015). It was assumed that due to the extremely high production of these phages, an excessive amount of copies of the major coat protein on the surface of the bacterial cell can cause this phenomenon. Moreover, by their accumulation on the surface of bacterial cells, filamentous phages can significantly affect aggregation (Addy et al., 2012) and hydrophobicity of cells (Chen et al., 2009). In some cases, a change in bacterial motility was also recorded after infection with this group of phages. Both swarming and swimming motilities were inhibited by filamentous phages, as proven in several different studies (Wang et al., 2013; Ahmad et al., 2014; Ahmad et al., 2017). Using pili, bacteria achieve twitching motility and this type of movement is also often inhibited by filamentous phages (Ahmad et al., 2014; Secor et al., 2016; Ahmad et al., 2017). All of the mentioned changes in the phenotype ultimately affect the initial phases of biofilm development.

However, among the most interesting bacteriophages from this family are those that lead to changes in existing and emergence of new traits of pathogenic strains. The best-known example is the already mentioned CTXφ phage of the *V. cholerae* bacterium, which enables it to

express a whole series of toxins (Faruque and Mekalanos, 2012). In addition to it, there are a few other filamentous bacteriophages that have been confirmed or are assumed to be involved in increasing the virulence of other bacteria such as *P. aeruginosa* (Webb et al., 2003; Webb et al., 2004; Rice et al., 2009), *E. coli* O18:K1:H7, *Yersinia pestis* biovar Orientalis (Gonzalez et al., 2002), *Ralstonia solanaccearum* (Yamada, 2013) and *Neisseria meningitidis* (Bille et al., 2005).

### 1.2.6. Filamentous bacteriophages of species *P. aeruginosa*

*P. aeruginosa* filamentous bacteriophages, most often designated as Pf, also belong to the *Inoviridae* family. It was found that Pf phages are significant in the virulence of *P. aeruginosa* and present in many clinical *P. aeruginosa* isolates. The presence of Pf specific genetic elements was determined in 56% of tested *P. aeruginosa* isolates from various sources (Knezevic et al., 2015). Also, Pf prophages were identified in 36.2% and 52.1% of *P. aeruginosa* strains isolated from two independent cohorts of patients with cystic fibrosis (Burgener et al., 2019).

Some Pf phages replicate episomally, while there are also integrative bacteriophages that persist in the form of prophages in the host chromosome (Kuo et al., 1994; Davis et al., 2002). Non-integrative filamentous Pf phages that are currently described are *Primolicivirus Pf1* and *Tertilicivirus Pf3* and they are recognized as separate genera and species by ICTV (Knezevic et al., 2021). Of the integrative Pf phages, only the *Primolicivirus Pf8* is currently recognized as a species. In addition to them, integrative bacteriophages such as Pf4 and Pf5, from the strains PAO1 and UCBPP-PA14, respectively, are described, and they are intact and possess a replicative form (Webb et al., 2004; Mooij et al., 2007; Rice et al., 2009). Integrative filamentous phages were also found in other reference *P. aeruginosa* strains, such as PfLES58 from LESB58 strain or Pf7 from the PA7 strain, but nothing has been investigated on them so far (King et al., 2012).

Pseudomonas phage Pf1 was detected in *P. aeruginosa* strain PAK (Takeya and Amako, 1966; Bradley, 1973) and Knezevic et al. (2015) determined the presence of Pf1 specific genetic elements in 18% of tested *P. aeruginosa* strains. This bacteriophage is 2000 nm long and 6 – 7 nm in diameter (Fig. 8) (Fauquet et al., 2005). As already mentioned, Pf1 replicates episomally. However, it has an incomplete gene identical to the integrase gene of other *P. aeruginosa* phages.

Namely, the Pf1 integrase contains only 100 amino-acids, which represents 1/3 of the integrase of other filamentous phages and is therefore considered non-functional (Torbet and Maret 1979; Tsuboi et al. 2010). The Pf1 genome is 7,349 nt long and makes up 12% of the mass of the virion (Fig. 8) (Hill et al., 1991).

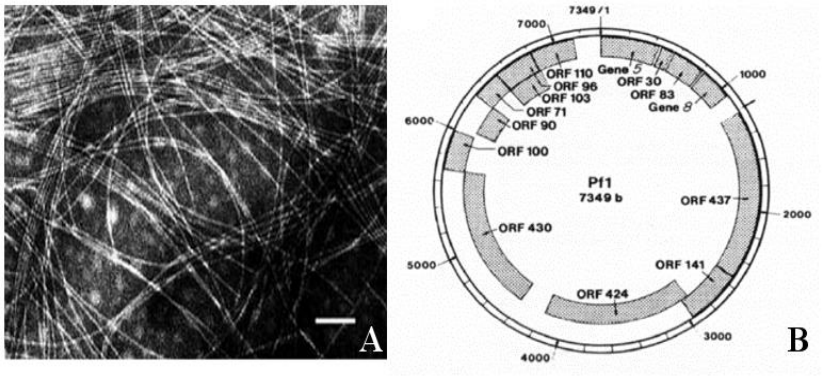


**Figure 8.** A - transmission electron micrograph of bacteriophage Pf1 (source of illustration: http://www.asla-biotech.com/products/pf1-phage-for-nmr-analysis). B – circular representation of the bacteriophage Pf1 genome (Hill et al., 1991).

It contains 7600 copies of the main capsid protein (CoaB) (Wiseman et al., 1976; Wiseman and Day, 1977). The main capsid protein consists of 46 amino-acids, and it was confirmed by spectroscopic techniques that the secondary structure is 100% in the form of an α-helix (Day, 1969; Thomas and Murphy, 1975). The CoaB protein of Pf1 phage is particularly interesting because it integrates into a membrane and replaces p5 during virion extrusion through cell envelopes (Park et al. 2010). Due to numerous studies on the structure of Pf1, the capsid structure of this phage has become a paradigm for the capsids of a number of filamentous phages (Goldbourt et al., 2010).

Pseudomonas phage Pf3 is 700 nm long and 6 nm in diameter and specifically infects the *P. aeruginosa* O strain containing IncP1 plasmids (Fig. 9) (Luiten et al., 1985). The phage Pf3 persists only extrachromosomally, and the genome size and organization is actually more similar to Enterobacteria phage M13, than to Pseudomonas phage Pf1. By comparing the genomes of Pf1 and Pf3, it was determined that the genomic organization of both phages is conserved, but they are very little similar to each other at the level of nucleotide and protein sequences (Mai-Prochnow et al., 2015). Pf3 phage has a GC content of 45% (Luiten et al., 1985), which is significantly different from Pf1 phage which has 61% (Hill et al., 1991). This data indicated that

Pf3 is more similar to Vibrio phages CTXϕ and VSK than to other Pf phages (Mai-Prochnow et al., 2015). It is assumed that the Pf3 phage originated from other species, and that in *P. aeruginosa* the genome was received through horizontal gene transfer. Pf3 genome is protected by a 2500 molecules of the major capsid protein (CoaB). Each CoaB protein consists of 44 amino-acids. Pf3 *coaB* gene does not encode a signal peptide, which is unexpected considering that the packaging of similar filamentous phages is associated with the cytoplasmic membrane, i.e. that it involves the replacement of ssDNA binding proteins with capsid proteins bound to the cell membrane (Luiten et al., 1983). M13 phages have a "stop-transfer" sequence within the major coat protein. This sequence comes into play after the initial partial vectorial discharge of the nascent polypeptide through the inner cell membrane. Its primary role is to anchor the coat protein molecule within the membrane. Upon closer examination of the Pf3 coat protein sequence, a region sharing similar characteristics to stop-transfer sequence and likely performing the same function was found. However, it remained unclear which specific region of the Pf3 coat protein is responsible for its vectorial discharge through the cell membrane. Luiten et al. (1983) hypothesized that there is a possibility that the signals for insertion and stop-transfer overlap with each other. This difference in the length of CoaB could further explain the phylogenetic distance of Pf3 phage compared to other Pf phages, regardless of the fact that this protein is highly conserved among filamentous phages. Lastly, X-ray diffraction showed that the Pf3 helix of the protein subunits has the same symmetry as the bacteriophage Pf1 helix. Although they have the same protein symmetry, for Pf3 it was determined that its DNA structure is inverted and the phosphate groups are inside and the bases are towards the outside of the virion.

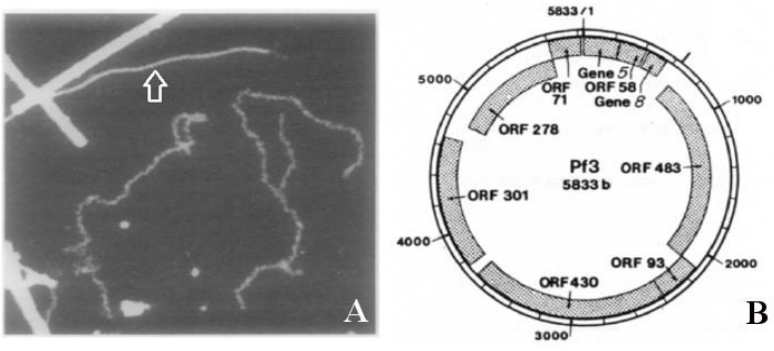


**Figure 9.** A – transmission electron micrograph of Pf3 bacteriophage; B – circular representation of the genome of bacteriophage Pf3 (Hill et al., 1991).

Pseudomonas phage Pf8 was found in the *P. aeruginosa* AUS411.500 isolate belonging to the international CF clone (Ambroa et al., 2020). The genome of this phage is about 10,000 bases long and has a total of 16 proteins and one tRNA coding region. Pf8 proteins are most similar to Pf4 and Pf5 phage proteins. What makes Pf8 very interesting is the presence of a toxin-antitoxin system and a methyltransferase in the genome. A TEM micrograph of this filamentous phage was obtained, which means that it produces virions.

Although a lot of research has been done on the Pf1 phage, the Pf4 phage is the best known of all *P. aeruginosa* filamentous phages. Its natural strain is PAO1. The projected length of Pf4 phage virions is about 3700 nm, which makes it the longest known Pf phage (King et al., 2011). The size of the Pf4 phage genome is predicted to be 12,437 nt. Pf4 is highly prevalent in *P. aeruginosa* strains and it was determined that 22% of strains have Pf4 specific genetic elements in their genomes (Knezevic et al., 2015). Pf4 is essential for multiple stages of biofilm development and contributes to the virulence of *P. aeruginosa in vivo*. Whiteley et al. (2001) noted that Pf4 genes are highly activated in *P. aeruginosa* biofilms. Moreover, the abundance of infectious virions was 100 – 1000 times higher in biofilm than in planktonic cultures. It is assumed that Pf4 changes to a superinfective form in the late stages of the biofilm and is able to infect and lyse *P. aeruginosa* strains (Webb et al., 2003; Rice et al., 2009; Tortuel et al., 2020). In this form, Pf4 facilitates the dispersal of a subpopulation of surviving cells (Rice et al., 2009), inhibits pyoverdine production and twitching motility (Secor et al., 2016). Superinfective Pf4 also participates in the formation of small-colony phenotypic variants (SCVs) during biofilm formation (Webb et al., 2004; Rice et al., 2009). On the surface of SCVs cells, high densities of filamentous phages were found. It is characteristic of SCVs cells that they attach very quickly to new surfaces and form microcolonies. The occurrence of SCVs represents a serious problem due to the connection with poor lung function and increased resistance to antibiotics in patients with cystic fibrosis (Haussler et al., 1999; Götz et al., 2004). Pf4 phages in the biofilm are spontaneously organized into a highly ordered liquid crystalline matrix that has a positive effect on bacterial fitness (Secor et al., 2015). Such structures facilitate adhesion and increase the biofilm's tolerance to desiccation and antibiotics. Rice et al. (2009) found that mice infected with a *P. aeruginosa* mutant, in which the Pf4 prophage was deleted, survived longer than those infected with an

isogenic wild-type strain. Pf4 causes the establishment of chronic infections because the presence of this phage in the biofilm-relevant quantities makes *P. aeruginosa* less invasive, less inflammatory, and more resistant to phagocytosis by macrophages (Secor et al., 2017).

*P. aeruginosa* strain UCBPP-PA14 possesses a Pf prophage that is highly homologous to the Pf4 phage, and is designated as Pf5. However, Pf5 does not possess a putative reverse transcriptase, a component of an ABC transporter system, a putative prevent-host-death (*phd*) antitoxin protein, and a plasmid stabilization toxin protein of the RelE/ParE family (Webb et al., 2004; Mooij et al., 2007). Moreover, there are three additional coding sequences (CDSs) in Pf5 that can not be found in Pf4. Finally, both Pf5 and Pf4 are integrated in different places in the genome of *P. aeruginosa*. Pf5 is the least prevalent in *P. aeruginosa* strains and only 7% of tested strains had its specific genetic elements (Knezevic et al., 2015). The estimated size of the Pf5 phage genome is 10,675 nt. The influence of the Pf5 phage on the virulence of its host has been poorly investigated. It was not determined that this phage possesses a superinfective form nor that it promotes the appearance of SCVs in static conditions (Mooij et al., 2007). On the other hand, whole transcriptome analysis of the *dppA1 P. aeruginosa* mutant indicated that greater biofilm formation occurs if the Pf5 phage genes are repressed (Lee et al., 2018).

Interestingly, Pf4 and Pf5 of *P. aeruginosa* possess their own excisionases, *xisF4* and *xisF5*, respectively (Li et al., 2019), which significantly promote prophage excision. The only difference between them is that only XisF5 is essential for Pf5 excision. XisF4 actually activates Pf4 phage replication by increasing phage initiator gene expression. In addition, *xisF4* and the neighboring phage repressor c gene *pf4r* are transcribed divergently and their 5'-untranslated regions overlap. XisF4 and Pf4r not only auto-activate their own expression but also repress each other. Pf4 phage production is additionally controlled by the type II toxin-antitoxin (TA) system, PfiT/PfiA (Li et al., 2020).

The PfLES58 prophage was found in a highly virulent epidemic *P. aeruginosa* strain, LESB58 (Cheng et al., 1996; Winstanley et al., 2009). This strain effectively displaces pre-existing populations of *P. aeruginosa* in the lungs of individuals with cystic fibrosis (McCallum et al., 2001). Chronic infections caused by LESB58 are associated with increased morbidity and mortality compared to other *P. aeruginosa* strains (Al-Aloul et al., 2004). LESB58 has an unusual phenotype,

characterized by early overexpression of virulence factors such as LasA, elastase and pyocyanin during growth (Winstanley et al., 2009). Comparing it with the PAO1 strain, the highly transmissible and aggressive LESB58 strain shows a wider spectrum of antibiotic resistance and a better ability to adapt in the lungs of CF patients (Wnorowska et al., 2015). In an experiment with rat chronic lung infection, *P. aeruginosa* strains such as PAO1, PA14 and LESB58 showed similar levels of *in vivo* growth, but different in terms of virulence. These differences were further confirmed by biofilm and motility assays, where LESB58 produced more biofilm and had lower mobility than PAO1 and PA14. Additionally, LESB58 isolates can easily mutate and develop resistance to antibiotics commonly used in therapies (Winstanley et al., 2009). Namely, LESB58 was initially discovered in *P. aeruginosa* isolates that showed resistance to ceftazidime in clinics where ceftazidime monotherapy was routinely used. Subsequent studies confirmed that LESB58 isolates can also acquire resistance to meropenem, aztreonam, tobramycin and ciprofloxacin. To determine the cause of such an important epidemic strain, Winstanley et al. (2009) sequenced the genome of LESB58. Sequencing indicated the presence of genomic islands, including five inducible prophage genomes (LESϕ2, LESϕ3, LESϕ4, LESϕ5 and LESϕ6), one defective prophage and five non-phage islands. LESϕ prophages 2, 3, 4, and 5 exhibited the highest similarity to phages belonging to the *Siphoviridae* family. However, LESϕ6 was most similar to Pf1 filamentous phage and was later named Pf-LESB58 (or PfLES58) (Winstanley et al., 2009; Knezevic et al., 2015). So far, it is not known whether PfLES58 forms a replicative form, produces infectious virions and in what way it affects the virulence of its host. However, LESϕ2, LESϕ3, and LESϕ5 prophages are assumed to influence the competitiveness of their strain *in vivo* (James et al., 2012). LESB58 mutants, with disrupted genes of these prophages, showed a 10- to 1000-fold reduced competitiveness in rats with chronic lung infection compared to LESB58 wild-types. Furthermore, LESϕ2 and LESϕ3 prophages, together with LESϕ4, can be induced by exposure to clinically relevant antibiotics e.g. ciprofloxacin and free LESB58 phages have been successfully detected in sputum.

Due to the wide distribution of filamentous phages in the genomes of *P. aeruginosa*, research is necessary on the influence of phages of this family on phenotypic traits, primarily on the virulence of *P. aeruginosa*. Knowing the role of these phages in bacterial virulence is

fundamental to understanding the host-phage relationship. In addition, these findings may help predict bacterial virulence based on the presence or absence of appropriate phages, but may also help control strain pathogenicity, if these phages decrease virulence or increase sensitivity to antibiotics. Phages of this family have a significant potential to be used in the control of the growth of host bacteria, which has been determined for phages Pf1 and Pf3, especially in combination with gentamicin and tetracycline. However, additional knowledge is needed in this context, because filamentous phages generally affect the virulence of bacteria by lysogenic conversion. Pf4 and Pf5 filamentous phages were investigated so far, but not to a large extent, while nothing is known about the nature of PfLES58 phages and their effect on the host (Webb et al., 2003; 2004; Rice et al., 2009).

Furthermore, filamentous bacteriophages, such as M13 phage, are used in the Phage Display technique, but also in vector cloning techniques (Rakonjac and Bennett, 2016). The reason why these phages are used in biotechnology is their ability to change the length of the virion depending on the size of the packaged DNA (King et al., 2012). Bacteriophages of this family could have wide application in medicine, as well as application in diagnostics if phages that specifically adhere to certain cells or tissues are selected (Sartorius et al., 2019). Also, these phages are highly immunogenic and this property can be used in the preparation of vaccines and stimulation of certain T lymphocyte lineages, but also as agents for delivery of targeted therapy, because only virions whose adhesion protein binds to receptors of interest can be selected (e.g. for certain molecules on cancer cells) (Prisco and Berardinis, 2012; Karimi et al., 2016; Gibb et al., 2021).

# 2. The study aims

Due to the importance and possible application of Pf filamentous phages, it is necessary to characterize these phages in detail and shed light on their biology. The aim of the study is to elucidate the properties and role of filamentous bacteriophages from the family *Inoviridae*, specific for *P. aeruginosa*, present in strains PAO1, UCBPP-PA14 and LESB58.

The aim will be achieved by obtaining the following results:

- Definition of phages morphological characteristics, genome characteristics and taxonomic position within the family *Inoviridae*
- Optimization of filamentous virion production, determination of lytic spectrum and infectivity of alternative hosts
- Definition of filamentous phage inducibility with antibiotics
- Definition of the role of filamentous phages in the virulence of host bacteria: changes in antibiotic sensitivity, biofilm formation, motility, growth rate, pigment production, as well as the role of phages in autoaggregation and hydrophobicity of cells.

# 3. Materials and methods

### 3.1. Bacterial and phage strains

In the study, three *P. aeruginosa* strains LESB58, PAO1, and UCBPP-PA14 which are naturally infected with filamentous prophages Pf4, Pf5 and PfLES58, respectively, were used. To determine the phage titer, strain TuD43 was used as a host, since it lacks any known Pf-like genetic elements (Knezevic et al, 2015) and is sensitive to all three phages. Beside the mentioned strains, additional 267 *P. aeruginosa* strains from various sources were used for determination of the lytic spectrum of Pf phage and PfLES58 prevalence (Table S1), deposited in PK Lab collection, University of Novi Sad. The strains are stored in glycerol stocks at -80°C and grown on Mueller-Hinton agar at 37 °C overnight. Additional cultivation steps were carried out using different types of media, including MH broth, Lysogeny broth (LB) agar with different agar percentages, as well as King's A or King's B medium, depending on the specific experiment.

*Pseudomonas virus JG024* was used to potentially trigger the induction of Pf prophages. This obligatory lytic bacteriophage belongs to the class *Caudoviricetes*, genus *Pbunavirus*. The phage JG024 was kept in SM buffer containing 10% glycerol at -80 °C.

### 3.2. Antimicrobial agents

Ciprofloxacin (CIP, 5 μg), gentamicin (GEN, 10 μg), tetracycline (TET, 30 μg) and streptomycin (STR, 300 μg) (Bioanalysis, Ankara, Turkey) were used for disc-diffusion method. In addition, solutions of CIP, GEN, TET, STR, ceftazidime (CAZ), chloramphenicol (CHL), polymyxin B (PMB) and mitomycin C (MMC) (Sigma Aldrich, St. Louis, MO, USA) were used to determine minimal inhibitory concentrations (MICs).

### 3.3. Sequence analysis of Pf prophages and PCR primers design

To analyze the sequences of Pf prophages and design PCR primers, the GenBank database was used to retrieve the genome sequences and key proteins of the Pf bacteriophages. The genomes of Pf1, Pf8, and Pf3 phages were previously sequenced (Access. No. NC_001331.1,

NC_073756.1, and NC_001418.1, respectively). PfLES58, Pf4 and Pf5 phages do not have a sequenced genome. Since these phages belong to the *Inoviridae* family, which has small genomes encoding only 10 or 11 proteins and lacks highly recognizable CDSs, predicting the precise location of the *att* site is challenging. However, identical regions at the beginning and end of the phage genomes that overlap during replicative form formation were already searched to determine the coordinates of all three Pf prophages in their bacterial host genomes (Knezevic et al., 2015). Information on the genome size and GC content of Pf phages was obtained using OligoCalc (Kibbe, 2007).

For visualizing the genomes of Pf phages and comparing the positions of key genes, nucleotide sequences in GenBank format were used. Gene positions were generated, visualized, and interactively colored using the Easyfig tool, a Python application that enables the creation of linear comparisons of multiple genomic loci (Sullivan et al., 2011).

Phylogenetic relationships of exemplar representatives of each genus from the *Inoviridae* family with PfLES58, Pf4, and Pf5 phages based on the nucleotide sequence of the genome were analyzed using the MEGA X program (Tamura et al., 2021). Nucleotide sequence alignment was performed in MUSCLE. The phylogenetic tree was constructed using the Maximum Likelihood method and the Kimura 2-parameter model. Nucleotide sequences of the genomes of filamentous phages were downloaded from the NCBI database using their unique accession numbers.

PCR primers for colony and qRT-PCR were designed using the PCR Primer Blast program (Table 2) (Ye et al., 2012). Additional quality checks of the designed primers were performed using the OligoAnalyzer (PrimerQuest™ program) and Bioinformatics PCR test.

**Table 2.** List of primers used in this study

| Purpose | Label of Primer | Gene function | Sequence (5' -> 3') | Amplicon (bp) | Ref. |
|---|---|---|---|---|---|
| RT-qPCR | RpoD-F | Sigma factor 70 | CGT TGA TCC CCA TGT CGT T | 155 | This study |
| | RpoD -R | | GCA ACA GCA ATC TCG TCT GA | | |
| | ProC-F | Pyrroline-5-carboxylate reductase | CAG GCC GGG CAG TTG CTG TC | 180 | Salvi et al., 2003 |
| | ProC -R | | GGT CAG GCG CGA GGC TGT CT | | |

| | | | | |
|---|---|---|---|---|
| Pf4CoaB-F | Major capsid protein (CoaB) | GCA ACG CAT CGC CAA GTT | 113 | This study |
| Pf4CoaB -R | | CGC TGG TGT CGA TCA CCC | | |
| Pf4Zot-F | Zonula occludens toxin (Zot) | GAT GTT CGG CGT GGT GAG | 180 | This study |
| Pf4Zot-R | | ATC TTC GAC GAA ACC CAA CTG | | |
| Pf4CoaA-F | Minor capsid protein (CoaA) | GTG CCG ATT CCT ACA CCT TC | 135 | This study |
| Pf4CoaA-R | | CCT TGA CGC AGG TAG TTC CC | | |
| PfLES58CoaB-F | Major capsid protein (CoaB) | CCC CCT TGG TGG TTT CGT | 87 | This study |
| PfLES58CoaB-R | | TCC GCA ACG CTT CCA TCG | | |
| PfLES58Zot-F | Zonula occludens toxin (Zot) | GAT GTT CGG CGT GGT GAG | 180 | This study |
| PfLES58Zot-R | | ATC TTC GAC GAA ACC CAA CTG | | |
| PfLES58CoaA-F | Minor capsid protein (CoaA) | ATC GAA CTT GGA AGC GAG GA | 110 | This study |
| PfLES58CoaA-R | | GCT CCT TCG ACA AGA CCA TC | | |
| Pf5CoaB-F | Major capsid protein (CoaB) | GCA ACG CAT CGC CAA GTT | 113 | This study |
| Pf5CoaB-R | | CGC TGG TGT CGA TCA CCC | | |
| Pf5Zot-F | Zonula occludens toxin (Zot) | CTG TCG ATC TAT AAC AAG ACC CTC | 189 | This study |
| Pf5Zot-R | | CGA GCC TTC GGA GAA CTG | | |
| Pf5CoaA-F | Minor capsid protein (CoaA) | CGT GTT GAT GAC GGA CGG TA | 134 | This study |
| Pf5CoaA-R | | GAG GAC AGC CAG GGT CAT TC | | |
| Pf4RF-F | Replicative form (RF) | CTT GGC AGG GTG ATT TGG A | 95 | This study |
| Pf4RF-R | | AGG AAC GCT TCA AAA CCC TA | | |
| PfLES58RF-F | Replicative form (RF) | ACG GCT CTG CAC TTC TAC G | 120 | This study |
| PfLES58RF-R | | CGA CAG TTC TTC GAC ACT TGC | | |
| Pf5RF-F | Replicative form (RF) | GCC TAG CGT TGA CCA GTT A | 78 | This study |
| Pf5RF-R | | CTT TGC CGA TTT GTG CGT A | | |

| | | | | | |
|---|---|---|---|---|---|
| PCR/RFLP | PfUb-F | Universal part of the Pf phages | TTG TGT ACG ACA GCG GGA A | 570 (Pf4, Pf5), 593 (PfLES58) | Knezevic et al., 2015 |
| | PfUb-R | | TCA ATT CGC CTT TTT CGG C | | |
| | PfLES58RF-F | Replicative form (RF) | ACG GCT CTG CAC TTC TAC G | 120 | This study |
| | PfLES58RF-R | | CGA CAG TTC TTC GAC ACT TGC | | |
| | PfLES58CoaA-F | Minor capsid protein (CoaA) | ATC GAA CTT GGA AGC GAG GA | 110 | This study |
| | PfLES58CoaA-R | | GCT CCT TCG ACA AGA CCA TC | | |
| | PfLES58Int-F | Integrase (Int) | GGT ATT CCG GCG ACA AAT | 447 | This study |
| | PfLES58Int-R | | ATC AGG TAG CGG TGT TGG | | |
| | PfLES58RF-F | Replicative form (RF) | ACG GCT CTG CAC TTC TAC G | 604 | This study |
| | PfLES58RF-R | | ATC AGG TAG CGG TGT TGG | | |
| | IntF4-F | Integrase (Int) | TCG AAT TCC GCT TCC ATC AC | 1001 | Knezevic et al., 2015 |
| | IntF4-R | | CCT GAT GCT TGG TCA GGT ACG | | |
| | Pf4RFc-F | Replicative form (RF) | AGC AGC GCG ATG AAG CAA T | 865 | Rice et al., 2009 |
| | Pf4RFc -R | | TAG AGG CCA TTT GTG ACT GGA | | |
| | Pf5CoaA-F | Minor capsid protein (CoaA) | CGG GAA TCG TAT TGA GCC GA | 440 | Knezevic et al., 2015 |
| | Pf5CoaA-R | | GAG GAC AGC CAG GGT CAT TC | | |
| | Pf5RF-F | Replicative form (RF) | ACG GTG GAA ACA TCC TGG | 725 | Mooij et al., 2007 |
| | Pf5RF-R | | AAC AGT GAA TTG CGG ACA AGG | | |
| | PfLES58-IS-F | PfLES58 integration site in PAO1 and PA14 genome | CGA CAG TTC TTC GAC ACT TGC | 258 | This study |
| | PfLES58-IS-R | | GAC AGG CAG ACC ATG ACC | | |
| | Pf4-IS-F | Pf4 integration site in LESB58 and PA14 genome | CAA TGG TCG TCA CGC AGA AC | 943 | This study |
| | Pf4-IS-R | | CCG CTC AAC CCG ATC TAC | | |
| | Pf5-IS-F | Pf5 integration site in LESB58 genome | GAT TCT TGG GCA TTC GTC GC | 243 | This study |
| | Pf5-IS-R | | GCC TAG CGT TGA CCA GTT A | | |

### 3.4. DNA and RNA isolation and PCR methods

The viral DNA was isolated using the phenol-chloroform method. First, dialyzed phages were treated with DNAse and RNAse for 24 hours at concentrations of 5 U $mL^{-1}$ and 10 mg $mL^{-1}$, respectively. Inactivation of the enzymes was achieved by incubation at 65 °C for 1 hour. After incubation, EDTA, SDS, and proteinase K were added to the phage suspensions in final concentrations of 20 mM, 0.5%, and 50 µL/mL, respectively. The phage samples were then incubated at 56 °C for 1 hour prior to the viral DNA isolation. Samples were treated with an equal volume of phenol (1:1) and centrifuged at 4000 x g for 5 minutes. The upper phase was transferred to a new tube, and phenol and chloroform were added in a ratio of 1:0.5:0.5. Centrifugation was repeated at 4000 x g for 5 minutes. The upper phase was transferred to a new tube and treated only with chloroform in a ratio of 1:1. The DNA was then precipitated with a double volume of 96% ice-cold ethanol and washed with 70% ice-cold ethanol. The samples were then centrifuged at maximum speed, at 4 °C, for 20 minutes. After removing the supernatant and drying, the resulting pellet was dissolved in 30 µL of Elution buffer (MiniPrep, 10 mM Tris-HCl, pH 8.5). The concentrations of isolated ssDNA were measured using BioSpec-nano (Shimadzu Biotech, Japan).

For colony PCR, multiple colonies were collected from each strain as samples, where one half of the colony was kept on agar for the possibility of successful confirmation of superinfection. Original host DNA were used as positive controls, while sterile distilled water was used as a negative control. The following parameters were used for all PCR analyses: initial cycle of 94 °C for 5 min followed by 35 cycles of 94 °C for 30 s, annealing at 53 – 58 °C for 20 s, and extension at 72 °C for 60 s with a final 7 min extension at 72 °C. DreamTaq Green PCR Master Mix (2x) (Thermo Fisher Scientific, Vilnius, Lithuania) was used for the PCR mixture. In order to visualize and document PCR products, agarose gel electrophoresis (0.7 – 1.5%) was used (BioDocAnalyze System, Biometra, Germany). Positive controls included either bacterial or viral DNA. Distilled water was used as a negative control. The presence of viral genomes was also checked on agarose gel using the method of Green and Sambrook (2017).

Isolation of RNA from the samples was performed in the following way. The collected samples were immediately centrifuged for 2 minutes at 12,000 g. The GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) was used to isolate RNA. To eliminate variance in growth intensity, the concentrations of extracted RNA from all samples were normalized and treated with DNAse I (1 U) for 30 minutes at 37 °C, followed by enzyme heat inactivation. The extracted RNA was subjected to reverse-transcription using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Bedford, MA, USA) in the following steps: 10 min at 25 °C, 120 min at 37 °C, and 5 min at 85 °C. The reaction mixture was then cooled to 4 °C.

RT-qPCR was performed in triplicate and in three independent repetitions, with the following cycling conditions: an initial cycle of 2 min at 50 °C, the next step at 95 °C for 10 min, then a step at 60 °C for 1 min, followed by a step at 55 °C for 5 s, and the final step lasted 5 min at 95 °C. The obtained Ct values represent the average $\pm$ S.E. of the results of replicates. No template controls were used as negative controls. Two primer pairs targeting two *P. aeruginosa* housekeeping genes, *rpoD* and *proC*, were utilized to normalize data (Table 2). The $2^{-DDCT}$ method was used to calculate transcriptional changes, and changes in relative gene expression of $\geq$1.5 or $\leq$0.67 were considered significant (Livak and Schmittgen, 2001).

### 3.5. Confirmation of PfLES58 production

As Pf4 and Pf5 are known to produce the replicative form actively, it was also necessary to confirm the same for PfLES58 prophage in *P. aeruginosa* LESB58 strain. For this purpose, primers were designed to confirm the presence of the replicative form (RF) based on the prophage coordinates previously predicted in the LESB58 genome (Table 2) (Knezevic et al., 2015). Universal Pf phage primers, PfUb (Knezevic et al., 2015), and a primer pair for the highly conserved *P. aeruginosa* gene, RpoD (Salvi et al., 2003), were used as positive controls (Table 2). The PAO1 strain, which does not have the PfLES58 prophage in its genome, was used as a negative control. As an additional verification step, the obtained PCR product was sequenced by the dideoxy method and the sequence was deposited in NCBI.

### 3.6. Prevalence of PfLES58 specific genetic elements in *P. aeruginosa* strains

Using colony PCR, 267 *P. aeruginosa* strains from the PK Lab collection were tested for the presence of PfLES58 specific genetic elements. To detect the presence of PfLES58 replicative form (RF) and *coaA* gene, two primer sets were used (Table 2).

### 3.7. Propagation and purification of Pf filamentous phages by two different methods

To increase the yield of PfLES58, Pf4, and Pf5 filamentous phages for characterization, two propagation methods were designed.

Method 1 involved inoculation of 6 L of LB with 1 mL of overnight *P. aeruginosa* culture and incubation at 37 °C with agitation (200 rpm) for two days. After incubation, the bacterial cultures were centrifuged at 10,000 x g for 10 min and then purified by filtration through 0.45 and 0.22 μm filters. The resulting filtrate containing phages was precipitated overnight with polyethylene glycol 8000 (PEG 8000) and NaCl at final concentrations of 4% and 0.5 M, respectively. The phages were then centrifuged at 12,000 x g, 15 min at 4 °C, and the resulting precipitate was dissolved in 2 – 3 mL of SM buffer. This step aims to increase the concentration of bacteriophages in relation to the initial volume.

In method 2, a 1 mL overnight culture of *P. aeruginosa* was used to inoculate 600 mL of LB broth and incubated at 37 °C for 2 days with agitation (Fig. 10). Bacterial cultures were purified only by centrifugation at 10,000 x g for 10 min, with the centrifugation step being repeated several times until the bacterial sediment could no longer be seen. Fresh medium was added to the purified supernatant to restore the initial volume of 600 mL, and then inoculated again with 1 mL of overnight bacterial culture. This process was repeated after the fourth and sixth day. Phages that multiplied over a period of six days were precipitated using the same PEG and NaCl concentration as in method 1. However, after decanting the supernatant, the cuvettes with precipitated phages were centrifuged again for 5 min to concentrate the phages at the bottom. The phage precipitate was then collected with 6 mL of SM buffer by vortexing.

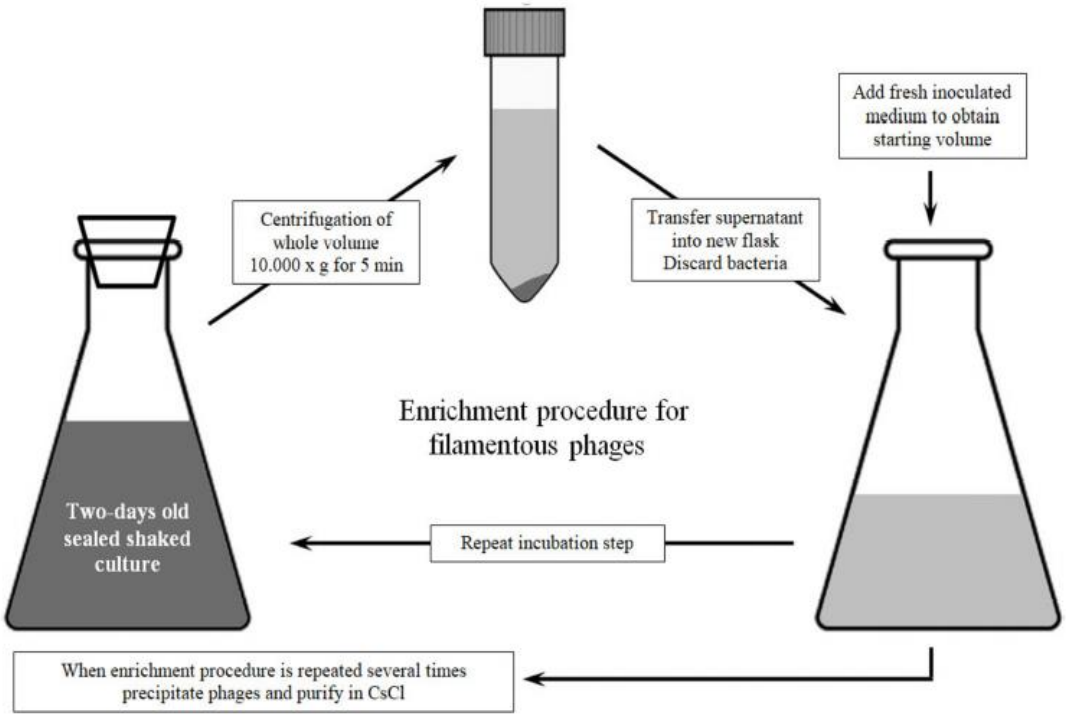


**Figure 10.** Schematic representation of method 2 for propagation of Pf bacteriophage

### 3.8. Comparison of two different methods for the propagation of Pf filamentous phages

In order to verify the effectiveness of both methods, PFU $mL^{-1}$ of bacteriophages was determined following ultracentrifugation and dialysis. Moreover, the quality of both methods was further verified by isolating and quantifying the ssDNA of the produced viruses.

Multiplied phages by method 1 and 2 were purified by equilibrium density gradient centrifugation [0.375 g $mL^{-1}$ Cesium chloride, Sigma-Aldrich (CsCl)] at 135,000 x g, for 42 h at 4 °C (Ti50 rotor, Beckman, United States). After ultracentrifugation, the resulting bands were harvested using a syringe and needle, and the phage suspensions were dialyzed in SM buffer. Each milliliter of phage suspension was dialyzed into 2 L of SM buffer. The quantification of dialyzed Pf phages was performed using the SPOT method, in which 10-fold phage dilutions were made in SM buffer. Then, 10 µl of each dilution was applied onto the TuD43 lawn and incubated at 37 °C. The following day, the number of formed plaques was counted and PFU $mL^{-1}$ was calculated.

In order to compare the effectiveness of both methods in producing Pf phages, viral DNA was extracted from all samples. The concentration of isolated DNA was determined. Finally, DNA was run on 0.7% agarose gel electrophoresis to confirm genome size.

### 3.9. Production of Pf phages and their plaque formation

Method 2 allowed for the continuous monitoring of Pf filamentous phage production during six days. The bacterial culture was purified every two days and a small fraction of the phage suspension was sampled and concentrated with PEG 8000 in a volume of 40 mL. The resulting phage pellet was dissolved with 500 µl of SM buffer. For phage quantification, the TuD43 strain was used again. Since the suspensions of filamentous phages obtained were not ultracentrifuged, the possibility of the presence of other prophages that were potentially released during propagation was eliminated by treating phage suspensions with 2% chloroform. Filamentous phages are sensitive to chloroform and after treatment with it, they become non-infectious (Griffith et al., 1981). Phage quantification was carried out using the SPOT method, both double- and single-layer method. For the double-layer method, 100 µL of overnight TuD43 culture was added to 3 mL λ top (0.65% agar), and then the contents were poured over LB agar (1.5% agar). The single-layer method involved the inoculation of an overnight culture of TuD43 in 10 mL of λ top, which was then poured into Petri plates. Treated and untreated phage suspensions were ten-fold diluted and applied to TuD43 lawn. Incubation was done overnight at 37 °C. The experiments were performed in triplicate in at least three independent repetitions. The next day, the plaques of treated and untreated samples were counted and further characterized.

The quantity of Pf phages (Nf) was determined by subtracting the number of tailed phages (Nt) obtained after treatment with chloroform from the total number of phages obtained without chloroform treatment (NT), which includes both filamentous and tailed phages: Nf = NT - Nt.

### 3.10. Transmission electron microscopy (TEM) imaging of Pf phages

To obtain an image of Pf1, PfLES58, Pf4, and Pf5 bacteriophages by transmission electron microscopy, samples with phages were negatively stained by the droplet method (Bozzola and Russel, 1998). Samples were coated with carbon for five minutes after transferring to copper grids provided with Formvar film. In this way, binding of viral particles to the film was enabled. The bound viral particles were then washed with an aqueous solution of uranyl acetate (0.5 – 1% w/v) for 1 min. The contrasted samples were then air-dried and examined with a Philips CM 100 transmission electron microscope (Philips, Netherlands). The image was obtained by Gatan Bioscan CCD camera, using Digital Micrograph software 3.4. (Gatan Inc., USA). The determined dimensions of Pf phage represent the mean value of at least 10 different measurements.

### 3.11. Pf phage DNA isolation and enzyme treatment

Filamentous bacteriophages carry ssDNA as their genome and to confirm this for Pf phages, their viral DNA was treated with different enzymes in order to prove the presence of ssDNA (Table 3). The phenol-chloroform method was used to isolate viral DNA from Pf phages, as previously described. To verify the success of the isolation process, the samples were treated with 5 U $mL^{-1}$ of DNAse and left to incubate overnight at 37 °C. The presence of ssDNA was confirmed by treating the samples with 0.1 µL (10 U) of S1 nuclease and 6 µl of 5X Reaction Buffer in a final volume of 30 µL per sample, followed by incubation at room temperature for 30 minutes. To further confirm the presence of ssDNA, a restriction enzyme that exclusively cuts double-stranded DNA was used. FastDigest EcoRI enzyme was used for all three viral DNA samples, with the reaction mixture consisting of 2 µL of restriction enzyme, 2 µL of 10x FastDigest Buffer, 1 µL of DNA (1 µg $µL^{-1}$), and 16 µL of nuclease-free water. The mixture was then incubated at 37 °C for 16 hours. Finally, to confirm that the isolated DNA was in circular form, 2 µL of ssDNA was treated with 1 µL of Exonuclease I and 1 µL of 10x Exonuclease I buffer, followed by incubation at 37 °C for 15 minutes. Exonuclease I cuts only linear strands of DNA.

The untreated and treated DNA samples were separated by gel electrophoresis on a 0.7% agarose gel containing ethidium bromide.

**Table 3.** Enzymes with differential nucleic acid cleavage activities

| | dsDNA | Linear ssDNA | Circular ssDNA | RNA |
|---|---|---|---|---|
| DNAse | +* | + | + | - |
| S1 nuclease | -** | + | + | + |
| EcoRI | + | - | - | - |
| Exonuclease I | - | + | - | - |

* susceptible to enzyme activity; ** not susceptible to enzyme activity

### 3.12. Protein characterization of Pf phages

To obtain proteins for SDS-PAGE analysis, ultracentrifuged and dialyzed Pf phages were used. For protein extraction and concentration, 400 µL of a phage suspension was mixed with ice-cold methanol and chloroform, and centrifuged at 18,000 x g, 4 °C for 10 minutes. After removing the upper layer, 400 µL of ice-cold methanol was added, and the samples were centrifuged again. The pellet was dissolved in 400 µL of ice-cold methanol, centrifuged, and the methanol was removed. The precipitate was dissolved in 50 µL of $dH_2O$, and the protein concentration was determined using the Bradford protein assay (Bradford, 1976). Proteins of the appropriate concentration were mixed with 2X SDS-PAGE sample buffer (v:v 1:1), boiled for 5 minutes at 98 °C, and immediately transferred to ice to prevent protein renaturation. The resulting proteins were then analyzed using SDS-PAGE.

In this experiment, a 15% gel was used to separate phage proteins. The gel was prepared using 4.5 mL of deionized water, 7.2 mL of 40% acrylamide, 4 mL of 1.5 M Tris at pH 8.8, 160 µL of 10% SDS, 160 µL of 10% APS, and 16 µL of TEMED. Freshly prepared APS was used for this gel. After the gel was loaded, isopropanol was placed on top of it, and it was allowed to polymerize. Following the polymerization of the separating gel, a concentrating gel was made using 5.8 mL of deionized water, 1.5 mL of 40% acrylamide, 2.5 mL of 0.5 M Tris at pH 6.8, 100 µL of 10% SDS, 100 µL of 10% APS, and 10 µL of TEMED. The gel was placed in an SDS-PAGE running buffer containing 1 L of deionized water, 3.03 g of Tris base, 14.44 g of glycine, and 1 g of SDS. The PageRuler™ Unstained Low Range Protein Ladder (Thermo Fisher Scientific, Vilnius, Lithuania) was loaded onto the gel, followed by phage protein samples with a final concentration of

approximately 10 µg. The proteins were run at 30 V until they reached the separating gel, at which point the voltage was raised to 200 V. Electrophoresis was stopped when the dye front approached the bottom of the gel, which took about 1.5 h. To preserve low mass proteins, the proteins were initially fixed with 5% glutaraldehyde for 10 minutes. The gel was then transferred to a 50 mL Gel Fix solution and left on a shaker for 15 min. This step was repeated once more with the new Gel Fix solution. Upon fixation, the gel was transferred to a Commasie Brilliant Blue staining solution containing 0.3 g of Brilliant Blue R250, 120 mL of methanol, and 150 mL of deionized water for 1 h. Excess dye was removed from the gel in a Destain Solution containing 415 mL of deionized water, 50 mL of methanol, and 35 mL of glacial acetic acid overnight. The next day, the protein bands were analyzed, and their molecular mass was determined.

### 3.13. Determination of the lytic spectrum of Pf phages on various *P. aeruginosa*

The lytic spectrum of Pf phages was determined on 267 different *P. aeruginosa* strains that are not original hosts of these phages. Plaque production by Pf phages was checked using a single layer SPOT method. Overnight cultures of bacteria were suspended in 1 mL of PBS buffer and then centrifuged at 6,000 x g for 2 minutes. The resulting supernatant was decanted, and the bacterial sediment was resuspended in PBS buffer. The centrifugation step was repeated, and the bacterial sediment was dissolved once again in PBS buffer to make a suspension of 0.5 McFarland density. To each 9.9 mL λ top, 100 µl of bacterial suspension was added, resulting in a final concentration of $10^6$ CFU $mL^{-1}$. The contents were vortexed gently and poured into a Petri dish, followed by the addition of 10 µl of phage ($10^6$ PFU $mL^{-1}$) to the bacterial lawn. The phage suspensions were checked for contamination by being applied to a poured λ top that had not been previously inoculated with bacteria. The plates were allowed to dry for approximately 20 minutes and then incubated at 37 °C for 18 hours. The appearance of lysis at the place of addition of the phage suspension confirmed that the given strain was sensitive to the Pf phage used.

The host range was characterized as narrow if the phages lysed up to 30% of the strains, moderate if they lysed from 30 to 50% or wide if lytic zones were recorded in over 50% of the strains. Host range was determined in three independent replicates.

### 3.14. Induction of Pf phages from their natural hosts *P. aeruginosa*

A quarter of the obtained MIC values for each agent (CIP 0.0625 µg $mL^{-1}$, GEN 0.5 µg $mL^{-1}$, CAZ 16 µg $mL^{-1}$, and MMC 0.5 µg $mL^{-1}$) were used to examine the impact of subinhibitory doses of antimicrobials on PfLES58, Pf4, and Pf5 gene expression in their natural hosts, LESB58, PAO1, and PA14, respectively. Also, all mentioned strains were infected with 0.25 MOI of obligatory lytic phage JG024, to determine gene expression after superinfection of strains by tailed phage (MIC equivalent 1 MOI).

In 50 mL of freshly made MH broth, 100 µL of the prepared bacterial suspension (0.5 McFarland density; $10^8$ CFU $mL^{-1}$) was added. The inoculated medium was incubated at 37 °C and 200 rpm, and the optical density of the contents was continually examined. After obtaining the appropriate optical density of 0.5 McFarland, each Erlenmeyer flask was amended with one antimicrobial agent or phage JG024, and the final volume in each flask was the same. Into flasks used as negative controls, the same volume of sterile distilled water was added. After 1 h, 6 h, and 24 h of treatment, samples were collected for bacterial RNA isolation to examine the effect of antimicrobial agents. However, in the case of treatment with JG024, samples were taken after 6 h, 12 h and 24 h of treatment.

RNA was immediately isolated from the collected samples and further subjected to reverse-transcription. Changes in gene expression were analyzed by qRT-PCR method using the obtained cDNA as a template. The newly designed primers for Pf *zot* and *coaB* genes (Table 2) were used in duplicate and in three independent repeats for the RT-qPCR.

### 3.15. Pf phage superinfection of *P. aeruginosa* strains

In order to examine the effect of Pf bacteriophages on the phenotype of alternative hosts, Pf phage infection was attempted on different *P. aeuginosa* strains. To achieve this, bacteria and phage were combined at MOI = 10 and incubated overnight at 37 °C. Infection with Pf4 phage was attempted on LESB58 and PA14 strain, while infection with PfLES58 phage was attempted on PAO1 and PA14 strains (Table 4). Pf5 infection was attempted on LESB58 and PAO1 strains. After incubation, the bacteria were centrifuged for 2 minutes at 6,000 g. The bacterial pellet was

resuspended in PBS buffer before being centrifuged a second time. This procedure was done once more to eliminate unattached Pf virions from the supernatant. One milliliter of MH broth was added to the bacterial pellet, and the bacteria were incubated overnight at 37 °C. To thoroughly remove Pf phages that did not infect the bacterial cells but may have remained in the medium or attached onto bacterial surface, the process of re-washing the bacterial cells and adding fresh medium was repeated twice. The bacteria were then inoculated on MH agar at 37 °C. The subcultivation process was carried out three times further in order to obtain stable chronic infection. To identify the existence of Pf prophages in various *P. aeruginosa* strains post-infection, two sets of primers were used (Table 2) to detect the presence of replicative form (RF) and the gene for Pf integrase (*int*) or CoaA (*coaA*).

**Table 4.** Sensitivity of different *P. aeruginosa* strains to Pf phage superinfection

| Strain designation | PfLES58 | Pf4 | Pf5 |
|---|---|---|---|
| LESB58 | x* | +** | + |
| PAO1 | + | x | -*** |
| PA14 | + | + | x |

* The strain is the natural host of the given Pf phage; ** The strain is sensitive to superinfection of a given Pf phage; *** The strain is resistant to superinfection of a given Pf phage

The acquired PCR products for the RF were cut using enzyme SduI, HpaII, or Csp6I depending on Pf phage (FastDigest, Thermo Fisher Scientific, Vilnius, Lithuania) to further confirm the presence of Pf prophages in the infected *P. aeruginosa* strains. According to *in silico* analyses, SduI cut the product of PfLES58 RF, giving fragments of 293, 223, and 88 bp. HpaII cut the product of Pf4 RF, giving fragments of 654 and 211 bp and Csp6I cut the product of Pf5 RF, giving fragments of 638 and 87 bp. A total of 10 µL of PCR product (~ 0.2 µg), 1 µL of FastDigest enzyme, 10 µL of FastDigest Green buffer, and 17 µL of $dH_2O$ were mixed together for digestion. The FastDigest mixture was then incubated at 37 °C for 5 minutes. The restricted PCR products were separated using 1% agarose gel with ethidium bromide at the end of the incubation.

We hypothesized that Pf phages were integrated into used *P. aeruginosa* strains utilizing the same *att* site in the bacterial genome. Phage DNA was incorporated into the bacterial genome *in silico* and primer pairs were created that included both bacterial and viral DNA (Table 2). ExoSAP Master Mix (100 µL of Exonuclease I (20 U/µL), 200 L of FastAP (1 U/µL), 60 µL of Exonuclease I Reaction Buffer 10x, and 240 µL of PCR water) was made for PCR product

purification. One microliter of ExoSAP Master Mix was added to a 5 µL PCR reaction, and the samples were incubated for 15 minutes at 37 °C. Enzyme inactivation was carried out for 15 minutes at 85 °C. A total of 5 µL of purified PCR product was combined with 5 µL of forward or reverse primer at a concentration of 5 µM for sequencing. Capillary electrophoresis was used to sequence PCR products on an ABI 3730 l Genetic Analyzer (Applied Biosystems). The alignment was performed out using the DNADynamo program, with a final manual re-check. The length of the examined sequences for both Pf4 infected strains, LESB58+Pf4 and PA14+Pf4, was 943 bp. For the PfLES58-infected strains, PAO1 and PA14, the sequence length was 258 bp, and for the Pf5-infected LESB58 strain, 243 bp.

### 3.16. Production of superinfective Pf and indigenous phages by different strains of *P. aeruginosa*

To confirm the production of Pf phages in *P. aeruginosa* hosts that were newly superinfected, both infected and wild strains were inoculated into 20 mL of MH broth with a final titer of $10^6$ CFU $mL^{-1}$. All strains were incubated for 12 hours at 37 °C and 200 rpm. After 6 hours of incubation, RNA was isolated from bacterial suspensions using the GeneJET RNA Purification Kit (Thermo Scientific, USA). The resultant RNA was equalized and purified with 1 µL of DNAse I (1 U) for 30 minutes at 37 °C. DNAse was inactivated with 1 µL of 50 mM EDTA at 65 °C for 10 minutes.

Furthermore, 10 mL of the bacterial suspensions has been purified after 12 hours of incubation by centrifugation (6,000 g, 10 min) followed by filtration through 0.22 µm pores. PEG6000 and NaCl at final concentrations of 4% and 0.5 M, respectively, were added to the purified suspensions and incubated overnight at 4 °C. Prior to isolating DNA from precipitated phages, DNase I was used to remove remaining DNA that was not from virions (Peng et al., 2017). Five units of DNase I ($\geq$2,500 units $mL^{-1}$) were added to 200 µL of each sample and incubated for 10 minutes at 37 °C. DNase I pre-treated phage samples were heat-denatured at 100 °C for 15 minutes to separate DNA from phage particles without the need for additional purification processes. The viral DNA was then diluted 100 times and used for qPCR to compare superinfective

Pf production with indgineous Pf phages in newly infected strains. PfLESB58, Pf4, and Pf5 RF qPCR primer pairs were created (Table 2).

### 3.17. Pf Influence on *P. aeruginosa* properties

Infection of different *P. aeruginosa* strains with Pf phages has been successful. Pf4 phage infected LESB58 and PA14 strains, resulting in strains denoted as LESB58+Pf4 and PA14+Pf4, respectively. PfLES58 infected PAO1 and PA14 strains, yielding strains PAO1+PfLES58 and PA14+PfLES58, respectively. Lastly, successful infection with Pf5 phage resulted in LESB58+Pf5 strain. The obtained infected strains were compared with the original wild strains to determine the phenotypic changes in *P. aeruginosa* that occurred after infection with the specific Pf bacteriophage.

#### 3.17.1. Growth Kinetics

Bacterial suspensions of the infected strains, with a density of 0.5 McFarland (~ $10^8$ CFU $mL^{-1}$), were prepared from overnight cultures. These suspensions were then diluted 100-fold in PBS buffer, followed by further dilution in double concentrated MH broth at a 1:1 ratio. In triplicate, 200 µL of the inoculated medium was added to the wells of a microtiter plate. The microtiter plates were shaken continuously and incubated for 24 hours at 37 °C in a spectrophotometer (Thermo Scientific™ Multiskan™ GO). Absorbance was measured every 30 minutes at 600 nm. The average values for infected strains were compared to non-infected strains. The experiment was repeated three times, and the results were averaged.

#### 3.17.2. Autoaggregation

To perform the autoaggregation test, the method described by Basson et al. (2007) was used. Bacterial cultures that were grown overnight were divided into 1 mL aliquots and centrifuged at 6,000 x g for 5 minutes. The resulting supernatant was removed, and the cells were

suspended in 1 mL of PBS, and this process was repeated twice. The washed cells were then used to prepare two series of bacterial suspensions. The first series consisted of 800 µL of PBS and 200 µL of the resuspended washed bacterial cells, which was left untreated and used immediately for measurements as controls. The second series also contained 800 µL of PBS and 200 µL of resuspended washed bacterial cells, but it was incubated for 1 hour at 37 °C. After incubation, the second series was centrifuged for 2 minutes at 650 x g. The resulting supernatant was divided into a microtiter plate in three replicates of 200 µL each. The optical density of the series was measured at 650 nm using a spectrophotometer (Thermo Scientific™ Multiskan™GO) to determine the degree of autoaggregation. The percentage of autoaggregation (%) was calculated using the equation [(OD0 - OD60)/OD0] x 100, where OD0 represents the initial optical density of strains (first series), and OD60 refers to the optical density after 60 minutes of treatment (second series). Bacterial strains were designated as highly aggregative (>50%), moderately aggregative (20–50%), and non-aggregative (<20%), based on the degree of autoaggregation (Malik et al., 2003). The average values obtained for infected strains were compared to wild strains. The experiment was conducted in at least three independent triplicates.

### 3.17.3. Cell surface hydrophobocity

To determine the hydrophobicity of the infected strains, the Bacterial Adherence to Hydrocarbons (BATH) method was employed (Mattos-Guaraldi et al., 2008). Firstly, 1 mL of overnight bacterial cultures were aliquoted into sterile tubes and then centrifuged for 2 minutes at 6,000 x g. The supernatant was removed and the cells were resuspended in 1 mL of PUM buffer ($K_2HPO_4$ + $4H_2O$ 22.2 g, $KH_2PO_4$ + $4H_2O$ 7.26 g, urea 1.8 g, $MgSO_4$ + $7H_2O$ 0.2 g, $dH_2O$ 1 L) which was repeated twice. Then, the washed cells were used to prepare two series of bacterial suspensions. The first series contained 900 µL of PUM buffer and 200 µL of resuspended washed bacterial cells and was left untreated. The second series contained 2.7 mL of PUM buffer and 600 µL of resuspended washed bacterial cells which had a bacterial count of $2 \times 10^8$ CFU $mL^{-1}$. To this series, 400 µL of hydrocarbon n-hexane was added, and the tubes were vortexed for 2 minutes and then left for 15 minutes at room temperature to separate the phases. The aqueous phase was transferred to new tubes and left in the refrigerator overnight for the remaining

hydrocarbons to evaporate. The samples were then aliquoted into a microtiter plate in three replicates of 200 µL each, and the optical density was read at 560 nm using a spectrophotometer (Thermo Scientific™ Multiskan™ GO) to determine the degree of hydrophobicity. The percentage of hydrophobicity (%) was calculated using the following equation: [(A0 - A1)/A0] × 100, where (A1) represents the optical densities of the treated suspensions compared to the control suspensions (A0). Depending on the degree of adhesion to the hydrocarbon, the strains were classified as extremely hydrophobic (>50%), moderately hydrophobic (20–50%), or hydrophilic (<20%) (Mattos-Guaraldi et al., 2008). The average values for infected strains were compared with wild strains. This experiment was conducted in at least three independent triplicates.

### 3.17.4. Motility tests

To assess the motility of *P. aeruginosa* strains, the following methods were employed. Twitching, swarming, and swimming motility were assessed by stabbing overnight cultures into LB plates containing 1.5%, 0.7%, and 0.3% agar, respectively. After 24 hours of incubation at 37 °C, the LB agar medium was removed for the determination of twitching and swarming motility for infected and wild strains. Crystal violet solution of 0.4% was then added inside each Petri dish to improve the visualization of the movement of the strains. Swimming motility was evaluated on the agar plate surface. The experiment was repeated in triplicate and at least three times independently.

### 3.17.5. Biofilm formation of polysterene surface

The method used to determine the biofilm formation by *P. aeruginosa* involved several steps (Knezevic et al., 2008). Overnight bacterial cultures were centrifuged for 2 min at 6,000 x g. The resulting supernatant was removed and the cells were resuspended in 1 mL of PBS buffer. These steps were repeated twice. Bacterial suspensions of 0.5 McFarland density (~ $10^8$ CFU $mL^{-1}$) were then prepared. The obtained bacterial suspensions were first diluted 100 times with PBS buffer (~ $10^6$ CFU $mL^{-1}$), and then additionally diluted with MH broth in a ratio of 1:1 (v/v). The inoculated medium was then transferred to microtiter plate wells in a volume of 300 µL. The

plate was incubated at 37 °C for 24 hours. After incubation, the medium was removed, followed by washing with PBS buffer. This step was repeated twice. The formed biofilm was fixed by adding 350 µL of methanol. After 15 mins of incubation at the room temperature, methanol was removed and the plate was allowed to dry at 44 °C. After that, the biofilm was stained by 350 µL of 0.4% crystal violet for 15 mins and then washed with tap water. The plate was left to dry at the room temperature. The dye was dissolved by adding 350 µL of 33% acetic acid and left at the room temperature for 20 mins. Finally, the optical density of each well was measured at 595 nm using a spectrophotometer (Thermo Scientific™ Multiskan™ GO).

The ODc value was used to assess cell adhesion, which represents the mean of the optical density of the negative control, increased by three standard deviations. *P. aeruginosa* strains were classified based on their OD value, with values greater than ODc considered highly adherent. The biofilm formation was compared between infected and wild strains, and the experiment was repeated in triplicate for three independent repetitions.

### 3.17.6. Pyocyanin production

The amount of pyocyanin produced by *P. aeruginosa* was measured using a modified method developed by Essar et al. (1990). Bacterial suspensions of 0.5 McFarland density (~ $10^8$ CFU $mL^{-1}$) were made from overnight cultures and then further diluted with PBS in a ratio 1:100. The resulting bacterial suspensions were then added to 10 mL of King's A broth and incubated at 37 °C with agitation for 24 hours. After incubation, the cultures were centrifuged and the resulting supernatants were collected. To 7.5 mL of supernatant, 4.5 mL of chloroform was added, mixed and then centrifuged. The blue layer at the bottom was transferred to a new tube, 1.5 mL of 0.2 M HCl and the tube content was mixed. After centrifugation, the blue color changed to pink and 1 mL of the pink layer was transferred to a new tube. The pyocyanin concentration was measured spectrophotometrically at 520 nm (Thermo Scientific™ Multiskan™GO), and the values obtained were multiplied by a constant of 17.072 to determine the concentration in µL/mL. The experiment was performed in triplicates and at least on three independent occasions.

### 3.17.7. Pyoverdine production

To determine the amount of pyoverdine produced, a modified method by Déziel et al. (1991) was used. Bacterial suspensions of 0.5 McFarland density (~ $10^8$ CFU $mL^{-1}$) were made from overnight cultures and then further diluted with PBS in a ratio 1:100. Each strain was then added to 2 mL of King's B Medium and incubated at 37 °C for 24 hours at 200 rpm. After incubation, 1 mL of the bacterial culture was used to determine the total number of bacteria. The remaining overnight cultures were centrifuged and the resulting supernatants were used to quantify the relative concentration of pyoverdine by measuring the fluorescence at 460 nm after excitation with wavelength 340 nm using a Fluoroscan Ascent FL instrument (Thermo Labsystems, Waltham, MA, USA). The experiment was performed in triplicates and repeated at least three independent occasions.

### 3.17.8. SCV production

Wild and Pf infected strains were added to 5 mL of LB and kept at 37 °C for five days without shaking. After incubation, the bacterial suspensions were centrifuged and the resulting pellet was washed twice with PBS. Then, serial dilutions were made, and the total number of wild-type and SCVs' colonies for each strain was determined by the spread plate method on LB agar containing 0.04% Congo Red (Friedman and Kolter, 2009). The colonies were counted after two days of incubation at 37 °C. The experiment was repeated three times, and the results were averaged.

### 3.17.9. Antibiotic susceptibility

To determine the antibiotic sensitivity of wild and Pf-infected *P. aeruginosa* strains, a disk diffusion method with CIP, GEN, TET, and STR (CLSI 2020) was used.

Further analysis of the antibiotic susceptibility of infected strains was done by determining the minimal inhibitory concentration (MIC) for CIP, GEN, STR, TET, CAZ, CHL, and PMB (CLSI 2020). Overnight bacterial cultures were centrifuged for 2 min at 6,000 x g. The resulting supernatant was removed and the cells were resuspended in 1 mL of PBS buffer. These

steps were repeated twice. Bacterial suspensions of 0.5 McFarland density (~ $10^8$ CFU $mL^{-1}$) were then made. The obtained bacterial suspensions were first diluted 100 times with PBS buffer (~ $10^6$ CFU $mL^{-1}$), and then additionally diluted with MH broth in a ratio of 1:1 (v/v). The inoculated medium was then transferred to microtiter plate wells in a volume of 100 µL. Antibiotics were then added in the same volume, with the final concentration of antibiotics in each subsequent well being double diluted, ranging from 128 to 0.0625 µg $mL^{-1}$. The microtiter plates were then incubated at 37 °C for 18 h. After incubation, the MIC value for each antibiotic was determined using 2,3,5-triphenyltetrazoliumchloride (TTC) as an indicator of bacterial viability.

The reference strain *E. coli* ATCC 25922 was used a control, and the experiment was repeated at least three times. The results were presented as the geometric mean. A decrease in the minimum inhibitory concentration (MIC) of one value after phage infection was not considered to be statistically significant. The infected and wild strains were classified as sensitive, intermediate sensitive, or resistant to antibiotics, based on the recommended criteria for *P. aeruginosa* or, if not available, for other non-enterobacteriace (for TET, STR, and CHL) (CLSI 2018).

# 4. Results

## 4.1. Sequence analysis of Pf prophages

In order to draw valid conclusions about the similarity between Pf phages, a more detailed analysis of their genomes and key proteins was conducted. This analysis involved comparing sequences between all phages. The percentage of identity in the genome sequences of Pf1, Pf4, Pf5, and Pf8 phages was above 50% in almost all combinations (Table 5). Values less than 50% were only recorded in combinations of Pf4 phage with Pf1 and Pf5, as well as Pf8 phage with Pf1. On the other hand, PfLES58 phages had a percentage of identity below 50% in all combinations with Pf4 and Pf5 phages, confirming the presence of significant differences in the genes of these phages. Similar results were obtained when comparing PfLES58 phage with Pf1, but in the combination Pf1-PfLES58 the percentage of identity was above 50%.

**Table 5.** Homology in genome sequences between Pf bacteriophages.*

| Pf phages | Pf1 | Pf4 | Pf5 | PfLES58 | Pf8 | Pf3 |
|---|---|---|---|---|---|---|
| Pf1 | 100 | 78.3 | 69.4 | 54.5 | 63.7 | 0 |
| Pf4 | 46.2 | 100 | 44.4 | 28.8 | 50.4 | 0 |
| Pf5 | 48.3 | 52.4 | 100 | 47.7 | 63.7 | 0 |
| PfLES58 | 38.0 | 34.2 | 49.6 | 100 | 37.5 | 0 |
| Pf8 | 46.5 | 62.0 | 68.6 | 38.4 | 100 | 0 |
| Pf3 | 0 | 0 | 0 | 0 | 0 | 100 |

*Homology determined using BLASTn and by calculating (% coverage x % identity) / 100.

A high degree of homology in terms of Zot protein sequences was present among all analyzed bacteriophages (>50%), with the exception of Pf3 phage (Table 6).

**Table 6.** Homology in amino-acid sequences of Zot protein between Pf bacteriophages. *

| Pf phages | Pf1 | Pf4 | Pf5 | PfLES58 | Pf8 | Pf3 |
|---|---|---|---|---|---|---|
| Pf1 | 100 | 98.1 | 86.3 | 54.1 | 85.7 | 6.2 |
| Pf4 | 98.1 | 100 | 86.6 | 52.5 | 86.3 | 6.2 |
| Pf5 | 85.0 | 86.0 | 100 | 54.9 | 99.8 | 12.5 |
| PfLES58 | 53.1 | 52.6 | 56.5 | 100 | 56.2 | 15.0 |
| Pf8 | 85.0 | 85.7 | 99.8 | 53.1 | 100 | 12.5 |
| Pf3 | 6.6 | 6.6 | 6.4 | 15.5 | 6.6 | 100 |

*Homology determined using BLASTp and by calculating (% coverage x % identity) / 100.

In the case of CoaA protein sequences, the similarity was below 50% in almost all combinations (Table 7). Only in the case of Pf4-Pf5, the percentage of identity was 52.6%, while in the combination Pf5-Pf4, it was much lower, at 18.7%.

**Table 7.** Homology in amino-acid sequences of CoaA protein between Pf bacteriophages. *

| Pf phages | Pf1 | Pf4 | Pf5 | PfLES58 | Pf8 | Pf3 |
|---|---|---|---|---|---|---|
| Pf1 | 100 | 32.6 | 22.4 | 7.0 | 22.2 | 0 |
| Pf4 | 32.6 | 100 | 52.6 | 7.8 | 22.8 | 0 |
| Pf5 | 18.9 | 18.7 | 100 | 7.2 | 33.6 | 0 |
| PfLES58 | 6.7 | 7.8 | 7.1 | 100 | 29.5 | 0 |
| Pf8 | 18.7 | 18.4 | 34.1 | 11.0 | 100 | 0 |
| Pf3 | 0 | 0 | 0 | 0 | 0 | 100 |

*Homology determined using BLASTp and by calculating (% coverage x % identity) / 100.

As in the case of Zot, the CoaB proteins of Pf1, Pf4, Pf5, and Pf8 bacteriophages showed a high degree of similarity (Table 8). Together with the Zot protein, the CoaB amino-acid sequence is important in defining the genus category, and phages belonging to the same genus have a similarity of both these proteins >50%. However, only PfLES58 showed CoaB protein similarity of less than 50% compared to other phages.

**Table 8.** Homology in amino-acid sequences of CoaB protein between Pf bacteriophages. *

| Pf phages | Pf1 | Pf4 | Pf5 | PfLES58 | Pf8 | Pf3 |
|---|---|---|---|---|---|---|
| Pf1 | 100 | 100 | 92.7 | 0 | 92.7 | 11.8 |
| Pf4 | 100 | 100 | 92.7 | 0 | 92.7 | 11.8 |
| Pf5 | 92.7 | 92.7 | 100 | 25.9 | 100 | 10.9 |
| PfLES58 | 0 | 0 | 29.2 | 100 | 29.2 | 0 |
| Pf8 | 92.7 | 92.7 | 100 | 25.9 | 100 | 10.9 |
| Pf3 | 24.7 | 24.7 | 17.4 | 0 | 17.4 | 100 |

*Homology determined using BLASTp and by calculating (% coverage x % identity) / 100.

The results of bacteriophage genome analysis are presented in Table 9. Pf4 has the largest genome among given Pf bacteriophages, while Pf3 has the smallest genome. The GC content varied between 45 and 61%, with the highest percentage recorded in the Pf1 phage. For most members, the number of genes was approximately the same (13 – 16). The Pf3 bacteriophage genome encodes only 8 genes, which correlates with its relatively small size. Pf3 phage was used in these analyses because it differs from other members of this group in its characteristics (outlier).

The genome sequences and key proteins of Pf1 bacteriophage were compared and a high homology was found between Pf1, Pf4, Pf5, and Pf8 bacteriophages (Table 9). Their genome sequences largely overlap (60 – 80%). Similar situations were observed with Zot and CoaB proteins (>50%). However, there was a significant difference in CoaA protein between Pf4 and Pf5 bacteriophages compared to Pf1 phages, with identity percentages significantly lower at 32.6% for Pf4 and 18.9% for Pf5 compared to CoaA of Pf1 phages. The largest sequence deviation from Pf1 was observed in Pf3 and PfLES58 phages. PfLES58 CoaA protein share only 6.7% identity with the CoaA protein of Pf1 bacteriophage. No significant similarity was found between these two phages regarding the amino-acid sequence of the CoaB protein. The genome identity is slightly higher at 54.5%, while the amino-acid sequence similarity is only above 50% in the case of the Zot protein.

**Table 9.** Homology between Pf bacteriophages based on genome and key protein amino-acid sequences: Zot, CoaA, and CoaB.

| Pf phages | Phage GenBank Accession/Prophage Coordinates | Genome Length (nt) | %GC | Gene Count *** | DNA (% of sequence identity)* | CoaA (%) | Zot (%)** | CoaB (%) |
|---|---|---|---|---|---|---|---|---|
| Pf1 | NC_001331.1 | 7349 | 61 | 14 | 100 | 100 | 100 | 100 |
| Pf4 | NC_002516.2 785311-797747 | 12437 | 56 | 14 | 78.3 | 32.6 | 98.1 | 100 |
| Pf5 | NC_008463.1 4345126-4355800 | 10675 | 57 | 16 | 69.4 | 22.4 | 86.3 | 92.7 |
| PfLES58 | NC_011770.1 4545190-4555758 | 10569 | 56 | 13 | 54.5 | 7 | 54.1 | 0 |
| Pf8 | NC_073756.1 | 10061 | 58 | 16 | 63.7 | 22.2 | 85.7 | 92.7 |
| Pf3 | NC_001418.1 | 5833 | 45 | 8 | 0 | 15.75 | 11.8 | 11.8 |

* Determined using BLASTn; ** Determined using BLASTp; *** Determined using CoreGenes 3.5.

The genomic comparisons of Pf bacteriophages revealed a consistent arrangement of key genes (Fig. 11). Most Pf phages possess an integrase gene (*int*) at the beginning of the genome. Following the integrase, genes involved in phage morphogenesis, such as the *zot* gene (*g1*), are present. After that, genes for structural proteins such as *coaA* (*g3*) and *coaB* (*g8*) can be found. Towards the end of the genome, the gene for ssDNA binding protein (*g5*) is located. In all Pf phages, a significant number of genes encoding proteins with unknown functions are present. Hypothetical proteins are often found between *int* and *zot* genes, as well as between *zot* and *coaA* genes. Other hypothetical proteins are often located at the end of the genome.

Pf4 phages exhibit additional genes, including those for the type II toxin-antitoxin system (RelE/ParE) at the start and reverse transcriptase genes at the end of the genome (Fig. 11). Pf8 phage has a gene for a putative toxin near its end, along with genes for replication initiation and excisionase. The most remarkable difference in terms of gene number and arrangement is observed in Pf3 phage. Pf3 phage does not have an integrase gene, and its *g5* gene is located on the opposite side, that is, at the beginning of the genome. Its gene for CoaA is significantly smaller compared to the same gene in other Pf phages. This phage also contains genes for p4 and TspB protein, which are not found in other Pf phages.


**Figure 11.** Visualization of linear comparisons of multiple genomic loci of Pf bacteriophages using EasyFig.

## 4.2. Phylogenetic analysis of Pf bacteriophages and their relationship with other filamentous phages

Phylogenetic tree was constructed based on genome sequences of filamentous phage from different genera from the family *Inoviridae* (Fig. 12).

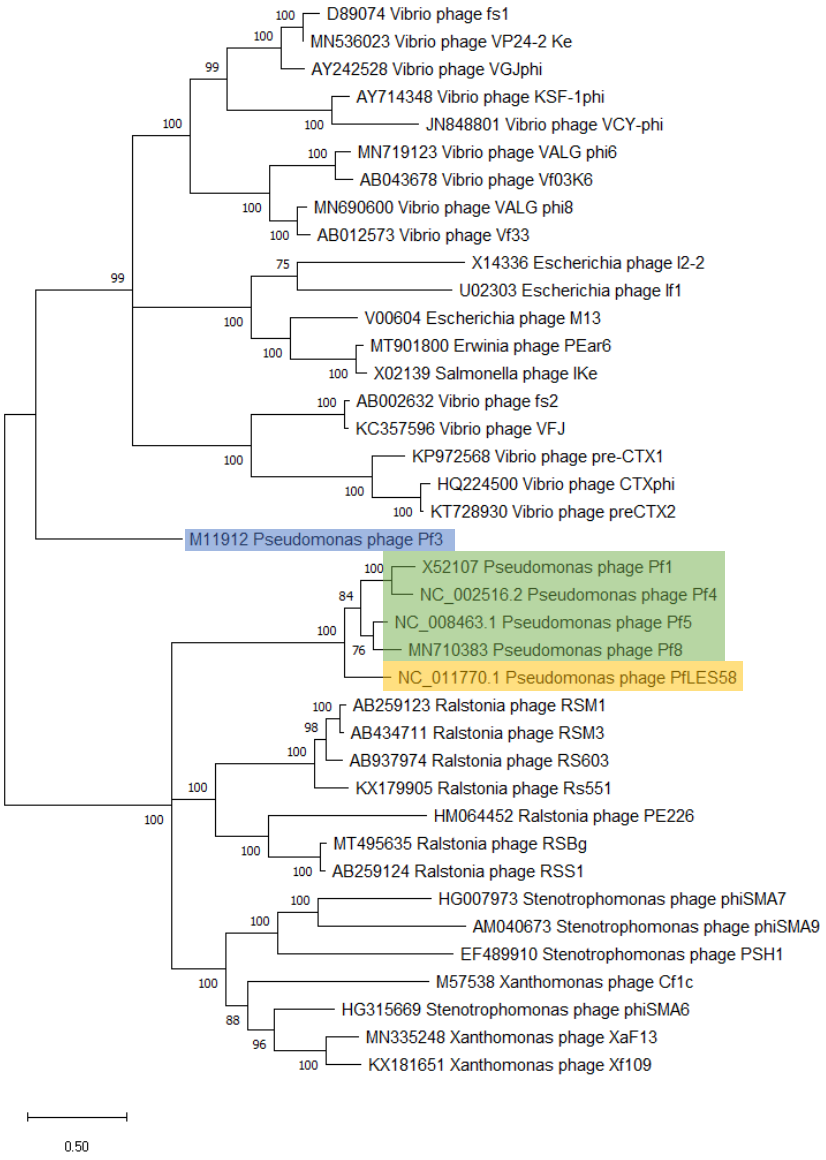


**Figure 12.** Phylogenetic relationships of recognized species of filamentous phages with Pf4, Pf5 and PfLES58 phages based on the nucleotide sequence of the genome. The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model (Kimura 1980). The tree with the highest log likelihood (-306316.39) is shown. The percentage of trees in which the associated taxa clustered together is shown

next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 39 nucleotide sequences. There were a total of 13684 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar et al., 2018).

According to the obtained results, PfLES58, Pf4, and Pf5 phages showed the closest similarity to filamentous phages infecting *Ralstonia*, *Stenotrophomonas*, and *Xanthomonas* bacteria (Figure 1). Pf1 and Pf4 phages belong to the same clade, as is the case with Pf5 and Pf8 phages. PfLES58 does not belong to any of these clades. The phage Pf3, which also infects *P. aeruginosa*, appeared distinct from other Pf phages and exhibited similarity to filamentous phages of *Vibrio* and phages that infect Enterobacteria.

### 4.3. Confirmation of PfLES58 production

The confirmation of the presence of PfLES58 replicative form (RF) was successful (Fig. 13A).

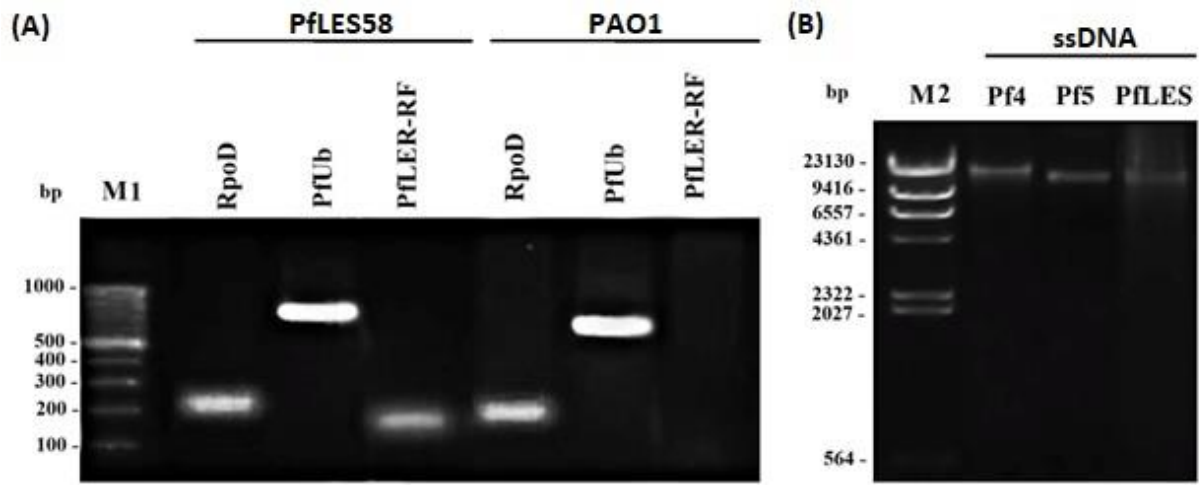


**Figure 13.** (A) - Agarose gel electrophoresis (0.7%) of PCR products that confirm the production of PfLES58 replicative form (RF). (B) - Agarose gel electrophoresis (0.7%) of ssDNA isolated from Pf virions with expected genome size of 12437 kb (Pf4), 10675 kb (Pf5), and 10569 kb (PfLES58); M2 – λ-HindIII ladder.

This was not previously confirmed in the literature for this prophage unlike Pf4 and Pf5. The expected RF product was obtained from total LESB58 DNA, as opposed to PAO1 where it was absent as expected. Both the housekeeping gene and universal genetic elements of the Pf phages were detected in the DNA of both strains. The obtained RF sequence for PfLES58 (MG783392.1) confirmed the previously predicted prophage ends, and additional experimental steps verified the production of both virions and plaques.

### 4.4. Prevalence of PfLES58 specific genetic elements in *P. aeruginosa* strains

The PCR method was used to examine the existence of PfLES58 specific genetic elements in 267 strains of *P. aeruginosa*. Out of these, 110 strains (41.2% of the total tested strains) were found to have *coaA* gene of PfLES58 phage (Fig. 14). However, only 56 strains (50.9%) produced virions successfully and were found to have RF of PfLES58 phage. A total of 157 strains (58.8%) did not have any PfLES58 genetic elements detected. PfLES58 was found in strains obtained from different sources. The highest percentage of isolates positive to PfLES58 were obtained from sputum (38.5%, 5/13) and patients with cystic fibrosis (42.9%, 3/7) (results are not shown). Furthermore, both PfLES58 genetic elements were detected in hospital strains and isolates from wound and urinary infections.

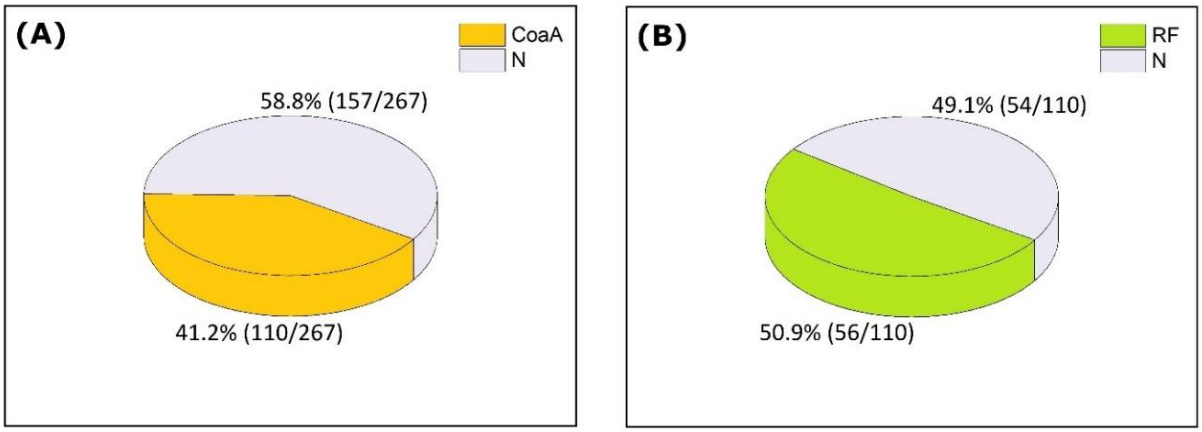


**Figure 14.** A PCR-based analysis of 267 *P. aeruginosa* strains for the presence of PfLES58 specific genetic elements. (A) - *coaA* positive strains. (B) - *coaA* positive strains producing virions with PfLES58 RF. N - absence of both, *coaA* gene and replicative form.

### 4.5. Comparison of method 1 and method 2 for Pf phage propagation

After using method 2 propagation, the bands of CsCl purified particles for Pf4, Pf5, and PfLES58 phages were notably thicker (Fig. 15). The number of virions in the harvested and dialyzed phage bands was significantly higher after applying method 2. Method 1 resulted in phage titers ranging from $10^5$ to $10^6$ PFU $mL^{-1}$, but after using method 2, phage titers ranged from $10^9$ to $10^{13}$ PFU $mL^{-1}$ (Fig. 16).

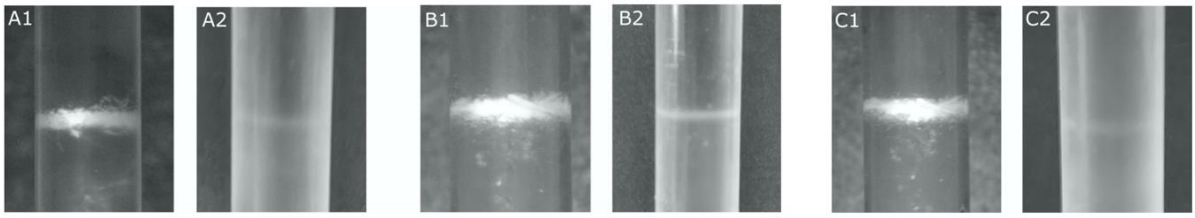


**Figure 15.** Pf bacteriophage bands after equilibrium density ultracentrifugation in CsCl at 135,000 x g and 4 °C for 42 hours. Method 2 (1) produced more distinct bands than method 1 (2) for all three phages (Pf4 (A), Pf5 (B), and PfLES58 (C)).

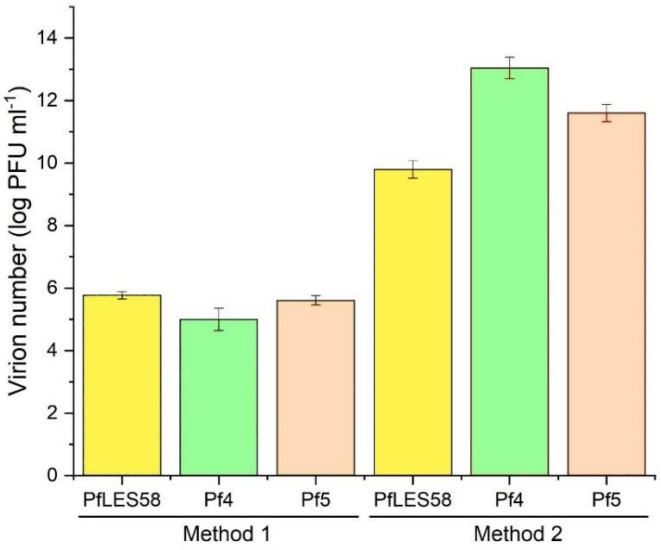


**Figure 16.** Phage count obtained using CsCl method following the application of method 1 and method 2.

Likewise, the yield of ssDNA obtained from nucleic acid isolation from virions was much higher using method 2, ranging from 25.3 to 65.93 ng $mL^{-1}$, depending on the phage, compared to method 1 which yielded between 2.94 – 3.33 ng $mL^{-1}$ (Table 10). The genome sizes of PfLES58,

Pf4, and Pf5 were confirmed to be approximately 10.5, 12.5, and 10.5 kb, respectively, based on the isolated DNA from virions (Fig. 13B).

**Table 10.** Concentrations of ssDNA obtained after CsCl purification of Pf phages and ssDNA isolation using two different propagation methods.

| | ssDNA (ng $mL^{-1}$) | | |
|---|---|---|---|
| | **Pf4** | **Pf5** | **PfLES58** |
| **Method 1** | 3.13 ± 0.2 | 2.94 ± 0.2 | 3.33 ± 0.8 |
| **Method 2** | 43.8 ± 4.9 | 65.93 ± 1.6 | 25.3 ± 1.8 |

### 4.6. Production of Pf virions and plaque formation

During a 6-day monitoring period after PEG8000 precipitation, the increase in phage numbers for PfLES58, Pf4, and Pf5 was measured. Chloroform treatment was used to confirm the presence of tailed phages in the suspension, and the results showed that PFU of filamentous phages was 50.8% greater than the total phage number in PAO1 supernatant and 85.5% in PA14 after 2 days of incubation (Table 11). As shown in Figure 17, phage titers varied significantly between days 2 and 4, depending on the Pf phage strain and bacterial strain, ranging from $10^4$ to almost $10^8$ PFU $mL^{-1}$. The only exception was Pf4, whose phage titer was similar in the given time interval. After 6 days, phage titers for all Pf phages were within the range of $10^9$ – $10^{10}$ PFU $mL^{-1}$, which was suitable for further phage purification.

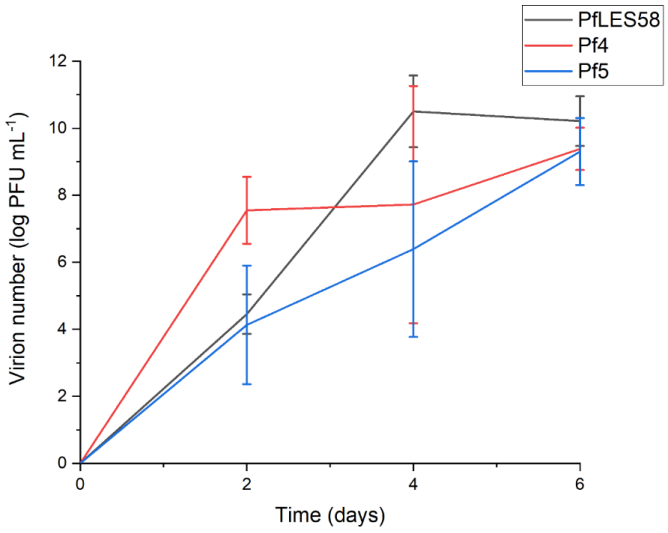


**Figure 17.** The Pf virion number per mililiter in supernatant during 6 days of incubation and PEG8000 precipitation.

**Table 11.** The percentage of filamentous phages in the total number of phages obtained from PEG8000 precipitation.

| Precipitated *P. aeruginosa* supernatant | Days | Filamentous phage plaques in total number (%) |
|---|---|---|
| PAO1 | 2 | 50.8 |
| | 4 | 60.3 |
| | 6 | 49.8 |
| UCBPP-PA14 | 2 | 85.8 |
| | 4 | 88.5 |
| | 6 | 96.3 |

Furthermore, an interesting occurrence was observed when using the single- or double-layer SPOT method to determine Pf phage titers. Specifically, after an 8-hour incubation, visible plaques were present with both techniques, but after 24 hours, the plaques on the double layer became faint and almost imperceptible (Fig. 18-I). Finally, we provide the first evidence that PfLES58 and Pf5 phages form plaques on bacterial lawns, which appear small (approx. 1 mm) and cloudy (Fig, 18-II).

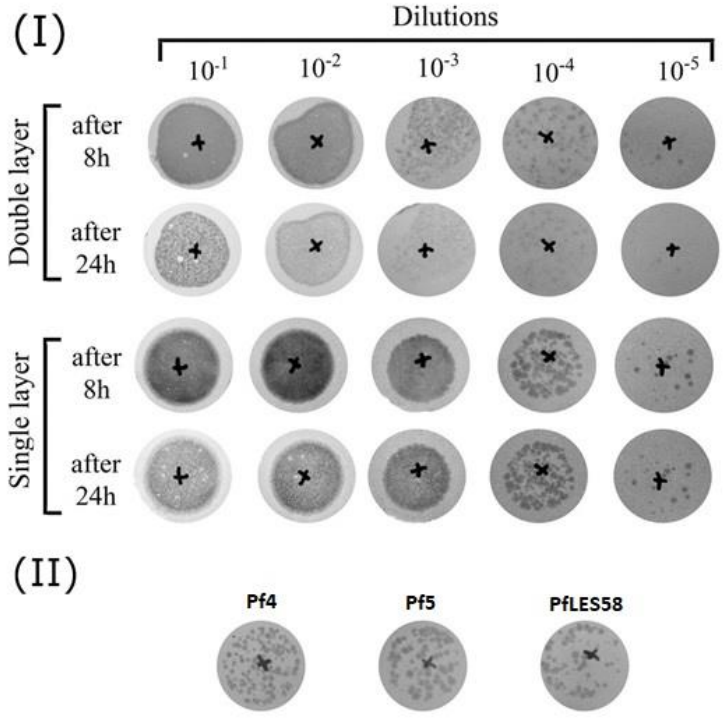


**Figure 18.** The morphology of filamentous phage plaques on TuD43 *P. aeruginosa* lawns: **(I)** Comparing plaque morphology of Pf4 virions using single- and double-layer SPOT methods after 6-day incubation with PEG8000 concentration. **(II)** Plaque morphology comparison of three Pf phages.

### 4.7. Transmission electron microscopy (TEM) imaging of Pf phages

Filamentous bacteriophages of appropriate morphology and dimensions were observed by transmission electron microscopy (Fig. 19). Approximately the length of Pf phage virions ranged from 2,000 to 3,800 nm. The diameter of virions was 6 – 7 nm. Among the studied phages, Pf1 exhibited the smallest size, while Pf4 stood out as the largest. These results are expected because the size of the Pf1 phage genome is the smallest, while that of Pf4 is the largest (Table 12). The dimensions of PfLES58 and Pf5 are similar, which also coincides with the size of their genomes.

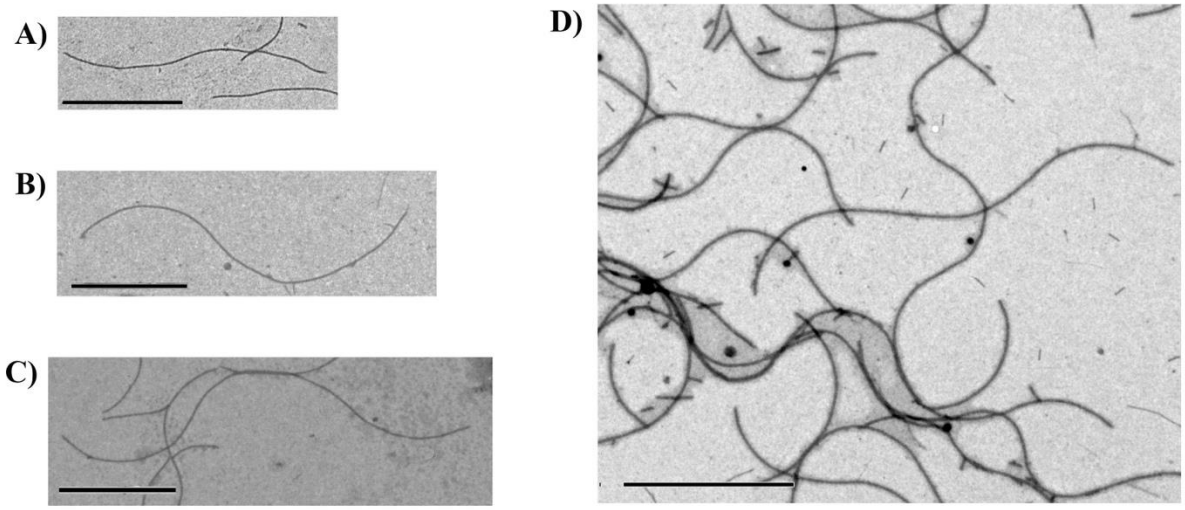


**Figure 19.** Transmission electron microscopy of filamentous phages: Pf1 (A), Pf5 (B), Pf4 (C) and PfLES58 (D); bar 1000 nm.

**Table 12.** Dimensions of Pf virions based on measurements of transmission electron microscopy images.

| Phage designation | Genome size (nt) | Approx. diameter (nm) | Approx. lenght (nm) |
|---|---|---|---|
| Pf1 | 7,349 | 6-7 | 2,000 – 2,100 |
| Pf4 | 12,437 | 6-7 | 3,700 – 3,800 |
| Pf5 | 10,675 | 6-7 | 2,900 – 3,100 |
| PfLES58 | 10,569 | 6-7 | 2,900 – 3,000 |

### 4.8. Confirmation of circular ssDNA in Pf virions

To confirm presence of circular ssDNA originating form Pf virions, and not from bacterial hosts, the isolated DNA was treated with four different enzymes (Fig. 20). The integrity of the

DNA was preserved following treatment with restriction enzyme EcoRI and exonuclease. However, DNA degradation was observed when treated with DNAse and S1 nuclease for all three Pf phages. These results confirm the presence of circular ssDNA in all three Pf phages.


**Figure 20.** Treatment of circular ssDNA from Pf filamentous phages with different enzymes. A – PfLES58, B – Pf4, C – Pf5, (+) - Positive control (phage DNA), (-) - Negative control ($dH_2O$).

## 4.9. SDS-PAGE analysis of Pf phage proteins

The analysis of proteins extracted from dialyzed Pf phages using SDS-PAGE revealed the existence of two main bands in all three Pf phages. Based on their size, the first band, which ranges from 30 to 100 kDa, is assumed to be the minor coat protein (CoaA) of the Pf phage (Fig. 21). On the other hand, the second band has a size of about 5 kDa, which is consistent with the major coat protein (CoaB) of the Pf phage.

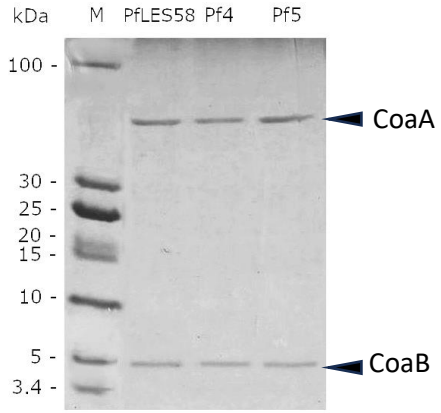


**Figure 21.** SDS-PAGE analysis of extracted proteins from dialyzed Pf phages showing two distinct bands corresponding to minor coat protein (CoaA) and major coat protein (CoaB). M - PageRuler™ Unstained Low Range Protein Ladder.

## 4.10. Pf bacteriophage lytic spectra

A total of 251 *P. aeruginosa* isolates were evaluated for their susceptibility to Pf bacteriophage, alongside 16 reference strains (267 in total) (Table S2). The largest number of

tested strains originated from wounds, urinary infections, and hospitals, but also in smaller numbers from water, soil, plants, cosmetic products, and production lines (Table S2). PfLES58 showed the widest lytic spectrum (90.6%, 242/267), followed by Pf5 (62.5%, 167/267) and Pf4 (46.1%, 123/267), respectively (Fig. 22). Looking at the lytic spectrum of all three Pf phages together, only 11 strains (4.1%) were not lysed with any Pf phages. All strains from the upper respiratory tract, including throat and nasal swabs and sputum, were susceptible to PfLES58, while hospital strains were the least sensitive (69%, 22/32) (results are not shown). Pf4 primarily infected strains obtained from ear swabs (62%, 13/21) and cystic fibrosis patients (57%, 4/7), whereas Pf5 showed similar efficacy on strains from different sources and was most effective on hospital strains (69%, 22/32) (results are not shown). Strains isolated from the natural environment were sensitive to all three Pf phages.

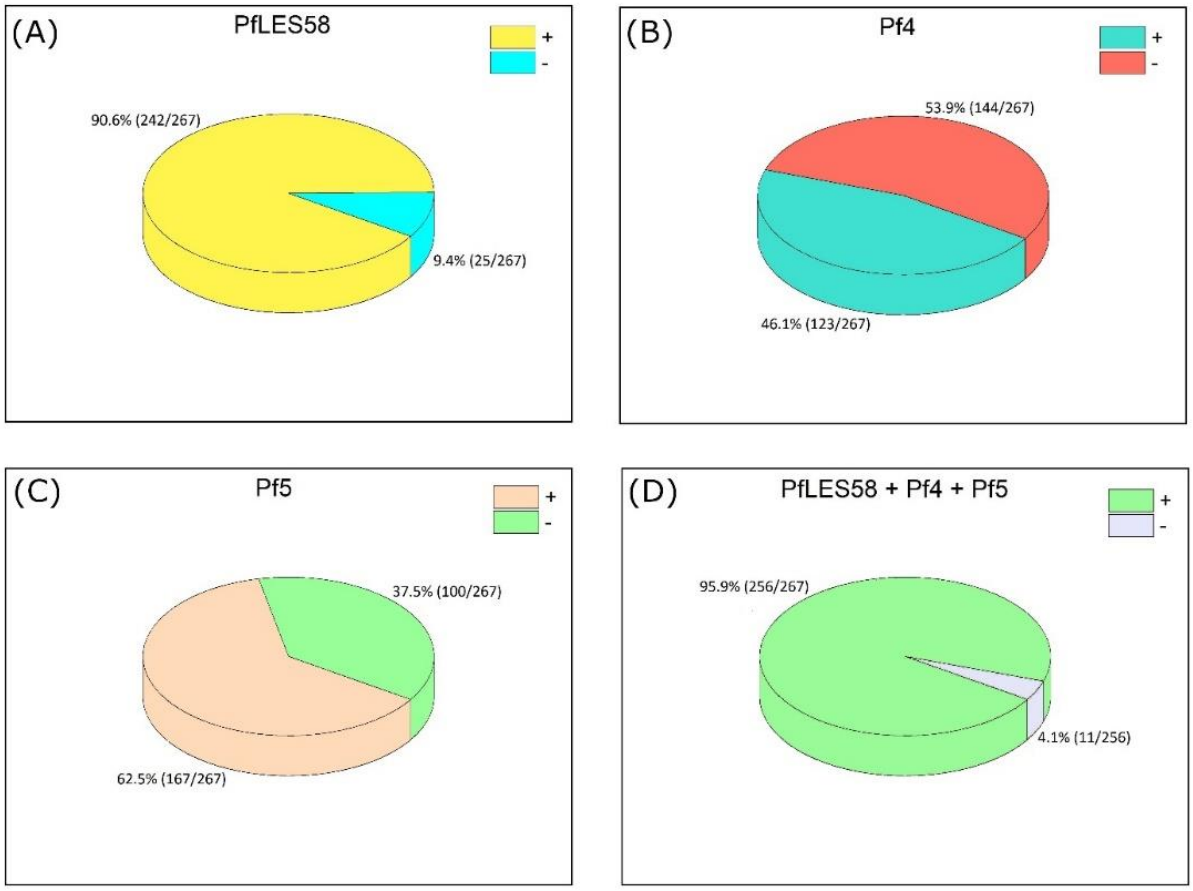


**Figure 22.** Susceptibility of *P. aeruginosa* strains to PfLES58 (A), Pf4 (B), Pf5 (C) and in combination with all three together (D). (+) susceptible to Pf phage; (-) not susceptible to Pf phage

### 4.11. Effects of antimicrobial treatments and obligatory lytic phage infection on Pf phage gene expression in natural hosts

The impact of antimicrobial treatments on the expression of Pf genes in their natural hosts was assessed by qRT-PCR analysis of *coaB* and *zot* gene expression (Fig. 23 I-A, I-B and I-C). Treatment with CIP induced the expression of PfLES58, Pf4, and Pf5 genes after the first hour of treatment (RFC 1.5, 1.68, and 1.52, respectively). However, the effect of CIP on phage induction was not maintained after 6 and 24 hours. Similar results were obtained with MMC for all three phages. PfLES58, Pf4, and Pf5 phage induction was observed after 1 hour of treatment, with RFC values of 1.95, 1.61, and 1.57, respectively. MMC further induced Pf4 and Pf5 phages after 6 hours of incubation (RFC 1.62 and 1.71, respectively). There were no significant changes in induction of the three Pf phages in the presence of GEN and CAZ. After 24 hours, RFC was close to 1 or even slightly lower for all four antimicrobial agents.

The impact of an obligatory lytic phage on the expression of *coaB* and *zot* genes in Pf phages was also examined. No significant changes were observed in the expression of PfLES58, Pf4, and Pf5 phage genes in the presence of JG024 after 6 hours of incubation. However, all three prophages were induced after 12 and 24 hours of incubation. PfLES58 gene expression had an RFC of 1.65 after 12 hours and 1.63 after 24 hours (Fig. 22 II-A). Pf5 had an RFC of 1.53 after 12 hours and 2.56 after 24 hours (Fig. 23 II-C). Lastly, for Pf4, the RFC was 4.33 after 12 hours and 4.57 after 24 hours (Fig. 23 II-B).

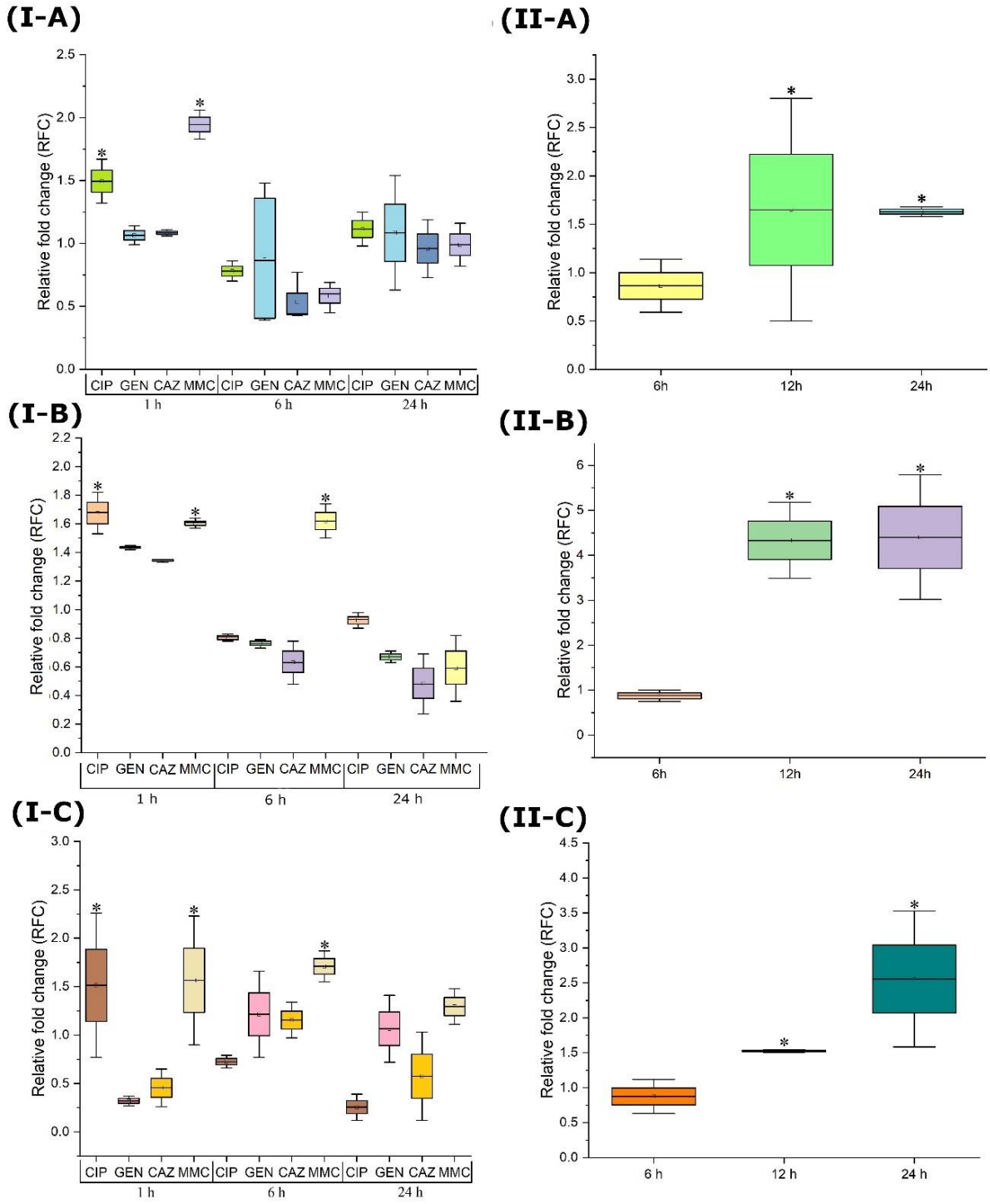


**Figure 23.** Change of Pf *coaB* and *zot* gene expression in their natural hosts after (I) treatment with 1/4 MIC of CIP, GEN, CAZ, and MMC and (II) infection with phage JG024 at MOI 0.25 (equivalent to 1/4 MIC). Results are average + S.E., n = 6; calculated using $2^{-\Delta\Delta CT}$ method, and cut-off for relative change in expression was ≥1.5 or ≤0.67; * ≥1.5. A – PfLES58, B – Pf4, C – Pf5.

### 4.12. Pf phage infection of other *P. aeruginosa* strains

The natural hosts of PfLES58 (LESB58), Pf4 (PAO1), and Pf5 (PA14) phages were infected with various Pf phages in a cross-infection experiment. PAO1 and PA14 strains were infected with PfLES58, while LESB58 and PAO1 were used for Pf5 infection. Finally, natural hosts of PfLES58 and Pf5, LESB58 and PA14, were infected with Pf4. Successful infections were confirmed by PCR amplification of a structural gene and the replicative form (Fig. 24). The presence of integrase was checked for PfLES58 and Pf4 phages, while *coaA* was checked for Pf5.

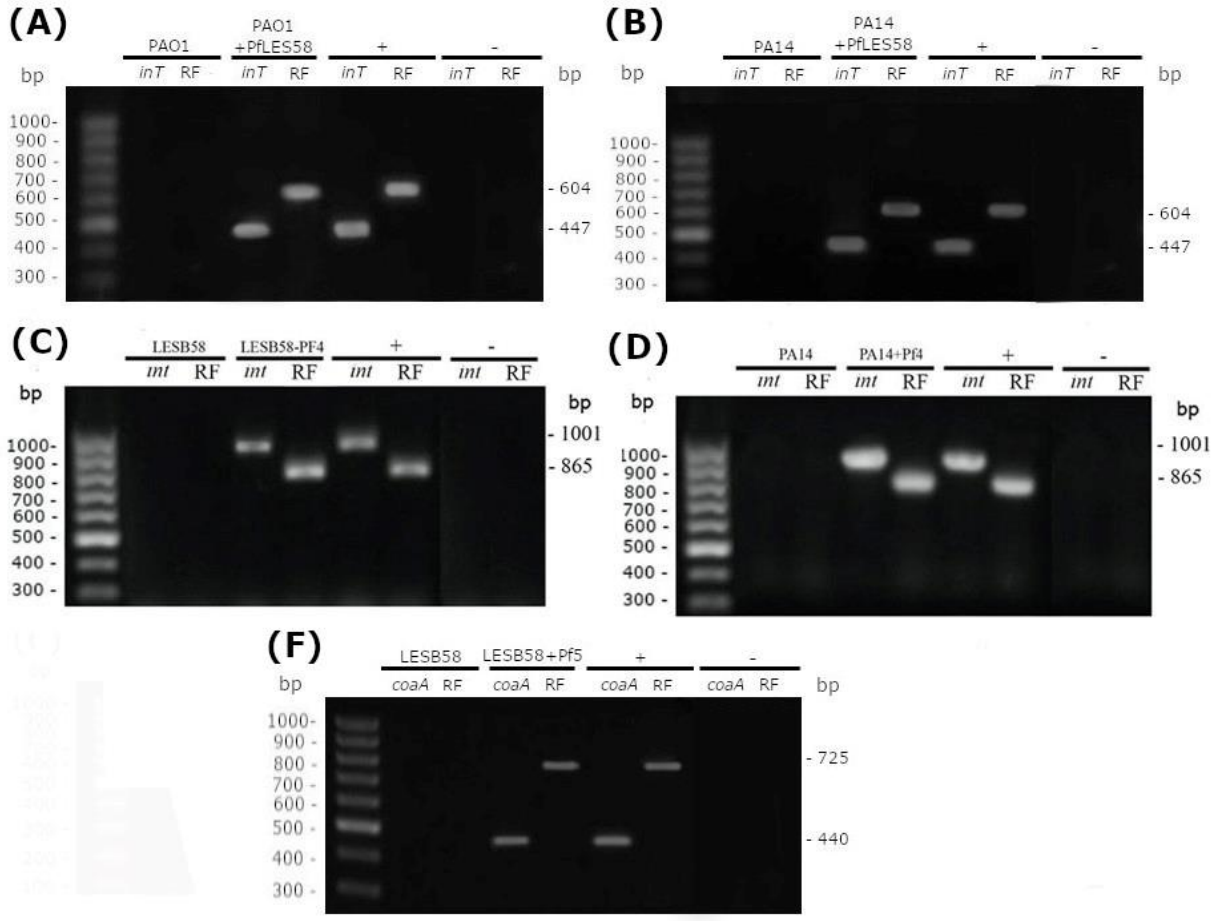


**Figure 24.** Cross-infection experiment of natural hosts of Pf phages with the same Pf phages. (+) Positive control (natural host of the given Pf phage), (-) Negative control ($dH_2O$).

The specificity of the obtained PCR products for the replicative form of PfLES58, Pf4, and Pf5 phages were verified by digestion with SduI, HpaII, and Csp6I, respectively (Fig. 25).


**Figure 25.** PCR products from Pf phage replicative forms digestion with specific restriction enzymes. A - The expected 293, 223, and 88 bp products were obtained after SduI enzyme digestion of the PfLES58 RF product. B - The expected 654 and 211 bp products were obtained after HpaII enzyme digestion of the Pf4 RF product. C - The expected 638 and 87 bp products were obtained after Csp6I enzyme digestion of the Pf5 RF product.

The expected bands were obtained, and the assumed *att* site for each Pf phage was confirmed by sequencing the corresponding PCR products using primer pairs that cover both bacterial DNA, *att* site, and phage genomes (Fig. 26). The expected size of PCR products for confirming the integration of PfLES58 phage in PAO1 and PA14 strains is 258 bp, while for Pf4 integration in LESB58 and PA14 strains it is 943 bp (Fig. 26A-B). Pf5 only infected the LESB58 strain, and the size of the PCR product for confirming its integration is 243 bp (Fig. 26C). The results showed that the DNA of Pf is integrated into the bacterial genomes as prophages. Pf4 was found integrated near tRNA-Gly in both cases, in LESB58 and PA14 strains (Table S3). On the other hand, PfLES58 and Pf5 integrated into the genome of all infected strains near the gene that codes tRNA 2-thiocytidine biosynthesis protein TtcA (Table S4 and S5, respectively).

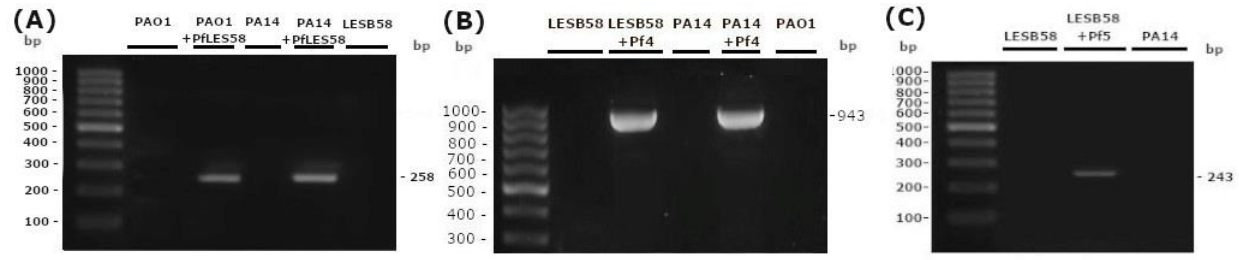


**Figure 26.** Confirmation of Pf bacteriophage integration in the genome of other *P. aeruginosa* strains.

### 4.13. Production of filamentous phages in different Pf superinfected strains of *P. aeruginosa*

The study successfully confirmed the production of both superinfective and indigenous phages from strains PAO1, PA14, and LESB58 by detecting specific *coaA* gene expression and presence of replicative forms using qPCR analysis. Upon infection with PfLES58, increased expression of Pf4 (RFC 4.01) and PfLES58 (RFC 2.76) phages was observed in the superinfected PAO1+PfLES58 strain (Fig. 27). However, no significant change in *coaA* gene expression was observed in the PA14+PfLES58 strain for Pf5 and PfLES58 phages. Pf5 had increased expression in the LESB58+Pf5 strain (RFC 3.97), and a significantly increased expression of PfLES58 phage was also recorded (RFC 2.12). Pf4 was also produced from the infected corresponding strains along with Pf5 or PfLES58. PfLES58 phage expression remained unchanged after infection of the LESB58 strain with Pf4 phage, while a 300-fold decrease in Pf5 gene expression was observed in the presence of Pf4 phage in PA14+Pf4 strain (RFC 0.003). The expression of Pf4 phage in the PA14 strain was significantly reduced compared to PAO1, while in the LESB58 strain, it remained unchanged. Wild strains were used as negative controls.

qPCR analysis of viral DNA of the PAO1+PfLES58 strain found that the presence of PfLES58 phage did not significantly affect the production of Pf4 phage (Fig. 28A). However, a significant inhibition of PfLES58 phage production was observed in this strain compared to the LESB58 strain ($p < 0.05$). The Ct value for the RF PfLES58 phage in the LESB58 strain was 28.96 $\pm$ 0.49, while in the PAO1+PfLES58 strain it was 34.55 $\pm$ 0.69. Similar results were observed in the PA14+PfLES58 strain, where there was a significant inhibition in the production of PfLES58 phage (35.06 + 0.57) ($p < 0.01$), while Pf5 production was not significantly affected by the presence of this phage (Fig. 28B). In the LESB58+Pf5 strain, PfLES58 was also inhibited (35.7 $\pm$ 0.08) ($p < 0.05$), while Pf5 production was not significantly changed (Fig. 28E). The production of PfLES58 phage remained stable and without significant statistical changes after Pf4 phage infection. In the wild LESB58 strain, the Ct value for RF PfLES58 phage was 28.96 $\pm$ 0.50, while in the Pf4 infected strain it was 32.94 $\pm$ 0.37 (Fig. 28C). There was significantly lower production of Pf5 phages in the infected strain in the presence of Pf4 phages. In the wild PA14 strain, the Ct value for RF Pf5 phage was 23.79 $\pm$ 0.5 compared to the Pf4 infected strain where the Ct value was 32.63 $\pm$ 0.9 (Fig. 28D).

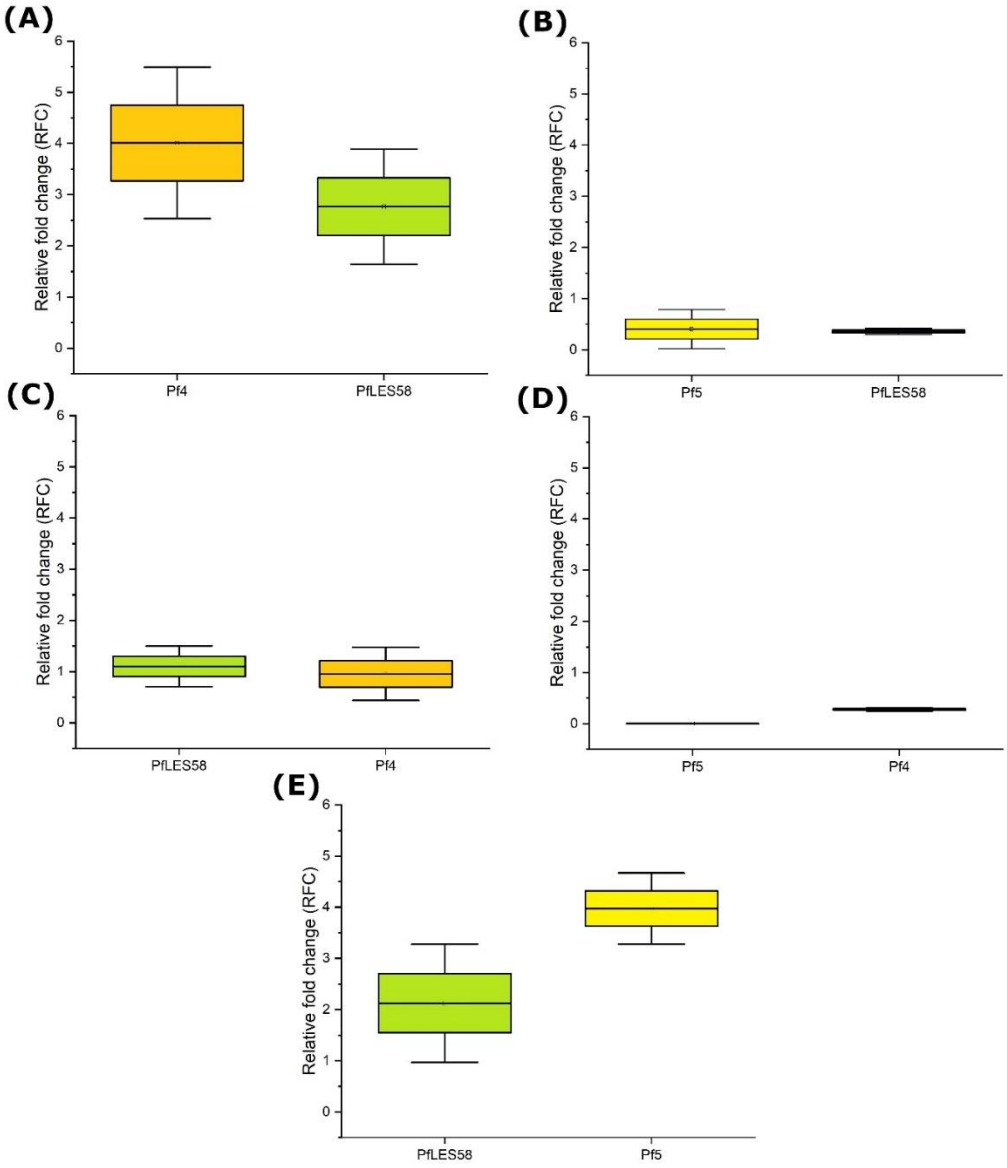


**Figure 27.** qRT-PCR analysis of superinfective and indigenous phages: A,B – Detection of PfLES58 *coaA* gene expression at the RNA level in PAO1 and PA14 strains, respectively, along with Pf4 and Pf5. C, D – Detection of Pf4 *coaA* gene expression at the RNA level in LESB58 and PA14 strains, respectively, along with PfLES58 and Pf5. E – Detection of Pf5 *coaA* gene expression at the RNA level in LESB58 strain, along with PfLES58. The results show the mean values with standard error of six replicates calculated using the $2^{-\Delta\Delta CT}$ method. The cut-off for relative change in expression is set at ≥1.5 or ≤0.67. The asterisk (*) denotes significant changes in expression above or below the cut-off.

These results corresponded with the relative change in PfLES58 and Pf5 phage gene expression at the RNA level in both infected strains. The Ct value of Pf4 in the PAO1 strain was an average of 24.73. However, in both infected strains, the production of Pf4 phage was lower. In PA14+Pf4, it was 29.96 $\pm$ 3.75 (Fig. 28D), and in LESB58+Pf4, it was 31.39 $\pm$ 0.83, which is a statistically significant difference ($p < 0.05$) (Fig. 28C).

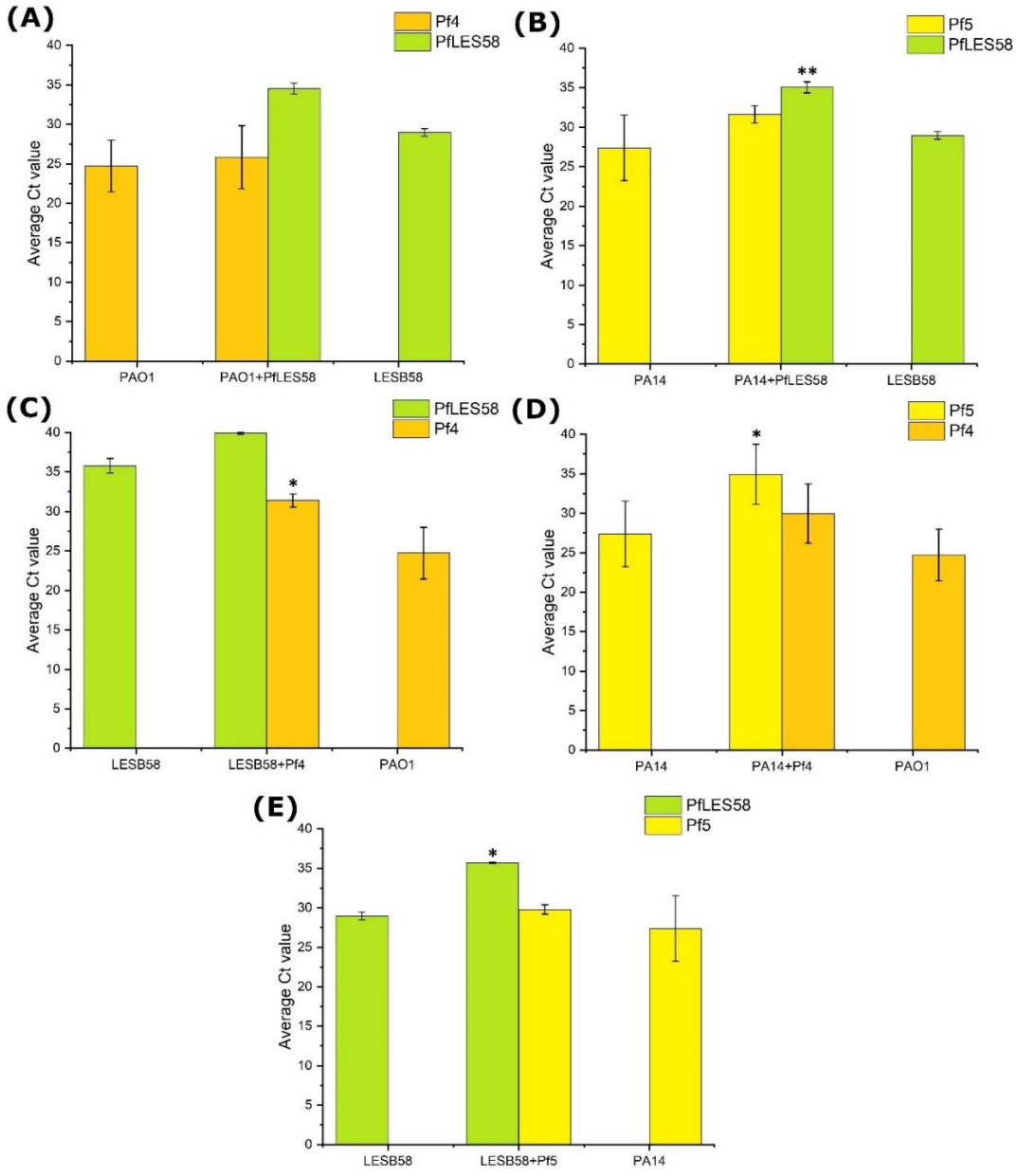


**Figure 28.** qPCR analysis of superinfective and indigenous Pf phage production at viral DNA using RF of bacteriophages after 24 h of incubation, respectively. Results are average Ct values + S.E., n = 6, calculated using Student's t-test; * $p \leq 0.05$; ** $p \leq 0.01$.

### 4.14. Pf influence on phenotype of *P. aeruginosa*

#### 4.14.1. Pf phage influence *P. aeruginosa* growth rate

The growth kinetics of both infected strains were affected to some extent by PfLES58 (Fig. 29).

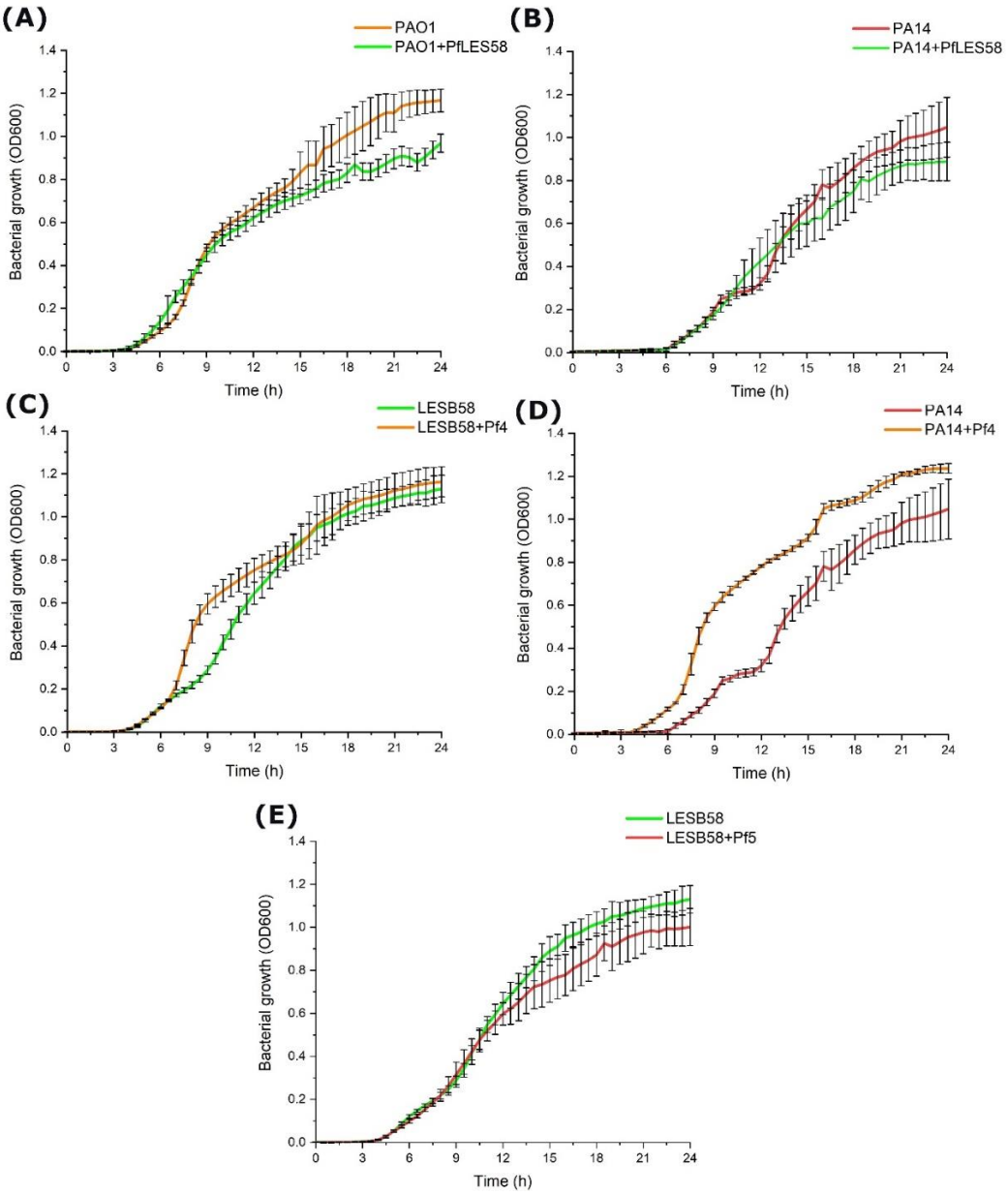


**Figure 29.** Growth of wild and Pf-infected *P. aeruginosa* strains, monitored every 30 min during 24 h, and expressed as OD600 (average + S.D.).

The growth of PAO1 and PA14 strains was inhibited upon infection with PfLES58, with a more pronounced effect observed in PAO1+PfLES58 (Fig. 29A). The LESB58+Pf5 strain also displayed a similar trend, exhibiting slower growth compared to the wild strain (Fig. 29E). In contrast, Pf4 had only a slight impact on the growth kinetics of infected strains. The growth of both infected strains was slightly stimulated by the phage Pf4 between 7 and 15 hours for LESB58 (Fig. 29C) and 7 and 21 hours for PA14 (Fig. 29D), but eventually, both infected and wild cultures reached the same optical density (OD).

### 4.14.2. Pf phages decrease *P. aeruginosa* autoaggregation

In infected strains, the cell autoaggregation was affected by PfLES58, resulting in a statistically significant decrease observed in PA14+PfLES58 ($p < 0.01$) (Fig. 30A). However, the PA14 strain remained moderately aggregative. Pf4 phage also had an effect on infected strains' autoaggregation ability, with both LESB58+Pf4 and PA14+Pf4 strains changing from moderately aggregative to non-aggregative upon infection ($p < 0.01$ and 0.001, respectively) (Fig. 30B). On the other hand, in the case of LESB58+Pf5, no statistically significant change was observed, and the strain remained moderately autoaggregative (Fig. 30C).

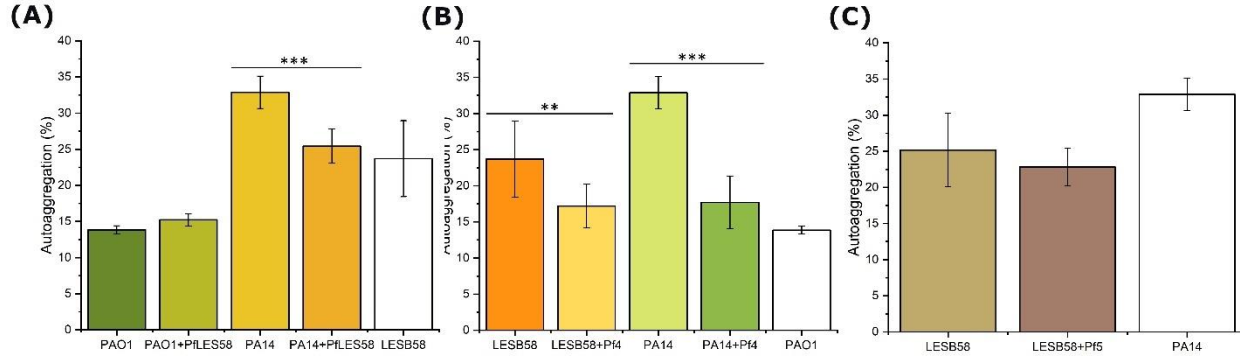


**Figure 30.** Effect of Pf phage infection on autoaggregation in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; *** $p < 0.001$; ** $p < 0.01$; tested by Wilcoxon signed rank test.

### 4.14.3. Pf phages increase cell surface hydrophobocity

The phage Pf4 caused an increase in hydrophobicity in both strains, but only PA14 exhibited a significant increase ($p < 0.001$), although it remained hydrophilic (Fig. 31B). Similarly, PfLES58 had a significant impact on cell hydrophobicity in both cases ($p < 0.01$ and $p < 0.01$). Both PAO1+PfLES58 and PA14+PfLES58 cell surfaces changed from hydrophilic to moderately hydrophobic (Fig. 31A). A significant increase in the hydrophobicity of LESB58+Pf5 cell surface was also observed ($p < 0.01$), but the strain remained moderately hydrophobic (Fig. 31C).

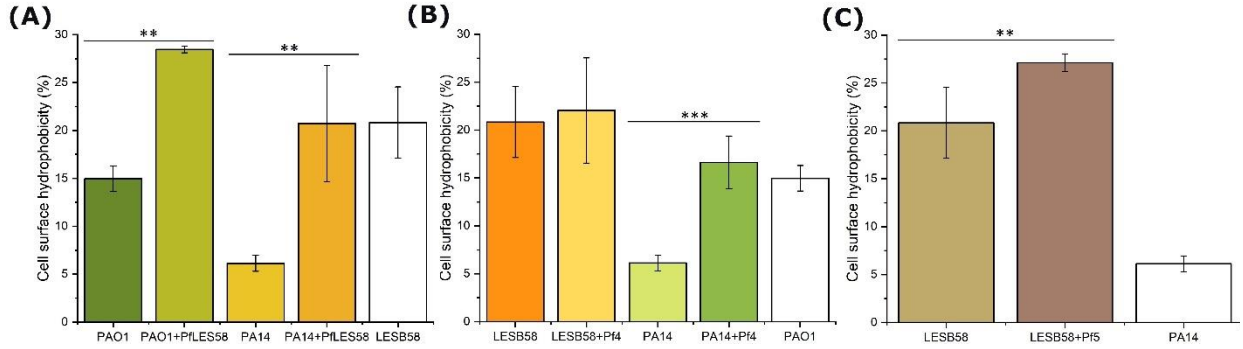


**Figure 31.** Effect of Pf phage infection on hydrophobicity in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; *** $p < 0.001$**; $p < 0.01$; tested by Wilcoxon signed rank test.

### 4.14.4. Motility tests

#### 4.14.4.1. Pf phages inhibit *P. aeruginosa* twitching motility

PfLES58 had a significant inhibitory effect on the twitching motility of PAO1 strain ($p < 0.01$) (Fig. 32A). The twitching motility of Pf4-infected PA14 was also reduced with a statistically significant difference ($p < 0.05$) (Fig. 32B). Furthermore, LESB58 strain exhibited a significant reduction in twitching motility ($p < 0.01$) in the presence of Pf5 phage compared to the wild strain (Fig. 32C).

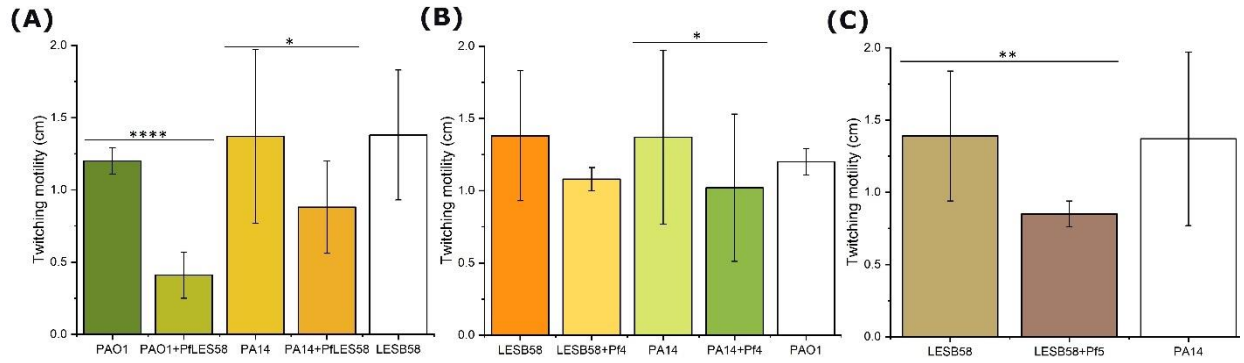


**Figure 32.** Effect of Pf phage infection on twitching motility in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; **** $p < 0.0001$; ** $p < 0.01$; * $p < 0.05$; tested by Wilcoxon signed rank test.

### 4.14.4.2. Pf phages change *P. aeruginosa* swarming motility

In the PAO1 strain infected with PfLES58, no significant changes in swarming motility were observed (Fig. 33A). Conversely, a statistically significant decrease in swarming motility was observed in the PA14 strain ($p < 0.05$). Similarly, the presence of Pf4 phage led to a reduction in swarming motility in both *P. aeruginosa* strains (Fig. 33B), and a significant difference was observed in the PA14+Pf4 strain ($p < 0.01$). On the other hand, Pf5 significantly increased swarming motility in the infected LESB58 strain ($p < 0.001$) (Fig. 33C).

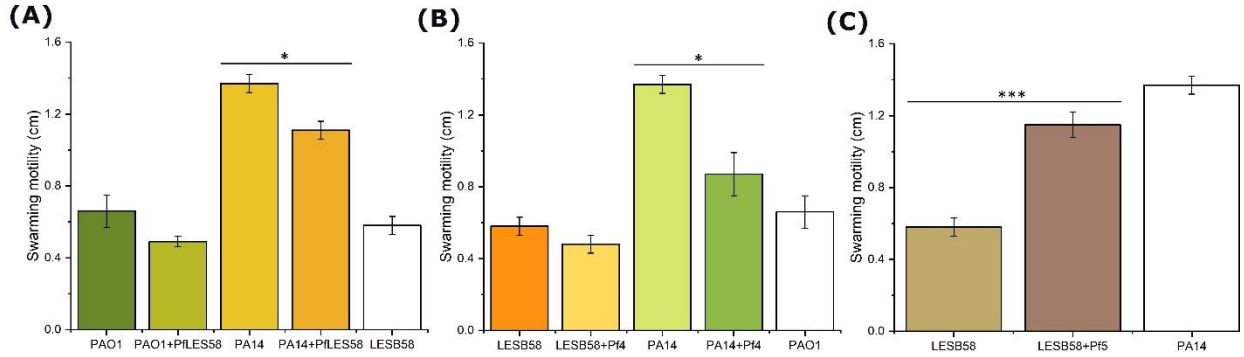


**Figure 33.** Effect of Pf phage infection on swarming motility in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; *** $p < 0.001$; * $p < 0.05$; tested by Wilcoxon signed rank test.

### 4.14.4.3. Pf phages increase *P. aeruginosa* swimming motility

The presence of Pf4 phages in both infected strains led to a significant increase in swimming motility on semi-solid medium (Fig. 34B). The radius of Pf4 infected strains motility ranged from 2.1 cm up to 2.58 cm on average, while wild strains had a radius of barely 0.7 cm during the same incubation period. This difference was statistically significant for both strains ($p < 0.0001$ or $p < 0.05$). Additionally, PfLES58 was found to significantly increase swimming motility in both infected strains ($p < 0.05$ and $p < 0.01$) (Fig. 34A). Lastly, Pf5 increased swimming motility in the LESB58 strain ($p < 0.05$) (Fig. 34C).

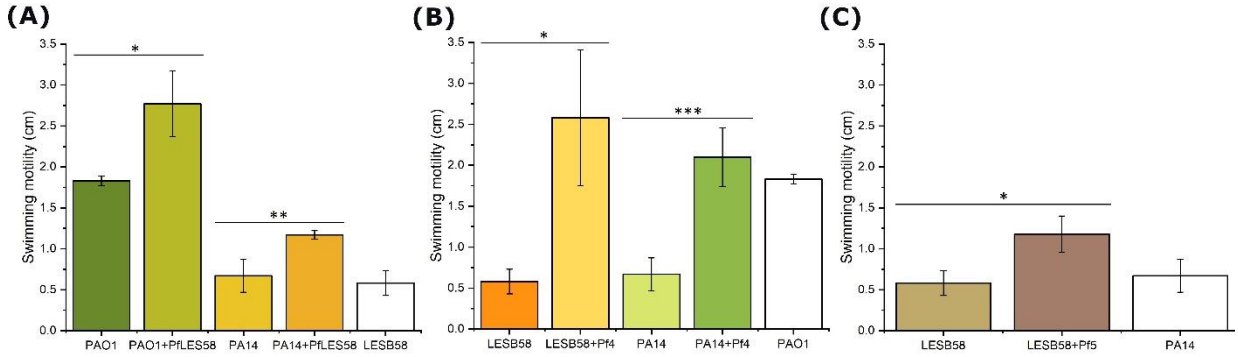


**Figure 34.** Effect of Pf phage infection on swimming motility in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; tested by Wilcoxon signed rank test.

### 4.14.4.4. Pf phages increase *P. aeruginosa* biofilm formation of polysterene surface

The infection with PfLES58, Pf4, and Pf5 phages resulted in alterations in biofilm production in all infected strains. The PAO1+PfLES58 strain exhibited significantly higher biofilm production at 3.64 $\pm$ 0.28 compared to the wild strain at 2.24 + 1.18 ($p < 0.05$) (Fig. 35A). PfLES58 increased biofilm production 1.6 times in PAO1 strain. Similarly, Pf5 increased biofilm production in the LESB58 strain 2.4 times, that is, from 0.85 $\pm$ 0.43 to 2.02 $\pm$ 0.44 ($p < 0.001$) (Fig. 35C). Biofilm production in both Pf4 infected strains was significantly greater than in wild strains ($p < 0.05$ or $p < 0.01$) (Fig. 35B). In both cases, biofilm production was increased approximately 1.7 times.

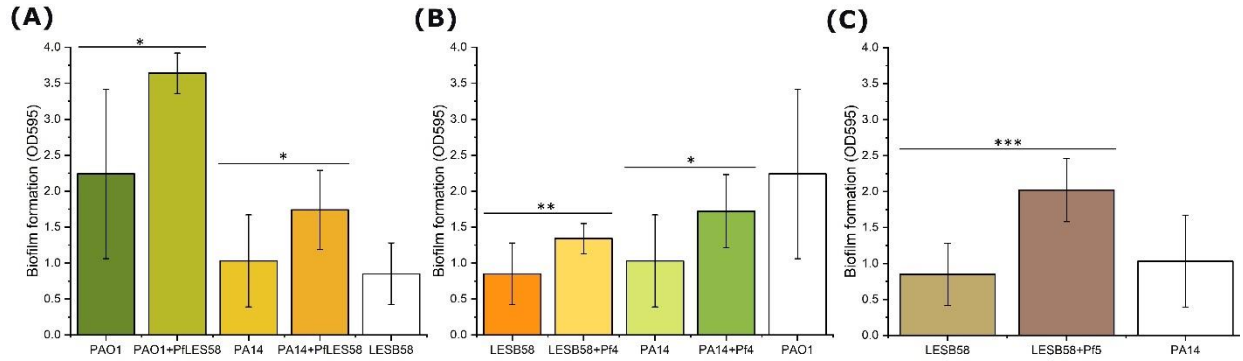


**Figure 35.** Effect of Pf phage infection on biofilm formation in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; tested by Wilcoxon signed rank test.

### 4.14.5. Pf phages decrease pyocyanin production by *P. aeruginosa*

In both strains, the presence of Pf4 phage had an inhibitory effect on pyocyanin production, with statistical significance ($p < 0.01$ or $p < 0.05$) (Fig. 36B). In PA14 strain, the presence of PfLES58 phage resulted in a significant decrease in pyocyanin production ($p < 0.05$), while no significant change was observed in PAO1+PfLES58 strain due to generally low pyocyanin production in PAO1 strain (Fig. 36A). Pf5 phage also significantly inhibited pyocyanin production in LESB58 strain ($p < 0.05$) (Fig. 36C).

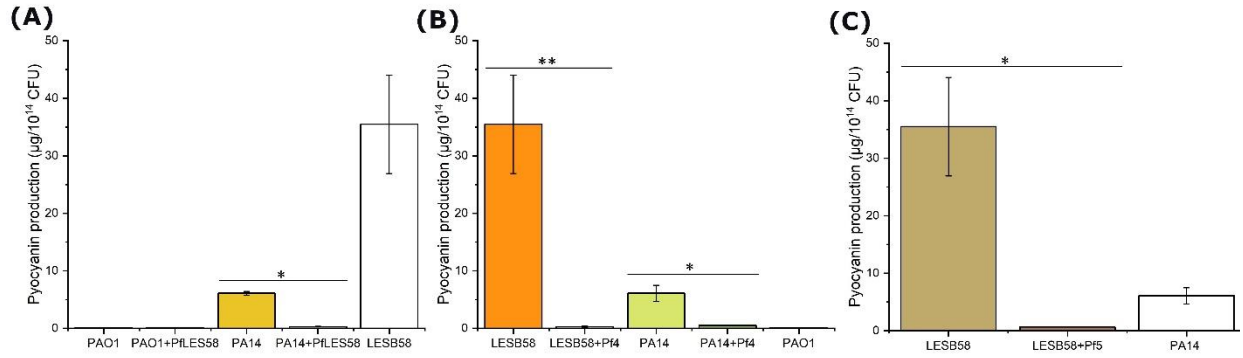


**Figure 36.** Effect of Pf phage infection on pyocyanin production in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; ** $p < 0.01$; * $p < 0.05$; tested by Wilcoxon signed rank test.

### 4.14.6. Pf phages decrease pyoverdine production by *P. aeruginosa*

Infection with PfLES58 phage did not result in any significant change in pyoverdine production in PAO1 and PA14 strains (Fig. 37A). However, in the infected LESB58 strain, Pf5 phage caused a significant reduction in pyoverdine production ($p < 0.01$) (Fig. 37C). Furthermore, both infected strains showed a statistically significant decrease of about 20% in pyoverdine production after Pf4 phage infection ($p < 0.01$ or $p < 0.05$) (Fig. 37B).

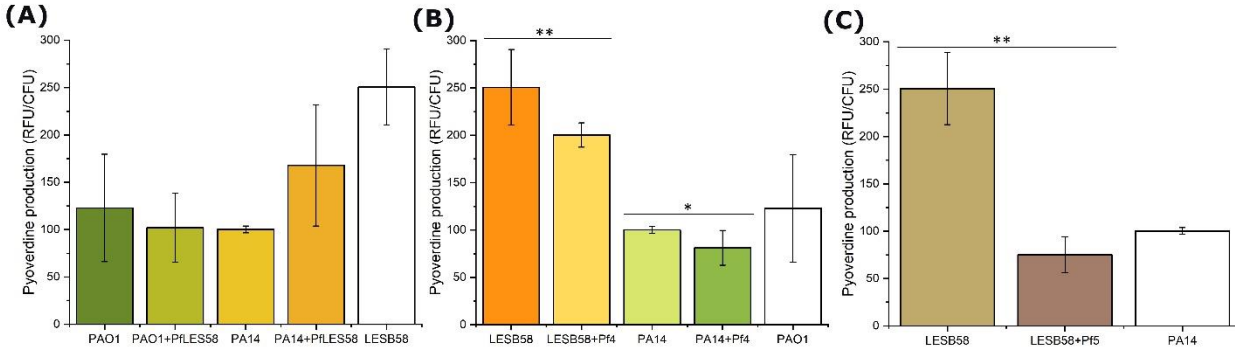


**Figure 37.** Effect of Pf phage infection on pyoverdine production in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; ** $p < 0.01$; * $p < 0.05$; tested by Wilcoxon signed rank test.

### 4.14.7. Pf phages increase SCV production

Following incubation for 5 days, both *P. aeruginosa* strains displayed an increased appearance of SCV after infection with Pf4 phage, although there was no significant difference observed ($p > 0.05$) (Fig. 38B). In addition, the presence of PfLES58 and Pf5 phages resulted in a greater number of SCV colonies compared to wild strains. However, only in the PAO1+PfLES58 strain, the presence of SCV was significantly increased by more than 10% ($p < 0.01$) (Fig. 38A).

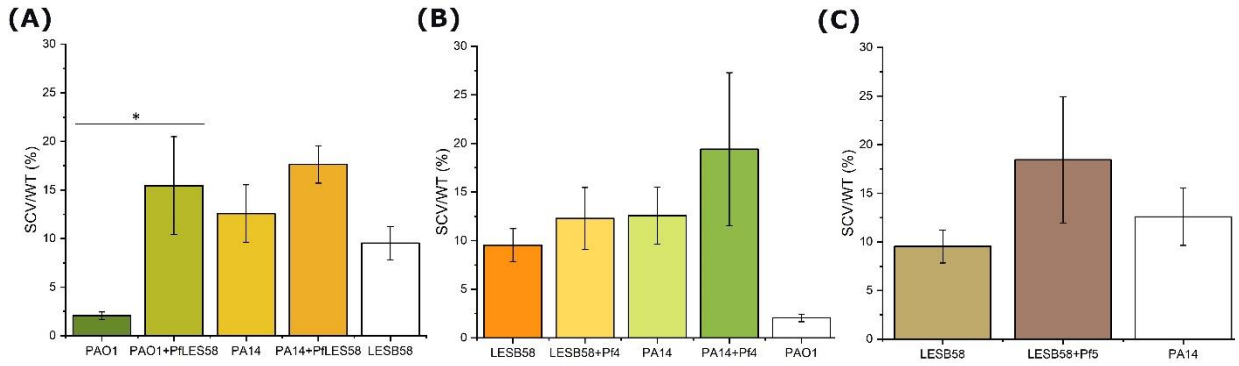


**Figure 38.** Effect of Pf phage infection on SCV prevalence in infected *P. aeruginosa* strains. The results are average + S.E. n = 9; * $p < 0.05$; tested by Wilcoxon signed rank test.

### 4.14.8. Pf phages increase *P. aeruginosa* susceptibility to antibiotics

The disk diffusion method was used to preliminarily verify changes in antibiotic susceptibility in infected strains. The LESB58 strain infected with Pf4 showed increased susceptibility to ciprofloxacin as determined by an approximate increase in inhibition diameter of 6.5 mm (Fig. 39). The same strain also showed changes in susceptibility to gentamicin with an increase in inhibition diameter of 3 mm. Additionally, LESB58+Pf5 had an increased zone of inhibition to CIP compared to the wild strain (Fig. 40).

Following the initial findings, a variety of antibiotics from different classes were tested against wild and Pf infected strains to determine their minimum inhibitory concentrations (MICs). Pf filamentous phages did not contribute to an increase in antibiotic resistance in their hosts. Moreover, increased susceptibility to specific antibiotics was observed in PfLES58 infected strains (Table 13). PfLES58 restored sensitivity in PAO1 to TET and in PA14 to STR. Similarly, Pf5 increased LESB58 strain sensitivity to STR, with a reduction in MIC from 64 μg $mL^{-1}$ to 8 μg $mL^{-1}$ in the infected strain (Table 13). Pf4 phage made LESB58 strain more susceptible to fluoroquinolones, aminoglycosides, and β-lactams, with a decrease in MIC by two values for GEN, STR, and CIP (Table 13). Notably, the largest difference was observed for CAZ, where the MIC was 64 μg $mL^{-1}$ for LESB58 and 16 μg $mL^{-1}$ for the infected strain. Pf4 also restored PA14 susceptibility to fluoroquinolones, with the MIC decreasing from 0.125 μg $mL^{-1}$ to <0.00625 μg $mL^{-1}$.

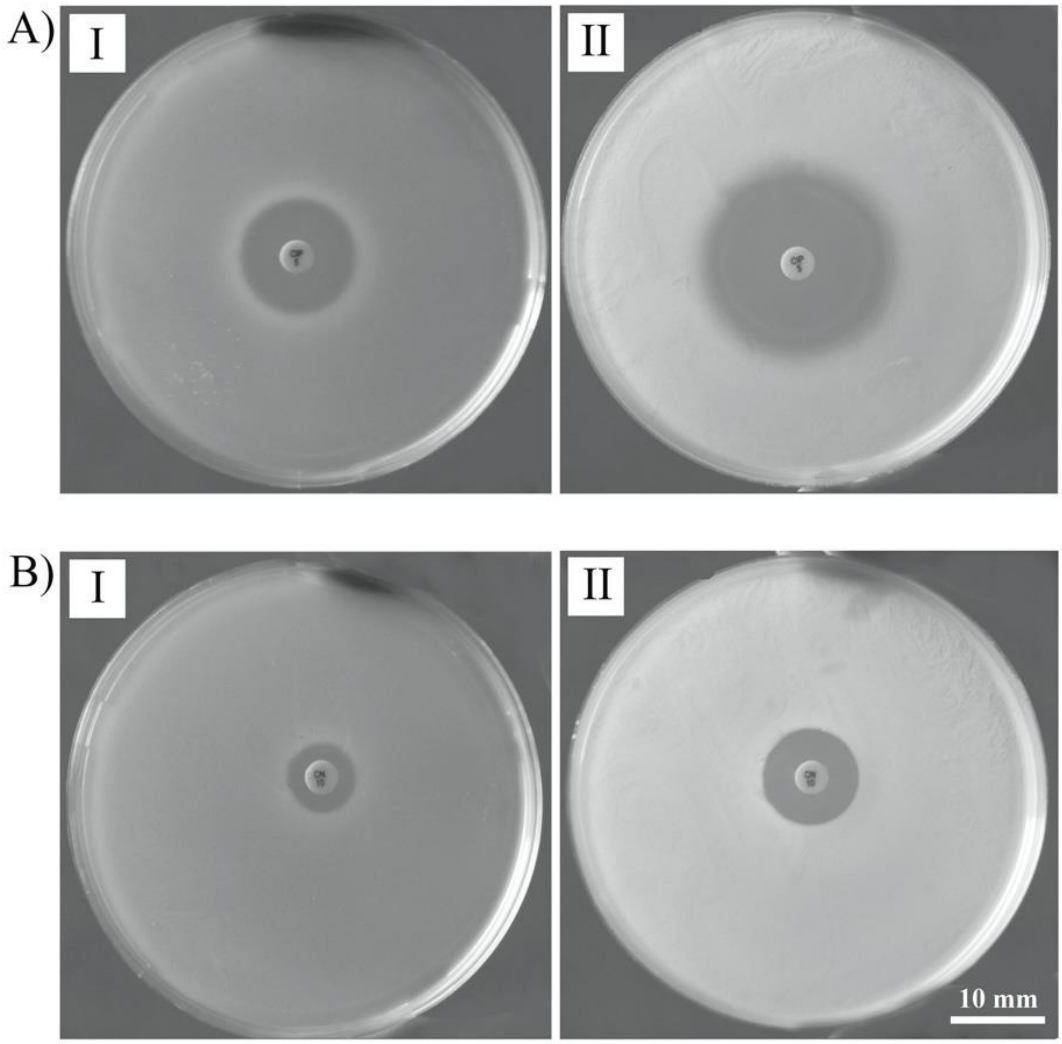


**Figure 39.** Changes in susceptibility of wild (I) and Pf4 infected (II) strain LESB58 to CIP (A) and GEN (B).

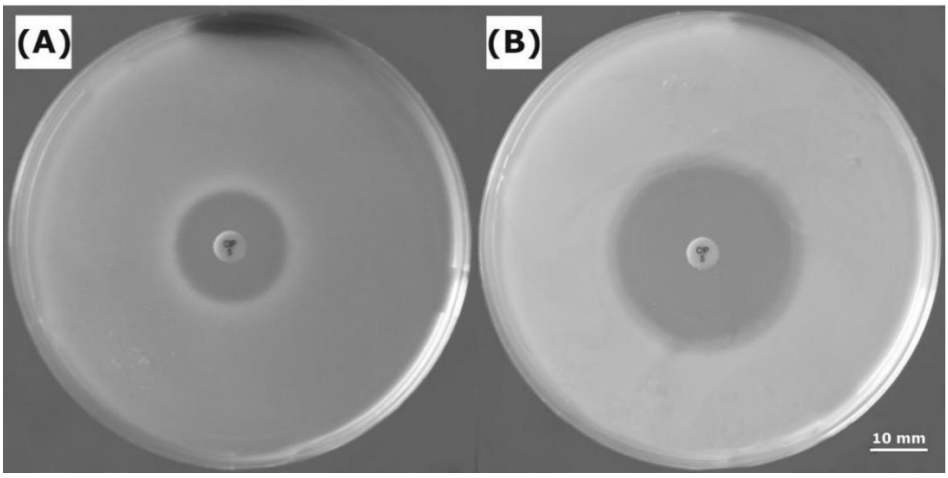


**Figure 40.** Changes in susceptibility of wild (**A**) and Pf5-infected (**B**) strain LESB58 to CIP.

**Table 13.** MIC of antibiotics for wild and PfLES58-infected *P. aeruginosa* strains

| ***P. aeruginosa* strains** | **MIC (μg mL$^{-1}$) [1]** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **CIP** | **GEN** | **TET** | **CAZ** | **STR** | **CHL** | **PMB** |
| LESB58 | 0.5 (S)[2] | 0.5 (S) | 8 (I) | >64 (R) | 64 (R) | 32 (R) | 1 (S) |
| LESB58+Pf4 | 0.125 (S) | 0.125 (S) | **4.0 (S)** | **16.0 (I)** | **16.0 (I)** | 32.0 (R) | 1.0 (S) |
| LESB58+Pf5 | 0.25 (S) | 0.5 (S) | 8 (I) | 32 (R) | **32 (S)** | 64 (R) | 0.25 (S) |
| PAO1 | <0.0625 (S)[2] | 1 (S) | 8 (I) | 16 (I) | 16 (I) | 32 (R) | 1 (S) |
| PAO1+PfLES58 | <0.0625 (S) | 1 (S) | **4 (S)** | 16 (I) | 16 (I) | 32 (R) | 0.5 (S) |
| PA14 | 0.125 (S) | 1 (S) | 16.0 (R) | 64.0 (R) | 16.0 (I) | 64.0 (R) | 1.0 (S) |
| PA14+PfLES58 | 0.125 (S) | 0.5 (S) | 16.0 (R) | 32 (R) | **8 (S)** | 64.0 (R) | 0.5 (S) |
| PA14+Pf4 | <0.0625 (S) | 0.5 (S) | 16.0 (R) | **16.0 (I)** | **8.0 (S)** | 32.0 (R) | 0.5 (S) |
| LESB58 [3] | 1 (I) | 32 (R) | 32 (R) | 256 (R) | 64 (R) | 64 (R) | 2 (I) |
| PAO1[3] | 0.0625 (S) | 1.0 (S) | 8.0 (R) | 16.0 (I) | 16.0 (S) | 32.0 (R) | 1.0 (S) |
| PA14 [3] | 0.125 (S) | 2 (I) | 64 (R) | 128 (R) | 64 (R) | 64 (R) | 2 (I) |
| *E. coli* ATCC 25922 | <0.0625 (S) | 1.0 (S) | 1.0 (S) | 1.0 (S) | 8.0 (S) | 4.0 (S) | 0.25 (S) |

[1] CIP—ciprofloxacin; GEN—gentamicin; TET—tetracycline; CAZ—ceftazidime; STR—streptomycin; CHL—chloramphenicol; PMB—polymyxin B. [2] (R)— resistant; (I) intermediate resistant; (S)—sensitive. [3] Determined for bacterial count $1 \times 10^8$ CFU mL$^{-1}$.

# 5. Discussion

Genome sequence analyzes of Pf bacteriophages revealed that Pf1, PfLES58, Pf4, Pf5, and Pf8 share certain similarity with each other. Also, a similar position of key genes was recorded in the genomes of all analyzed Pf bacteriophages. However, besides Pf3 phage, PfLES58 had the lowest percentage of identity with the above-mentioned bacteriophages. Furthermore, the similarity in CoaA protein of Pf phages was below 50% in all comparisons. The CoaA sequence is important for species definition as different species typically exhibit distinct lytic spectra, which are determined by the amino-acid sequence of this adsorption protein (Knezevic et al., 2021). Based on the obtained results and according to the established criteria for defining species in bacteriophages (<95% DNA sequence similarity), PfLES58, Pf4, and Pf5 are candidates for new species. Additionally, since PfLES58 demonstrates less than 50% similarity in CoaB protein compared to other phages, it is a candidate not only for a new species but also for a new genus (Knezevic et al., 2021). On the other hand, Pf1, Pf4, Pf5 and Pf8 had a percentage of identity in terms of the amino-acid sequence of the CoaB protein above 90% in all combinations, which makes them members of the same genus. These results were further confirmed by the great similarity of the Zot amino-acid sequence in all four mentioned Pf phages. Accordingly, Pf4 and Pf5 should be ascribed to the genus *Primolicivirus*. The specific characteristics of CoaB protein in PfLES58 phage, as well as CoaA, are of particular importance as they suggest PfLES58 phages belongs to a distinct genus and species, which is further supported by genome analysis at the nucleotide sequence level. A new genus *Secondolicivirus* should be established with PfLES58 as representative.

Considering the results of homology determination and the reliability of branch in the phylogenetic trees, it can be inferred that Pf1, Pf4, and Pf5 bacteriophages belong to the same genus, as both CoaB and Zot amino-acid sequences are similar >50%. Additionally, a phylogenetic connection between PfLES58 phage and these phages was indicated, although the branch reliability was weak.

PfLES58 has been found to generate a replicative form and actively produce virions, similar to what has been observed for Pf4 and Pf5 prophages (Webb et al., 2004; Mooij et al., 2007; Rice et al., 2009). However, it is worth noting that chromosomally integrated phages generally exhibit a significantly reduced production of new virions, even under inducing

conditions, with an average ratio of approximately 1 phage per 10 – 100 cells, as is the case with *Vibrio* phages (Davis et al. , 2002). Estimations suggest that around 3 Pf4 phage particles are produced per 10,000 bacterial cells (Secor et al., 2017). McElroy et al. (2014) reported that Pf4 phage particles were not initially detected *in vitro* after four days of incubation but reached concentrations of $10^{10}$ – $10^{11}$ PFU $mL^{-1}$ only by day 11. Similarly, Webb et al. (2003) found Pf4 phage in 10-day-old biofilms, with total phage numbers ranging from $10^6$ to $10^8$ PFU $mL^{-1}$. However, Rice et al. (2009) demonstrated that Pf4 is present even during the early stages of biofilm formation but can only be detected when it transitions into the superinfective form, which occurs during cell death and biofilm dispersal. The limited abundance of filamentous phages poses a significant challenge for further research on these bacteriophages and their involvement in the pathogenesis of *P. aeruginosa* hosts.

It is well-documented that Pf filamentous bacteriophages are widely distributed among *P. aeruginosa* isolates, as approx. 60% of the strains harbored at least one Pf-specific genetic element (Knezevic et al., 2015). Among these strains, Pf4 was the most prevalent (22%), followed by Pf1 (18%), and finally Pf5 (7%). PfLES58 phage showed the prevalence similar to Pf4 phage. Presence of PfLES58 CoaA protein and replicative form was observed in 21% of the tested *P. aeruginosa* strains. PfLES58 was most commonly found in strains originating from sputum and cystic fibrosis patients. These results align with the fact that the natural host for PfLES58 phage is the Liverpool epidemic strain (LES) (Winstanley et al., 2009). LES strain is the most prevalent isolate among cystic fibrosis patients. In a study encompassing 1200 isolates from 31 cystic fibrosis centers in England and Wales, LES strain was present in 48% of the centers and 11% of the isolates (Scott and Pitt, 2004). LES strain exhibits a higher level of aggressiveness compared to other *P. aeruginosa* strains as it can effectively replace already established strains of *P. aeruginosa* and infect non-CF parents of CF patients with a high morbidity rate (McCallum et al., 2001; McCallum et al., 2002; Salunkhe et al., 2005, 29). Additionally, CF patients confirmed to have LES isolates experienced greater morbidity compared to patients with non-epidemic strains of *P. aeruginosa* (Al Aloul et al., 2004).

To enhance Pf phage production, two methods for the propagation and concentration of integrative filamentous phages have been designed. Method 1 involved utilizing a larger bacterial

culture volume (6 L) with a shorter incubation period (48 hours), while method 2 employed a smaller culture volume (600 mL) with an extended incubation period (6 days). The analysis of various parameters indicated that method 2 was superior to method 1. Method 2 resulted in more distinct and visible phage bands following CsCl purification, making phage band harvesting easier. Also, the number of virions in the purified phage suspension, after propagation using method 2, exhibited an increase ranging from 3.5 to 7.4 logs. Lastly, the concentration of ssDNA isolated from the virions was 7.6 to 22.4 times higher compared to method 1.

In both methods, the strains were subjected to agitation during incubation to prevent the growth of stationary biofilms, including the formation of surface pellicles. This was done because filamentous phages have a tendency to become trapped in the biofilm matrix, contributing to its structure (Secor et al., 2016a). Agitation also increased the likelihood of bacteriophages adhering to host cells and potentially causing superinfection, which is known to enhance phage production (Rice et al., 2009). As the number of repetition steps increased, the viscosity of the medium containing the phages also increased. Although this may initially seem like a limitation of the method, it has been previously demonstrated that the production of Pf phages is actually stimulated in viscous environments (Yeung et al., 2009). Following the centrifugation step, the supernatant was supplemented with fresh medium to achieve a starting volume of 600 mL (approximately an addition of 100 – 200 mL). However, the nutrient availability remained limited and such conditions can further stimulate Pf phage production, as previously confirmed for phage Pf5 (Lee et al., 2018).

In method 2, multiple cycles of bacterial culture centrifugation were employed instead of filtration. This decision was based on evidence that filtration significantly reduces the number of filamentous particles, with a recovery rate of less than 0.01% (Piekarowicz et al., 2020). Consequently, the titer of Pf4 phages in CsCl purified suspensions (approximately $10^5$ PFU $mL^{-1}$) was lower than that in the original centrifuged and PEG-precipitated stocks after 48 hours (approximately $10^7$ PFU $mL^{-1}$). By omitting the filtration step and instead using multiple culture centrifugation to remove bacterial cells, the desired outcome was achieved. This resulted in phage suspensions free from bacterial contamination and suitable for subsequent virion concentration and purification steps.

In method 1, the culture volume is 10 times larger, amounting to 6 L, compared to method 2 which utilizes a volume of 600 mL. The 10-fold reduction in volume is advantageous for method 2; however, it does require an extended incubation period, resulting in a 4-day delay, which can be seen as a limitation. Nonetheless, the application of method 2 is justified by the significantly higher phage titer obtained. Over the course of 6-days incubation, the production of phage particles displayed a gradual increase. For PfLES58, Pf4, and Pf5 phages, the increase was approximately 5.6, 1.8, and 5.2 log units, respectively. It is important to note that filamentous phages can coexist with tailed phages, which may be present in the form of prophages within bacterial strains. If the phage suspensions are not purified in CsCl, they will likely contain both filamentous and tailed phages (Smith and Scott, 1993; Boulnager, 2009; Mauritzen et al., 2020; Knezevic et al., 2021). Accordingly, it is crucial to determine the total number of phages in the suspension and subsequently treat them with chloroform. Filamentous phages are sensitive to this organic solvent, so the chloroform treated suspensions will contain only tailed phages. The precise count of filamentous phages can only be obtained by subtracting these values. It is evident that Pf4 reached a high titer within 2 days, PfLES58 within 4 days, and Pf5 within 6 days. Thus, a 6-day incubation period is recommended for isolating an unknown filamentous phage when the host bacterium has a fast growth rate. However, if the host is a slow-growing bacterium, it should be considered to prolong the incubation period accordingly.

Distinct differences in the appearance of plaques were observed for all three phages when comparing the single-layer and double-layer techniques. Both methods employed λ top medium with a consistent agar content of 0.65%, eliminating agar concentration as the sole cause of the observed variation. The findings suggest that plaques formed by filamentous phages are more likely a result of reduced bacterial growth rather than actual cell lysis. It is possible that the presence of LB agar in the bottom layer of the double-layer method provides additional support for bacterial growth. Consequently, the plaques formed under these conditions appear paler and tend to fade over time. Based on these observations, for an accurate determination of filamentous phage numbers, the recommendation is to count Pf plaques after 8 hours when employing the double-layer method, and after 8 to 24 hours when using the single-layer method.

PfLES58, Pf4 and Pf5 bacteriophages together with Pf1 phage have a morphology characteristic of the *Inoviridae* family. Images obtained by transmission electron microscopy indicate the presence of virions in the form of filaments. As expected, Pf4 bacteriophage was the one with the longest filaments. PfLES58 and Pf5 phages were of similar dimensions, which coincides with the size of their genomes.

The viral DNA from ultracentrifuged and dialyzed Pf phages was isolated using the phenol-chloroform method. Treatment with various enzymes revealed that PfLES58, Pf4, and Pf5 phages possess circular ssDNA, which is characteristic of filamentous phages. Additionally, proteins were extracted from the dialyzed Pf phages using the methanol-chloroform method, and the presence of a ~5 kDa protein corresponding to the major capsid protein (CoaB) was confirmed. The role of CoaB is to encapsidate phage DNA in numerous copies. Additionally, SDS-PAGE analysis indicated the presence of another protein band, which, based on its size, most likely represents the minor coat protein (CoaA) of filamentous phages, i.e. adhesion protein.

Given that PfLES58, Pf4, and Pf5 prophages actively produce virions capable of forming plaques on a bacterial lawn, the host range has been determined for all three bacteriophages. Based on the obtained results, PfLES58 and Pf5 exhibited a wide host range, while Pf4 showed a moderate host range. Interestingly, the closely related Pf1 phage has an extremely limited host range. Among the 49 strains tested, only one strain (PAK strain) was found to be susceptible to the Pf1 phage (Takeya and Amako, 1966). The reason for this can be the specific biology of this phage. Namely, this phage cannot integrate in bacterial genome as a prophage and its persistence and/or multiplication in cell can be impaired with lack of a prophage form (Torbet and Maret 1979; Tsuboi et al. 2010). These relatively broad lytic spectra of other phages are particularly significant in terms of bacterial virulence, as phages possess the ability to infect new *P. aeruginosa* strains and influence bacterial genotype and phenotype through processes such as horizontal gene transfer and lysogenic conversion. These mechanisms can contribute to the enhancement of virulence in pathogenic bacteria (Little, 2005).

The inducibility of these phages and their ability to infect other strains of *P. aeruginosa* have also been examined.

Subinhibitory concentrations of CIP (ciprofloxacin) and MMC (mitomycin C) have successfully demonstrated increased expression of PfLES58, Pf4, and Pf5 genes after 1h of application, and in the case of Pf4 and Pf5 even after 5 hours of incubation. After 24 hours of incubation, there was no significant increase in the expression of Pf phage genes in the presence of these antimicrobial agents. It is well known that both CIP and MMC generally can trigger the SOS response in the bacterium, thus, leading to the expression of Pf phages (Torres-Barceló, 2018; Serment-Guerrero et al., 2020). The contemporaneous nature of this induction may be attributed to bacterial adaptation to a stressful environment and termination of the SOS response (Torres-Barceló 2018). Previous studies have utilized mitomycin C to induce Pf4 phages in the PAO1 strain, revealing the release of superinfective Pf4 forms at concentrations higher than 10 μg $mL^{-1}$, as determined by plaque assays (Webb et al., 2003). The inducibility of Pf4 is confirmed in the present study, but it is also confirmed for the first time for Pf5 and PfLES58. Thus, the three examined phages belong to the group of mitomycin C-inducible phages.

The induction of PfLES58, Pf4, and Pf5 phages was investigated in the presence of the obligatory lytic phage JG024. When using a 0.25 MOI, similar expression patterns were observed for all three phages. A significant increase in gene expression of Pf phages was observed only after 12 hours, and this effect persisted up to 24 hours of incubation. At a low MOI, as observed in this case, approximately 25% of the cells become infected at the initial stage of the treatment. Considering that the latent period of the JG024 phage is approximately 55 minutes, and roughly 180 phages are generated per cell (Garbe et al., 2010), the observed increase in the expression of the tested Pf genes after 12 hours of treatment can be explained in this way. Moreover, it can be hypothesized that the presence of the obligatory lytic phage influences cellular metabolism and/or the emergence of mutated cells, indirectly impacting the regulatory mechanisms of Pf phages and leading to increased production.

The finding that subinhibitory concentrations of CIP and infection by tailed phages stimulate production of Pf phages is particularly important from the aspect of phage therapy, particularly when phages are combined with antibiotics. Such therapies can promote lysogenic conversion of new strains. It is also important to notice that superinfection of two or more Pf phages are possible, and these phages not only can bring some bacterial genes due to imprecise

excision, but also provide new genetic material for mutation, drift and selection to act upon, contributing significantly to bacterial evolution. One of the solutions, when CIP is used for therapy, is to use inhibitory or superinhibitory concentrations of the agent during treatment.

Filamentous phages possess a superinfection exclusion protein (PfsE) that confers resistance to Pf infections in bacterial hosts (Schmidt et al., 2022). Additionally, the minor capsid protein (p7) of filamentous phages has been shown to inhibit Pf phage adsorption (Wang et al., 2022). Both PfsE and p7 bind to the type IV pilus of the bacterial host and thereby prevent successful infection. However, despite these defense mechanisms, PfLES58, Pf4, and Pf5 phages were able to establish chronic productive infections in *P. aeruginosa* strains that are not naturally infected with these phages. This suggests that there is no superinfection exclusion between these three Pf phages, as demonstrated by the cross-superinfection of PAO1, PA14, and LESB58 strains. This further explains the fact that *P. aeruginosa* strains can carry several different or even the same prophages from the family *Inoviridae* (Knezevic et al., 2015). Furthermore, sequencing results have indicated that Pf4 phages integrate near the tRNA-Gly gene, while PfLES58 and Pf5 were found near 2-thiocytidine biosynthesis protein TtcA.

The results of monitoring the expression of Pf genes and production of virions in different *P. aeruginosa* strains after superinfection with another Pf phage indicated a complex interaction of filamentous bacteriophages. In some cases, Pf phages had a pronounced production, while in other there was no obvious change in Pf production. Detailed investigations are necessary to determine how filamentous bacteriophages interact within the same host and why one is superior to another in terms of production.

Extensive research has been conducted on the filamentous phage Pf4 and its impact on the original host PAO1. However, little is known about Pf4 impact on other strains of *P. aeruginosa*. Similarly, limited information is available regarding the effects of Pf5 on *P. aeruginosa*. The involvement of PfLES58 in the virulence of the LESB58 strain remains also largely unknown. Therefore, investigations have been conducted to assess the effects of PfLES58, Pf4, and Pf5 phages on the phenotype of *P. aeruginosa*.

After infection, PfLES58 and Pf5 phages slightly inhibited the growth of *P. aeruginosa* strains. These results were expected since it is known that filamentous bacteriophages exhibit

either inhibitory effects on bacterial growth or no effect at all, as documented by several studies (Kamiunten et al., 1981; Horabin et al., 1986; Kuo et al., 1987; Derbise et al. al., 2007; Yamada et al., 2007; Wan and Goddard, 2012; Ahmad et al., 2014; Yu et al., 2015; Ahmad et al., 2017; Goehlich et al., 2019; Yeh, 2020). In contrast, Pf4 transiently stimulated the growth of its infected strains. A possible explanation behind this phenomenon can potentially be found in the Pf4 phage accessory genes. Pf4 possesses an antitoxin with a conserved Phd_YeFM sequence (Li et al., 2020), which binds to its toxin counterparts, and can interact with DNA through its N-terminus, and represses the expression of operons containing genes for the toxin and antitoxin. This domain complexes with Txe toxins with various domains and is present in *P. aeruginosa* (Marchler-Bauer et al., 2017), potentially accounting for the slight growth stimulation. However, after 24 hours of incubation, no discernible difference in growth was observed, indicating that Pf4 infection has no long-term influence on the growth of PA14 and LESB58 strains. Interestingly, PfLES58 and Pf5 phages lack a toxin-antitoxin system in their genome, which could be a potential explanation for the growth stimulation observed with Pf4 infected strains. Lastly, inhibition of bacterial growth can potentially indicate a burdened and slow metabolism of the bacteria, where certain groups of antibiotics would potentially have a weaker effect in removing bacterial infections.

PA14 and LESB58 cells showed a reduced possibility of autoaggregation after infection with Pf4 phage. Wild strains exhibited moderate autoaggregation, but after phage infection, they became non-aggregative. Similarly, PfLES58 phage significantly decreased autoaggregation in PA14 + PfLES58 cells, although the strain still displayed moderate autoaggregation. On the other hand, no significant difference in autoaggregation was observed in PAO1+PfLES58 and LESB58+Pf5 infected strains. Contrary to the obtained results, the presence of filamentous phages on the surface of bacterial cells has been proposed to contribute to changes on the surface of the cell membrane and promote cell-to-cell interactions, potentially leading to increased cell aggregation during biofilm formation (Addy et al., 2012). These findings suggest that the impact of filamentous phages on autoaggregation is influenced by bacterial strains and species, indicating the involvement of multiple factors in the complex phenomenon of cell autoaggregation. Autoaggregation is typically mediated by self-recognizing surface structures

such as proteins and exopolysaccharides, collectively referred to as autoagglutinins (Trunk et al., 2018). However, it is also affected by fimbriae and pili, and these structures are important for the initial phase of attachment to surfaces that precedes biofilm formation (Schembri et al., 2002;). It is known that SCVs of *P. aeruginosa* bacteria are abundant with type IV pili and that such cells are prone to stronger aggregation (O'Toole and Kolter, 1998; Déziel et al., 2001; Besse et al., 2023). Given that the results indicate a significantly reduced twitching motility of *P. aeruginosa* strains after infection with Pf phages, the occupation of type IV pili during infection by filamentous phages or their reduced expression due to the presence of additional prophages in the host can potentially explain the reduced aggregability of cells. The molecular mechanisms underlying bacterial autoaggregation are diverse (Misawa and Blaser, 2000; Farrell and Quilty, 2002), and further detailed research is needed to determine the precise mechanism of filamentous phage impact on cell autoaggregation.

Furthermore, Pf4 infection of different strains resulted in increase in cell hydrophobicity. However, there was no qualitative change in hydrophobicity, as the strains remained either hydrophilic or moderately hydrophobic. Similarly, Pf5 phage did not induce qualitative changes in the hydrophobicity of LESB58 cells, although a statistically significant increase in hydrophobicity was observed in this specific infected strain. In the case of PfLES58, a significant increase in hydrophobicity was noted in infected strains PAO1+PfLES58 and PA14+PfLES58, accompanied by a qualitative change in hydrophobicity. These two infected strains became moderately hydrophobic upon exposure to PfLES58 phage, whereas the original strains were hydrophilic. It can be postulated that filamentous phages, through their infection, impact the outer membrane lipids of cells, leading to alterations in the relative concentration of phospholipids (Bayer and Bayer, 1986). The hydrophobicity of bacterial cell surfaces plays a critical role in their interaction with different surfaces and various factors, such as outer membrane proteins and lipids, surface fibrils, fimbriae, and core oligosaccharides, influence the degree of hydrophobicity exhibited by microorganisms (Borecká-Melkusová and Bujdáková, 2008; Bujdáková et al., 2013; Krasowska and Sigler, 2014). Given that filamentous phages significantly influence such important factors for binding of bacterial cells to the surface, it can

be concluded that filamentous phages are heavily involved in the initial stages of biofilm formation of the bacterium *P. aeruginosa*.

Additional evidence of the influence of filamentous phages on the phenotype of *P. aeruginosa* is the changed motility of the strains after infection with Pf phages. Pf phages increased swimming motility in all infected strains of *P. aeruginosa*. Interestingly, this is the first case that filamentous phages stimulate swimming motility, because most phages from this family known to date either have no impact on swimming motility, or reduce it (Jian et al., 2013; Ahmad et al., 2014; Secor et al., 2017 ).

PfLES58 phage decreased swarming motility in both infected strains, although statistically significant differences were only observed in the case of PA14+PfLES58. Similar effects were observed with Pf4 phage and filamentous phages from other bacterial species (Jian et al., 2013; Ahmad et al., 2014; Ahmad et al., 2017; Akremi et al., 2020). In contrast to PfLES58 and Pf4 phage, Pf5 phage doubled the swarming motility of the infected LESB58 strain. This represents the first case of a filamentous phage increasing swarming motility in its host. Besides swimming motility, swarming motility is another flagellum-dependent form of movement observed in *P. aeruginosa*. Reciprocal regulation of swarming motility and biofilm formation involves overlapping sets of regulators (Shrout et al., 2005; Caiazza et al., 2007). For instance, strains lacking GacA, which is responsible for biofilm formation and EPS production, exhibited increased swarming motility (Parkins et al., 2001), while strains with enhanced EPS production showed reduced swarming motility (Caiazza et al., 2007). This phenomenon was also recorded in the case of Pf4 and PfLES58 infections. Both phages lowered swarming motility, but in their presence there was a greater biofilm production. Only in the case of Pf5 infection, both swarming motility and biofilm production were increased. The results obtained for swimming and swarming motility suggest a complex interaction where Pf filamentous phages potentially influence the genes responsible for motility in *P. aeruginosa*. Further detailed research is required to fully understand these interactions.

Bacteria also utilize type IV pili for twitching motility, enabling movement on solid surfaces (Bradley, 1980). This form of motility is associated with the formation of microcolonies (O'Toole and Kolter, 1998) and plays a crucial role in bacterial interactions with mammalian cells

(Zolfaghar et al., 2003). In the presence of PfLES58, the PAO1 strain exhibited a significant reduction in twitching motility compared to the wild strain, while PA14+PfLES58 showed slight inhibition. Moreover, Pf5 phage significantly inhibited twitching motility in the LESB58 strain. Similar results were observed with Pf4 phage infection in the LESB58 and PA14 strains. These findings are in accordance with previous reports of Secor et al. (2017), who demonstrated minimal twitching motility in the PAO1 strain superinfected with Pf4 phage. Reductions in twitching motility have also been observed with filamentous phages from other bacterial species, including Xanthomonas phage XacF1 (Ahmad et al., 2014), Ralstonia phage RSS51 (Ahmad et al., 2017), and Ralstonia phage RSM3 (Addy et al., 2012). Schmidt et al. (2022) concluded that Pf4 phage produces a PfsE protein that inhibits pili extension, which is consistent with the obtained results. Given that twitching motility contributes to cell autoaggregation and the formation of microcolonies (O'Toole and Kolter, 1998), the observed findings are in line with the results obtained for the autoaggregation of Pf infected strains. Given that type IV pili serve as the primary receptors for infection by filamentous phages (Bradley 1973), their decreased number on the host cell may reduce the possibility of infection by other similar phages.

The obtained results are of great importance because bacterial motility is a crucial factor in the formation of a biofilm (O'Toole and Kolter, 1998; Stickler, 1999; Li et al., 2011). Such changes in the bacterial phenotype can be decisive in the first stages of biofilm formation and the onset of chronic infections.

During chronic infections, *P. aeruginosa* undergoes significant adaptation, leading to increased persistence, reduced virulence, and notable phenotype diversification, often characterized by the emergence of small colony variants (Malone, 2022). Since the occurrence of *P. aeruginosa* SCVs was associated with Pf4 phages (Webb et al., 2004; Rice et al., 2009), the influence of Pf filamentous phages on occurrence of SCVs in other *P. aeruginosa* strains was examined. PfLES58 increased the presence of SCV colonies in both infected strains after 5 days of incubation, with a statistically significant difference observed in the PAO1+PfLES58 strain, where approximately 7.5 times more SCV colonies were observed. Similarly, Pf4 and Pf5 also increased the occurrence of SCV colonies, although without statistical significance. Notably, the presence of Pf5 prophage in the PA14 strain had not previously been linked to SCV formation

(Mooij et al., 2007). It was previously shown that increased expression of the *xisF4* gene in PAO1 resulted in higher production of Pf4 phage and a subsequent 55% increase in the number of SCV colonies (Li et al., 2019). Detailed analysis of PAO1 revealed that SCVs are exclusively produced by the wild-type strain during the stages of dispersal and cell death, coinciding with the potential superinfection caused by Pf4, which correlates with the appearance of SCVs (Rice et al., 2009). This superinfective form of Pf4 phage was detected in 5- to 6-day-old biofilms. It is plausible that PfLES58 and Pf5 phages may also transition to a similar form, contributing to SCV formation in different strains of *P. aeruginosa*. While the findings on twitching motility suggest the absence of type IV pili in the infected strains, it is crucial to highlight that Congo Red staining only confirms the presence of these structures on the cell surface, without providing information about their functionality. Moreover, it is important to note that twitching motility assessment was conducted after 24 hours of incubation, whereas the examination of SCV colonies was performed after 5 days of incubation.

In order to further confirm the involvement of filamentous phages in the development of chronic *P. aeruginosa* infections, the biofilm formation potential of *P. aeruginosa* strains after infection with Pf phages was checked. The presence of PfLES58, Pf4, and Pf5 phages resulted in a substantial enhancement of biofilm formation in their respective infected strains. The findings regarding the impact of Pf5 phage on biofilm formation contrast with those reported by Lee et al. (2018). Authors demonstrated that the substrate binding protein DppA1 can enhance biofilm formation by repressing the Pf5 prophage. However, their observations were made after 4 hours of incubation, whereas in the present study, the quantification of biofilm formation was conducted after 24 hours of incubation. Moreover, the study specifically focused on the interaction between Pf5 and its natural host strain PA14. In this case, the LESB58 strain was already infected with another Pf phage, suggesting a potential combined effect of both Pf phages in promoting enhanced biofilm production of this strain.

In the case of the PAO1 strain, extensive research has demonstrated the Pf4 phage involvement in the formation and persistence of biofilms (Webb et al., 2004; Rice et al., 2009; Secor et al., 2016). While type IV pili are known to facilitate cell-surface bridging and attachment, the presence of a higher number of filamentous phages on bacterial cell surfaces could have a

similar impact on biofilm formation (Webb et al., 2004; Marschal 1985), contributing to the expansion of microcolonies (Rice et al., 2009). However, this alone cannot fully explain these findings, as Pf4-infected strains exhibited decreased autoaggregation and twitching motility, both of which are considered important for biofilm formation. Additionally, enhanced cell surface hydrophobicity and swimming motility played a role in promoting better interactions between cells and the surface, potentially leading to more extensive biofilm development. Previous studies have documented the involvement of Pf4 in the structure of the biofilm matrix (Rice et al., 2009; Secor et al., 2016), as well as its impact on biofilm volume and thickness (Tortuel et al., 2020) in the PAO1 strain. Cell lysis caused by Pf4 phage could further contribute to improved biofilm production, as observed in the original host, PAO1 (Webb et al., 2003). Cell lysis releases nutrients into the biofilm matrix (Tolker-Nielsen et al., 2000; Sauer et al., 2002), while the released extracellular DNA (eDNA) contributes to the structure of the matrix (Alipour et al., 2009). Consequently, strains infected with Pf phage produced larger quantities of biofilm compared to wild counterparts, likely due to the development of more intricate biofilm matrix architecture during later stages of biofilm formation.

It has been estimated that biofilm-related infections account for 60% of all *P. aeruginosa* infections (Costerton et al., 1999; Davies 2003), and there is compelling evidence supporting the crucial role of biofilms in persistent infections, such as in the lungs of CF patients. As can be seen from the obtained results, the presence of filamentous bacteriophages contributes to the emergence of such infections and makes effective treatment of patients difficult. This is further supported by multiple studies that provide additional evidence that *P. aeruginosa* filamentous phages, such as Pf4, contribute to the establishment of chronic infections by reducing *P. aeruginosa* invasiveness, inflammatory response, and enhancing resistance to macrophage phagocytosis when present in biofilm-relevant quantities (Webb et al., 2004; Rice et al., 2009; Secor et al., 2017).

Moreover, Pf filamentous phages can influence the toxicity of infected *P. aeruginosa* strains, as evidenced by changes in exopigment production. The results reveal that PfLES58, Pf4, and Pf5 phages significantly suppress the production of pyocyanin. Ismail et al. (2021) demonstrated that PAO1 lacking Pf4 phage exhibited reduced pyocyanin production compared

to the wild-type strain after 24 hours of incubation. Although it appeared that Pf4 phage stimulated pyocyanin production in its native host, it actually decreased pyocyanin production in PA14 and LESB58 strains of *P. aeruginosa*. The results are significant because pyocyanin is a human cytotoxin and is a crucial virulence factor secreted by *P. aeruginosa* (Hall et al., 2016). Furthermore, a significant reduction in pyoverdine production in infected strains infected with Pf4 and Pf5 phages was observed. On the other hand, it was observed that a PAO1 mutant lacking the Pf4 prophage displayed a notable increase in pyoverdine production compared to the wild-type strain (Ismail et al., 2021). However, the introduction of Pf4 did not have any impact on pyoverdine levels in the mutant strain. PfLES58 did not have a significant impact on pyoverdine production in infected PAO1 and PA14 strains. Pyoverdine is an iron-chelating molecule, a water-soluble siderophore, which can activate virulence factors such as exotoxin A and protease PrpL when it is in form of ferripyoverdine (Kang et al., 2019). In addition to enabling chronic infections, filamentous bacteriophages make *P. aeruginosa* less virulent in this way.

Infection with Pf filamentous phages can contribute to increased antibiotic susceptibility in bacteria, and this phenomenon appears to be dependent on the bacterial strain and the specific antibiotic used. A noticeable increase in antibiotic activity against Pf4-infected LESB58 and PA14 strains, particularly for ciprofloxacin, gentamicin, ceftazidime, and streptomycin was observed. It is interesting to note that the sensitivity of both LESB58 and PA14 strains to ceftazidime changed from resistant to intermediate sensitive upon Pf4 infection, indicating a significant re-sensitization to this antimicrobial agent. Additionally, LESB58 demonstrated a change in sensitivity from intermediate sensitive to sensitive for streptomycin upon Pf4 infection, while in PA14, the change was from resistant to intermediate resistance. Moreover, original LESB58 showed intermediate resistance to tetracycline, but became sensitive to this antibiotic upon Pf4 infection. In the case of PfLES58 infection, qualitative changes were observed, with PAO1 becoming sensitive to tetracycline and PA14 becoming sensitive to streptomycin, whereas the wild strains exhibited intermediate resistance to these antibiotics. Similarly, LESB58 was initially resistant to streptomycin, but became sensitive to this antibiotic after Pf5 infection.

As Pf phages contribute to phage-enhanced growth in the form of a biofilm, and considering that bacteria in biofilms can be up to 1000 times more resistant (Rasmussen and

Givskov, 2006), it might be expected that sensitivity to antibiotics would decrease, but results indicated the opposite. However, the method used comprises immediate treatment of planktonic bacteria with antibiotics, allowing the antibiotic to exert its activity before biofilm formation could occur. The increased sensitivity upon filamentous phage infection has been previously documented for other phage-host systems. For instance, Wang et al. (2013) found that Vibrio phage VEJ increased the sensitivity of *V. cholerae* to ampicillin. Hagens et al. (2006) also demonstrated that filamentous phages, in combination with low doses of antibiotics, can inhibit and even kill *P. aeruginosa* strains. Moreover, two different PAO1 strains, one carrying a gentamicin resistance plasmid and the other carrying a tetracycline resistance plasmid, became more susceptible to their respective antibiotics when used in combination with filamentous phages and antibiotics. The authors hypothesized that the extrusion pores influenced the permeability of the host cell membrane, making it less effective as a barrier against antibiotic penetration. However, the multimeric tightly-gated channels formed by the extrusion pores do not allow molecule inflow into the cell under regular conditions (Marciano et al., 2001). Namely, cryo-electron microscopy studies have revealed that the central pore of the multimeric channel has a diameter of 6.0 – 8.8 nm, and it is tightly closed in the middle of the channel (Opalka et al., 2003). Despite this, the increased susceptibility to various antibiotics support the notion that Pf phages alter cell permeability upon infection. Another possible mechanism is that *Pseudomonas* filamentous phages can change the membrane potential of cells, as previously documented for Escherichia phage f1 (Lin et al., 2001). This change in membrane potential could subsequently affect membrane permeability by influencing the function of specific channels in the outer membrane, including integrated phage extrusion machinery, allowing antibiotics to enter the cells. Whether the extrusion pores can be opened prior to phage extrusion and if they can allow molecule inflow into the cell should be revealed in future studies. These findings suggest a new potential approach in phage therapy—treating multidrug and pandrug resistant strains with filamentous phages to re-sensitize bacteria to specific antibiotics. However, this strategy must be considered with precaution, in terms of the advantages and disadvantages it would have, particularily in the context of lysogenic conversion prevent. The selection of phages should focus

on those that do not contribute to biofilm formation and that significantly increase strain susceptibility to antibiotics.

In summary, filamentous bacteriophages can slow down the metabolism of *P. aeruginosa* and reduce toxin production. Their presence influences specific phenotypic characteristics, contributing to the biofilm formation. Increased biofilm production due to Pf infection and the higher frequency of SCV colonies significantly contribute to the development of serious infections. These factors lead to the emergence of less virulent *P. aeruginosa* strains, but due to biofilm, more persistent and challenging-to-treat chronic infections. Moreover, the extensive characterization of these bacteriophages opens up diverse possibilities for their application. Similar to other established filamentous phages utilized in different phage techniques, *P. aeruginosa* filamentous phages could find similar use. Furthermore, this group of bacteriophages holds substantial potential in medical and diagnostic fields. Notably, filamentous bacteriophages' high immunogenicity makes them suitable for vaccine development and stimulation of specific T lymphocyte lineages, as well as serving as agents for targeted therapy delivery.

# 6. Conclusion

In the present study, *Pseudomonas aeruginosa* specific phages belonging to family *Inoviridae* – Pf4, Pf5 and PfLES58, originating from strains PAO1, PA14 and LESB58, have been characterized in detail and their biology has been elucidated.

Based on the obtained results and in line with the study objectives, the following can be concluded:

- The phages PfLES58, Pf4, and Pf5 belong to the family *Inoviridae*, since they share morphological, genome and protein properties with other phages from the family. The analysis showed that these three phages are different species and that phages Pf4 and Pf5 belong to the same genus, together with Pf1 and Pf8 (*Primolicivirus*).
- The prophage PfLES58 actively replicates in *P. aeruginosa*, as demonstrated by the presence of replicative form in bacterial cells, and subsequently by virion production. Based on properties of key genes/proteins, PfLES58 belongs to a different genus (proposed name *Secondolicivirus*). Its genetic elements have high prevalence among *P. aeruginosa* strains (41.2%) of PK Lab culture collection.
- The phage yield is much higher, for approx. 4 – 8 logs, when a smaller culture volume is used and the incubation lasts longer than when a large culture volume is used with a shorter incubation. Further modification of the method is possible, so it can be adapted for filamentous phages that infect bacteria other than *P. aeruginosa*.
- Beside Pf4 phage, which is known to produce plaques, this property was confirmed for PfLES58 and Pf5 phages. The plaques are small (approx. 1mm) and opaque and can be easier detected when single-layer agar method is applied.
- Pf phages can be induced by subinhibitory concentrations of fluoroquinolones and mitomycin C, which almost doubled expression of phage genes, or by infection with obligatory lytic tailed phages from *Pbunavirus* genus, which elevate Pf gene expression up to 5 times. This is particularly important from the aspect of phage therapy or combined phage-antibiotic treatment.
- Pf phages can establish chronic productive infections in different *P. aeruginosa* strains, and their expression can exceed in some instances the expression of indigenous filamentous prophages (almost 2 times more). The superinfection exclusion was not

observed, so the multiple superinfections can provide new genetic material for mutations, drift or natural selection, i.e. evolution of *P. aeruginosa*. This is particularly important in the context of phage moderate to broad lytic spectra: Pf4 can infect 46.1% of strains, Pf5 62.5% and PfLES58 infect 90.6% strains when PK Lab culture collection was used.

- Pf phages can influence virulence related phenotypic properties of newly infected *P. aeruginosa* strains:
    - Some phages slightly decrease bacterial growth after infection;
    - Decrease autoaggregation, changing it qualitatively from moderately aggregative to non-aggregative in some instances; this is related with decreased virulence of strains;
    - Increase cell surface hydrophobicity, changing it qualitatively from hydrophilic to moderately hydrophobic in some instances; this contributes to better bacterial adhesion to hydrophobic surfaces;
    - Decrease twitching motility, decrease or increase swarming motility and increase swimming motility, depending on a specific phage-bacterial strain combination.
    - Pf phages almost completely inhibit the production of toxic pyocyanin (approx. from 10 to 130 times), which makes these infected strains less toxic.
    - Pf phages also decrease pyoverdine production, impairing iron chelation and reducing the bacterial fitness. As pyoverdine activates production of some toxin, Pf phages decrease general strain toxicity.
- Bacterial infection with bacteriophages of this group significantly increased biofilm production, from 1.6 to 2.4 times. Such effects lead to the emergence of *P. aeruginosa* strains that are more prone to establishing chronic infections. Additional proof of this is the more frequent appearance of SCV colonies after infection with Pf phage, but also decreased virulence that can make the infections less severe.
- Filamentous phages have the potential to re-sensitize newly infected strains to antibiotics, such as tetracycline, ceftazidime and streptomycin, offering a promising therapeutic option. Also, these phages, since here characterized in details, can be

considered for other phage based techniques (detection of *P. aeruginosa*, phage typing, phage display etc).

The results clearly indicate that the examined Pf phages contribute to bacterial virulence and evolution, have many unique properties and possess great potential for various application, so they deserve further research.

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# 8. Supplementary

**Table S1.** List of used *P. aeruginosa* strains

| Strain designation | Origin | Sample |
|---|---|---|
| 1A | Aleksinac (Republic of Serbia) | Urine |
| 2A | Aleksinac (Republic of Serbia) | Throat swab |
| 3A | Aleksinac (Republic of Serbia) | Urine |
| 4A | Aleksinac (Republic of Serbia) | Wound |
| 6A | Aleksinac (Republic of Serbia) | Nasal swab |
| 7A | Aleksinac (Republic of Serbia) | Sputum |
| 8A | Aleksinac (Republic of Serbia) | Ear swab |
| 9A | Aleksinac (Republic of Serbia) | Eye swab |
| 11A | Aleksinac (Republic of Serbia) | Throat swab |
| 12A | Aleksinac (Republic of Serbia) | Throat swab |
| 13A | Aleksinac (Republic of Serbia) | Wound |
| 14A | Aleksinac (Republic of Serbia) | Urine |
| 15A | Aleksinac (Republic of Serbia) | Wound |
| 16A | Aleksinac (Republic of Serbia) | Throat swab |
| 18A | Aleksinac (Republic of Serbia) | Nasal swab |
| 19A | Aleksinac (Republic of Serbia) | Wound |
| 20A | Aleksinac (Republic of Serbia) | Wound |
| 21A | Aleksinac (Republic of Serbia) | Wound |
| 22A | Aleksinac (Republic of Serbia) | Urine |
| 23A | Aleksinac (Republic of Serbia) | Urine |
| 24A | Aleksinac (Republic of Serbia) | Wound |
| 26A | Aleksinac (Republic of Serbia) | Wound |
| 27A | Aleksinac (Republic of Serbia) | Vaginal swab |
| 28A | Aleksinac (Republic of Serbia) | Gum swab |

| | | |
|---|---|---|
| 29A | Aleksinac (Republic of Serbia) | Sputum |
| 30A | Aleksinac (Republic of Serbia) | Wound |
| 31A | Aleksinac (Republic of Serbia) | Wound |
| 32A | Aleksinac (Republic of Serbia) | Wound |
| 33A | Aleksinac (Republic of Serbia) | Wound |
| 34A | Aleksinac (Republic of Serbia) | Urine |
| 35A | Aleksinac (Republic of Serbia) | Sputum |
| 36A | Aleksinac (Republic of Serbia) | Wound |
| 37A | Aleksinac (Republic of Serbia) | Wound |
| 38A | Aleksinac (Republic of Serbia) | Throat swab |
| 39A | Aleksinac (Republic of Serbia) | Ear swab |
| 41A | Aleksinac (Republic of Serbia) | Urine |
| 42A | Aleksinac (Republic of Serbia) | Ear swab |
| 44A | Aleksinac (Republic of Serbia) | Eye swab |
| 47A | Aleksinac (Republic of Serbia) | Urine |
| 48A | Aleksinac (Republic of Serbia) | Wound |
| 49A | Aleksinac (Republic of Serbia) | Urine |
| 50A | Aleksinac (Republic of Serbia) | Wound |
| 1B | Aleksinac (Republic of Serbia) | Urine |
| 2B | Aleksinac (Republic of Serbia) | Urine |
| 4B | Aleksinac (Republic of Serbia) | Wound |
| 7B | Aleksinac (Republic of Serbia) | Wound |
| 8B | Aleksinac (Republic of Serbia) | Urine |
| 9B | Aleksinac (Republic of Serbia) | Urine |
| 10B | Aleksinac (Republic of Serbia) | Wound |
| 11B | Aleksinac | Throat swab |

| | | |
|---|---|---|
| | (Republic of Serbia) | |
| 12B | Aleksinac (Republic of Serbia) | Wound |
| 14B | Aleksinac (Republic of Serbia) | Sputum |
| 15B | Aleksinac (Republic of Serbia) | Wound |
| 16B | Aleksinac (Republic of Serbia) | Wound |
| 17B | Aleksinac (Republic of Serbia) | Wound |
| 18B | Aleksinac (Republic of Serbia) | Wound |
| 20B | Aleksinac (Republic of Serbia) | Wound |
| 23B | Aleksinac (Republic of Serbia) | Wound |
| 24B | Aleksinac (Republic of Serbia) | Wound |
| 25B | Aleksinac (Republic of Serbia) | Urine |
| 26B | Aleksinac (Republic of Serbia) | Urine |
| 27B | Aleksinac (Republic of Serbia) | Wound |
| 28B | Aleksinac (Republic of Serbia) | Urine |
| 29B | Aleksinac (Republic of Serbia) | Wound |
| 30B | Aleksinac (Republic of Serbia) | Urine |
| 32B | Aleksinac (Republic of Serbia) | Wound |
| 33B | Aleksinac (Republic of Serbia) | Wound |
| 34B | Aleksinac (Republic of Serbia) | Sputum |
| 35B | Aleksinac (Republic of Serbia) | Sputum |
| 37B | Aleksinac (Republic of Serbia) | Wound |
| 39B | Aleksinac (Republic of Serbia) | Wound |
| 40B | Aleksinac (Republic of Serbia) | Wound |
| 42B | Aleksinac (Republic of Serbia) | Wound |
| 43B | Aleksinac (Republic of Serbia) | Wound |
| 44B | Aleksinac (Republic of Serbia) | Wound |

| | | |
|---|---|---|
| 45B | Aleksinac (Republic of Serbia) | Wound |
| 46B | Aleksinac (Republic of Serbia) | Wound |
| 47B | Aleksinac (Republic of Serbia) | Wound |
| 48B | Aleksinac (Republic of Serbia) | Wound |
| 49B | Aleksinac (Republic of Serbia) | Wound |
| 50B | Aleksinac (Republic of Serbia) | Wound |
| 51B | Aleksinac (Republic of Serbia) | Unknown |
| Aa245 | Aahen (Germany) | Burn wound |
| ATCC 9027 | Sydney (Australia) | Ear swab |
| ATCC 15692 | Unknown | Wound |
| ATCC 27853 | Boston (USA) | Blood |
| ATCC 15442 | Unknown | Water bottle in animal room |
| ATCC 10145 | Unknown | Unknown |
| Be128 | Beverwijk (Netherlands) | Sputum |
| Be133 | Beverwijk (Netherlands) | Burn wound |
| Be136 | Beverwijk (Netherlands) | Sputum |
| BG-M-17 | Belgrade (Republic of Serbia) | Unknown |
| Bo546 | Boston (USA) | Burn wound |
| Bo548 | Boston (USA) | Burn wound |
| Bo559 | Boston (USA) | Burn wound |
| BP213 | Novi Sad (Republic of Serbia) | Surface water - river |
| Br229 | Brussels (Belgium) | Hospital |
| Br257 | Brussels (Belgium) | Rhizosphere |
| Br642 | Brussels (Belgium) | Hospital |
| Br667 | Brussels (Belgium) | Burn wound |
| Br680 | Brussels (Belgium) | Burn wound |
| Br735 | Brussels (Belgium) | Burn wound |

| | | |
|---|---|---|
| Br906 | Brussels (Belgium) | Nasal swab |
| Br908 | Brussels (Belgium) | Throat swab |
| Br993 | Brussels (Belgium) | Sputum |
| BU004 | Budapest (Hungary) | Throat swab |
| C | Hanover (Germany) | Cystic fibrosis |
| C1 | Hanover (Germany) | Cystic fibrosis |
| C2 | Hanover (Germany) | Cystic fibrosis |
| C13 | Hanover (Germany) | Cystic fibrosis |
| C17 | Hanover (Germany) | Hospital |
| C18 | Hanover (Germany) | Hospital |
| C19 | Hanover (Germany) | Cystic fibrosis |
| CFA | Unknown | Unknown |
| Co7388-2 | Kali (Colombia) | Urine |
| Co380791 | Kali (Colombia) | Blood |
| CPHL 10622 | London (United Kingdom) | Unknown |
| Dsol 25865 | Unknown | Unknown |
| EMZHUW | Leuven (Belgium) | Hospital |
| EMZIBE | Leuven (Belgium) | Hospital |
| Esp06B | Brussels (Belgium) | Clinical non cystic fibrosis |
| EXB-V28 | Unknown | Unknown |
| GHB2 | Unknown | Unknown |
| GHB15 | Unknown | Unknown |
| GYZ7QW | Leuven (Belgium) | Hospital |
| H4S6IU | Leuven (Belgium) | Hospital |
| HPA4 | Unknown | Unknown |
| HPA17 | Unknown | Unknown |
| HYGGIJ | Leuven (Belgium) | Hospital |
| IMJLMG | Leuven (Belgium) | Hospital |
| Is573 | Istanbul (Turkey) | Burn wound |

| | | |
|---|---|---|
| Is579 | Istanbul (Turkey) | Burn wound |
| Is580 | Istanbul (Turkey) | Burn wound |
| Is586 | Istanbul (Turkey) | Burn wound |
| IEEBY2 | Leuven (Belgium) | Hospital |
| JR-18 | Vrsac (Republic of Serbia) | Production line |
| PAK | Unknown | Unknown |
| KAT3529 | Athens (Greece) | Wound |
| KBSNKD | Leuven (Belgium) | Hospital |
| KJXBJG | Leuven (Belgium) | Hospital |
| L6TBJD | Leuven (Belgium) | Hospital |
| LESB58 | Liverpool (United Kingdom) | Cystic fibrosis |
| LMG2107 | Kanas (Puerto Rico) | Water from the well |
| LMG5031 | Kanas (Puerto Rico) | Chinese evergreen |
| LMG10643 | Pusakanagara (Indonesia) | Leaf |
| LMG14083 | Unknown (Romania) | Unknown |
| Li004 | Lisbon (Portugal) | Cystic fibrosis |
| Li009 | Unknown | Unknown |
| Lo049 | London (United Kingdom) | Burn wound |
| Lo050 | London (United Kingdom) | Burn wound |
| Lo053 | London (United Kingdom) | Burn wound |
| Lw1047 | Livro (Kongo) | Blood |
| Mi151 | Michigan (USA) | Burn wound |
| Mi162 | Michigan (USA) | Burn wound |
| MSODFG | Leuven (Belgium) | Hospital |
| MSUDFA | Leuven (Belgium) | Hospital |
| ME4HFU | Leuven (Belgium) | Hospital |
| OB7025 | Unknown | Ear swab |

| | | |
|---|---|---|
| OT-3 | Unknown | Ear swab |
| OT-4 | Unknown | Ear swab |
| OT-5 | Unknown | Ear swab |
| OT-5A | Unknown | Ear swab |
| OT-8 | Unknown | Ear swab |
| OT-8A | Unknown | Ear swab |
| OT-13 | Unknown | Ear swab |
| OT-14 | Unknown | Ear swab |
| OT-35 | Unknown | Ear swab |
| OT-41B | Unknown | Ear swab |
| OT-45 | Unknown | Ear swab |
| OT-45A | Unknown | Ear swab |
| OT-51 | Unknown | Ear swab |
| OT-M2 | Unknown | Ear swab |
| PA6 | Brussels (Belgium) | Urine |
| PA7 | Buenos Aires (Argentina) | Unknown |
| UCBPP-PA14 | Boston (USA) | Wound |
| PA35554 | Novi Sad (Republic of Serbia) | Wound |
| PA36994-1 | Novi Sad (Republic of Serbia) | Wound |
| PA37304 | Novi Sad (Republic of Serbia) | Wound |
| PA40014 | Novi Sad (Republic of Serbia) | Wound |
| PA-M1 | Unknown | Surface water - river |
| PA-M2 | Unknown | Surface water - river |
| PA-XX | Novi Sad (Republic of Serbia) | Cosmetic product |
| PA-Z1 | Novi Sad (Republic of Serbia) | Land |
| PA-4U | Unknown | Mud |
| PAO1 | Melbourne (Australia) | Wound |
| PAO23 | Karachi (Pakistan) | River |
| PAO29 | Karachi (Pakistan) | River |
| PHDW6 | Tacloban (Philippines) | Wound |
| PKS685 | Leuven (Belgium) | Hospital |
| PER5 | Unknown | Unknown |
| PER9 | Unknown | Unknown |
| Pr335 | Prague (Czech Republic) | Hospital |
| PT31M | Mülheim (Germany) | Drinking water |

| | | |
|---|---|---|
| PUE4ZM | Unknown | Unknown |
| RB2915 | Novi Sad (Republic of Serbia) | Wound |
| Ro124 | Rotterdam (Netherlands) | Burn wound |
| QEBXI | Leuven (Belgium) | Hospital |
| RGWNEQ | Leuven (Belgium) | Hospital |
| RNZ4JM | Leuven (Belgium) | Hospital |
| SG-17-M | Ruhr (Germany) | Sputum |
| SG-50-M | Mülheim (Germany) | Pool water |
| SHK26U | Leuven (Belgium) | Hospital |
| SIS3740 | Athens (Greece) | Sputum |
| So092 | Sofia (Bulgaria) | Burn wound |
| So095 | Sofia (Bulgaria) | Burn wound |
| So098 | Sofia (Bulgaria) | Wound |
| So099 | Sofia (Bulgaria) | Burn wound |
| Sp1709-21 | Unknown | Unknown |
| SV1783 | Subotica (Republic of Serbia) | Water network |
| SV1784 | Subotica (Republic of Serbia) | Water network |
| SV1787 | Subotica (Republic of Serbia) | Water network |
| T10JUK | Leuven (Belgium) | Hospital |
| TuD43 | Tunis (Tunisia) | Unknown |
| TuD47 | Tunis (Tunisia) | Ascites |
| TuD199 | Tunis (Tunisia) | Sputum |
| U24XK3 | Leuven (Belgium) | Hospital |
| UB1 | Novi Sad (Republic of Serbia) | Urine |
| UB3577 | Novi Sad (Republic of Serbia) | Urine |
| UB5283 | Novi Sad (Republic of Serbia) | Urine |
| UB5296 | Novi Sad | Urine |

| | | |
|---|---|---|
| | (Republic of Serbia) | |
| UB7027 | Novi Sad (Republic of Serbia) | Urine |
| UB7084 | Novi Sad (Republic of Serbia) | Urine |
| UB7114 | Novi Sad (Republic of Serbia) | Urine |
| UB7156 | Novi Sad (Republic of Serbia) | Urine |
| US447 | San Antonio (USA) | Urine |
| US448 | San Antonio (USA) | Urine |
| US449 | San Antonio (USA) | Sputum |
| US450 | San Antonio (USA) | Burn wound |
| UD2CIF | Leuven (Belgium) | Hospital |
| V2KB6Q | Leuven (Belgium) | Hospital |
| V9BI2K | Leuven (Belgium) | Hospital |
| VC80KS | Leuven (Belgium) | Hospital |
| VNKYUI | Leuven (Belgium) | Hospital |
| NCTC 12951 | Unknown | Unknown |
| NCTC 6749 | London (United Kingdom) | Urine |
| XUG8JK | Leuven (Belgium) | Hospital |
| W68047-1 | Unknown | Unknown |
| JU1 | Kula (Republic of Serbia) | Ear swab |
| JU2 | Zrenjanin (Republic of Serbia) | Urine |
| JU4 | Novi Sad (Republic of Serbia) | Wound |
| JU5 | Subotica (Republic of Serbia) | Wound |
| JU6 | Subotica (Republic of Serbia) | Urine |
| JU7 | Subotica (Republic of Serbia) | Urine |
| JU8 | Kula (Republic of Serbia) | Urine |
| JU9 | Novi Sad (Republic of Serbia) | Urine |
| JU10 | Novi Sad (Republic of Serbia) | Urine |

| | | |
|---|---|---|
| JU11 | Novi Sad (Republic of Serbia) | Urine |
| JU12 | Stara Pazova (Republic of Serbia) | Vulva |
| JU13 | Stara Pazova (Republic of Serbia) | Nail |
| JU14 | Novi Sad (Republic of Serbia) | Wound |
| JU15 | Sombor (Republic of Serbia) | Wound |
| JU17 | Becej (Republic of Serbia) | Wound |
| JU18 | Novi Sad (Republic of Serbia) | Urine |
| JU19 | Bac (Republic of Serbia) | Wound |
| JU20 | Novi Sad (Republic of Serbia) | Urine |
| JU21 | Sombor (Republic of Serbia) | Wound |
| JU23 | Becej (Republic of Serbia) | Urine |
| JU24 | Novi Sad (Republic of Serbia) | Urine |
| JU25 | Becej (Republic of Serbia) | Ear swab |
| JU26 | Novi Sad (Republic of Serbia) | Wound |
| JU27 | Novi Sad (Republic of Serbia) | Urine |
| JU28 | Novi Sad (Republic of Serbia) | Urine |
| NE-PA-1 | Unknown | Unknown |
| NE-PA-2 | Unknown | Unknown |
| KCV-1 | Novi Sad (Republic of Serbia) | Wound |
| W7 | Unknown | Unknown |
| *Pseudomonas fluorescens* JR-16 | Vrsac (Republic of Serbia) | Production line |
| *Pseudomonas putida* JR-17 | Vrsac (Republic of Serbia) | Production line |
| *Escherichia coli ATCC 13706* | Unknown | Unknown |
| *Staphylococcus aureus* ATCC 11632 | Unknown | Unknown |

**Table S2.** Host range of PfLES58, Pf5, and Pf4 bacteriophages against different bacterial species and *P. aeruginosa* strains from various sources.

| Strain designation | Origin | Sample | Susceptibility of strains to Pf phages | | |
|---|---|---|---|---|---|
| | | | PfLES58 | Pf4 | Pf5 |
| 1A | Aleksinac (Republic of Serbia) | Urine | + | - | - |
| 2A | Aleksinac (Republic of Serbia) | Throat swab | + | + | + |
| 3A | Aleksinac (Republic of Serbia) | Urine | + | - | - |
| 4A | Aleksinac (Republic of Serbia) | Wound | - | - | - |
| 6A | Aleksinac (Republic of Serbia) | Nasal swab | + | - | + |
| 7A | Aleksinac (Republic of Serbia) | Sputum | + | - | + |
| 8A | Aleksinac (Republic of Serbia) | Ear swab | + | + | + |
| 9A | Aleksinac (Republic of Serbia) | Eye swab | + | - | + |
| 11A | Aleksinac (Republic of Serbia) | Throat swab | + | + | + |
| 12A | Aleksinac (Republic of Serbia) | Throat swab | + | + | + |
| 13A | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 14A | Aleksinac (Republic of Serbia) | Urine | + | - | - |
| 15A | Aleksinac (Republic of Serbia) | Wound | + | + | - |
| 16A | Aleksinac (Republic of Serbia) | Throat swab | + | - | + |
| 18A | Aleksinac (Republic of Serbia) | Nasal swab | + | - | - |
| 19A | Aleksinac (Republic of Serbia) | Wound | - | - | + |
| 20A | Aleksinac (Republic of Serbia) | Wound | - | - | - |
| 21A | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 22A | Aleksinac (Republic of Serbia) | Urine | + | + | + |
| 23A | Aleksinac (Republic of Serbia) | Urine | + | - | - |
| 24A | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 26A | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 27A | Aleksinac (Republic of Serbia) | Vaginal swab | + | + | + |
| 28A | Aleksinac | Gum swab | + | + | + |

| | | | | | |
|---|---|---|---|---|---|
| | (Republic of Serbia) | | | | |
| 29A | Aleksinac (Republic of Serbia) | Sputum | + | + | + |
| 30A | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 31A | Aleksinac (Republic of Serbia) | Wound | + | + | - |
| 32A | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 33A | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 34A | Aleksinac (Republic of Serbia) | Urine | + | - | + |
| 35A | Aleksinac (Republic of Serbia) | Sputum | + | - | - |
| 36A | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 37A | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 38A | Aleksinac (Republic of Serbia) | Throat swab | + | - | - |
| 39A | Aleksinac (Republic of Serbia) | Ear swab | + | + | - |
| 41A | Aleksinac (Republic of Serbia) | Urine | + | + | + |
| 42A | Aleksinac (Republic of Serbia) | Ear swab | + | - | - |
| 44A | Aleksinac (Republic of Serbia) | Eye swab | + | + | + |
| 47A | Aleksinac (Republic of Serbia) | Urine | + | - | - |
| 48A | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 49A | Aleksinac (Republic of Serbia) | Urine | + | + | - |
| 50A | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 1B | Aleksinac (Republic of Serbia) | Urine | + | - | - |
| 2B | Aleksinac (Republic of Serbia) | Urine | + | - | - |
| 4B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 7B | Aleksinac (Republic of Serbia) | Wound | - | + | + |
| 8B | Aleksinac (Republic of Serbia) | Urine | + | + | + |
| 9B | Aleksinac (Republic of Serbia) | Urine | + | + | + |
| 10B | Aleksinac (Republic of Serbia) | Wound | + | + | + |

| | | | | | |
|---|---|---|---|---|---|
| 11B | Aleksinac (Republic of Serbia) | Throat swab | + | - | + |
| 12B | Aleksinac (Republic of Serbia) | Wound | + | - | - |
| 14B | Aleksinac (Republic of Serbia) | Sputum | + | - | + |
| 15B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 16B | Aleksinac (Republic of Serbia) | Wound | + | - | - |
| 17B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 18B | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 20B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 23B | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 24B | Aleksinac (Republic of Serbia) | Wound | + | - | - |
| 25B | Aleksinac (Republic of Serbia) | Urine | + | + | - |
| 26B | Aleksinac (Republic of Serbia) | Urine | + | - | - |
| 27B | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 28B | Aleksinac (Republic of Serbia) | Urine | + | - | + |
| 29B | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 30B | Aleksinac (Republic of Serbia) | Urine | + | - | + |
| 32B | Aleksinac (Republic of Serbia) | Wound | + | + | - |
| 33B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 34B | Aleksinac (Republic of Serbia) | Sputum | + | - | + |
| 35B | Aleksinac (Republic of Serbia) | Sputum | + | - | + |
| 37B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 39B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 40B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 42B | Aleksinac (Republic of Serbia) | Wound | + | - | - |
| 43B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 44B | Aleksinac | Wound | + | - | - |

| | | | | | |
|---|---|---|---|---|---|
| | (Republic of Serbia) | | | | |
| 45B | Aleksinac (Republic of Serbia) | Wound | + | - | - |
| 46B | Aleksinac (Republic of Serbia) | Wound | + | - | + |
| 47B | Aleksinac (Republic of Serbia) | Wound | - | - | - |
| 48B | Aleksinac (Republic of Serbia) | Wound | + | + | + |
| 49B | Aleksinac (Republic of Serbia) | Wound | + | - | - |
| 50B | Aleksinac (Republic of Serbia) | Wound | + | - | - |
| 51B | Aleksinac (Republic of Serbia) | Unknown | + | - | - |
| Aa245 | Aahen (Germany) | Burn wound | + | - | - |
| ATCC 9027 | Sydney (Australia) | Ear swab | + | - | - |
| ATCC 15692 | Unknown | Wound | + | + | + |
| ATCC 27853 | Boston (USA) | Blood | + | + | + |
| ATCC 15442 | Unknown | Water bottle in animal room | + | + | + |
| ATCC 10145 | Unknown | Unknown | + | + | + |
| Be128 | Beverwijk (Netherlands) | Sputum | + | + | + |
| Be133 | Beverwijk (Netherlands) | Burn wound | + | + | + |
| Be136 | Beverwijk (Netherlands) | Sputum | + | + | + |
| BG-M-17 | Belgrade (Republic of Serbia) | Unknown | + | + | + |
| Bo546 | Boston (USA) | Burn wound | + | + | + |
| Bo548 | Boston (USA) | Burn wound | + | - | + |
| Bo559 | Boston (USA) | Burn wound | + | + | + |
| BP213 | Novi Sad (Republic of Serbia) | Surface water - river | + | + | + |
| Br229 | Brussels (Belgium) | Hospital | + | - | - |
| Br257 | Brussels (Belgium) | Rhizosphere | + | + | + |
| Br642 | Brussels (Belgium) | Hospital | + | - | - |
| Br667 | Brussels (Belgium) | Burn wound | + | - | - |
| Br680 | Brussels (Belgium) | Burn wound | + | + | - |

| | | | | | |
|---|---|---|---|---|---|
| Br735 | Brussels (Belgium) | Burn wound | + | - | + |
| Br906 | Brussels (Belgium) | Nasal swab | + | + | + |
| Br908 | Brussels (Belgium) | Throat swab | + | - | - |
| Br993 | Brussels (Belgium) | Sputum | + | - | - |
| BU004 | Budapest (Hungary) | Throat swab | + | - | - |
| C | Hanover (Germany) | Cystic fibrosis | + | - | - |
| C1 | Hanover (Germany) | Cystic fibrosis | - | - | - |
| C2 | Hanover (Germany) | Cystic fibrosis | - | + | + |
| C13 | Hanover (Germany) | Cystic fibrosis | + | - | - |
| C17 | Hanover (Germany) | Hospital | - | + | + |
| C18 | Hanover (Germany) | Hospital | + | + | - |
| C19 | Hanover (Germany) | Cystic fibrosis | + | + | + |
| CFA | Unknown | Unknown | + | - | - |
| Co7388-2 | Kali (Colombia) | Urine | + | - | + |
| Co380791 | Kali (Colombia) | Blood | + | - | - |
| CPHL 10622 | London (United Kingdom) | Unknown | + | - | + |
| Dsol 25865 | Unknown | Unknown | + | - | - |
| EMZHUW | Leuven (Belgium) | Hospital | + | - | - |
| EMZIBE | Leuven (Belgium) | Hospital | + | - | + |
| Esp06B | Brussels (Belgium) | Clinical non cystic fibrosis | + | + | + |
| EXB-V28 | Unknown | Unknown | + | - | - |
| GHB2 | Unknown | Unknown | - | - | + |
| GHB15 | Unknown | Unknown | + | - | - |
| GYZ7QW | Leuven (Belgium) | Hospital | - | - | - |
| H4S6IU | Leuven (Belgium) | Hospital | + | - | + |
| HPA4 | Unknown | Unknown | + | - | + |
| HPA17 | Unknown | Unknown | + | - | + |
| HYGGIJ | Leuven (Belgium) | Hospital | + | - | - |
| IMJLMG | Leuven (Belgium) | Hospital | + | - | + |

| | | | | | |
|---|---|---|---|---|---|
| Is573 | Istanbul (Turkey) | Burn wound | + | + | + |
| Is579 | Istanbul (Turkey) | Burn wound | + | - | + |
| Is580 | Istanbul (Turkey) | Burn wound | + | + | + |
| Is586 | Istanbul (Turkey) | Burn wound | + | - | - |
| IEEBY2 | Leuven (Belgium) | Hospital | - | - | + |
| JR-18 | Vrsac (Republic of Serbia) | Production line | + | + | + |
| PAK | Unknown | Unknown | + | - | + |
| KAT3529 | Athens (Greece) | Wound | + | - | - |
| KBSNKD | Leuven (Belgium) | Hospital | + | - | + |
| KJXBJG | Leuven (Belgium) | Hospital | - | + | + |
| L6TBJD | Leuven (Belgium) | Hospital | - | + | + |
| LESB58 | Liverpool (United Kingdom) | Cystic fibrosis | + | + | + |
| LMG2107 | Kanas (Puerto Rico) | Water from the well | + | + | + |
| LMG5031 | Kanas (Puerto Rico) | Chinese evergreen | + | - | - |
| LMG10643 | Pusakanagara (Indonesia) | Leaf | + | + | - |
| LMG14083 | Unknown (Romania) | Unknown | + | + | + |
| Li004 | Lisbon (Portugal) | Cystic fibrosis | + | + | - |
| Li009 | Unknown | Unknown | + | - | - |
| Lo049 | London (United Kingdom) | Burn wound | + | - | + |
| Lo050 | London (United Kingdom) | Burn wound | + | + | + |
| Lo053 | London (United Kingdom) | Burn wound | + | - | - |
| Lw1047 | Livro (Kongo) | Blood | + | - | - |
| Mi151 | Michigan (USA) | Burn wound | + | - | + |
| Mi162 | Michigan (USA) | Burn wound | + | - | - |
| MSODFG | Leuven (Belgium) | Hospital | + | - | + |
| MSUDFA | Leuven (Belgium) | Hospital | + | + | - |
| ME4HFU | Leuven | Hospital | - | - | - |

| | | | | | |
|---|---|---|---|---|---|
| | (Belgium) | | | | |
| OB7025 | Unknown | Ear swab | + | - | - |
| OT-3 | Unknown | Ear swab | - | + | + |
| OT-4 | Unknown | Ear swab | + | - | - |
| OT-5 | Unknown | Ear swab | + | + | + |
| OT-5A | Unknown | Ear swab | + | - | - |
| OT-8 | Unknown | Ear swab | + | + | + |
| OT-8A | Unknown | Ear swab | + | + | + |
| OT-13 | Unknown | Ear swab | + | - | + |
| OT-14 | Unknown | Ear swab | + | + | + |
| OT-35 | Unknown | Ear swab | + | + | + |
| OT-41B | Unknown | Ear swab | + | + | + |
| OT-45 | Unknown | Ear swab | + | - | + |
| OT-45A | Unknown | Ear swab | + | - | - |
| OT-51 | Unknown | Ear swab | + | + | + |
| OT-M2 | Unknown | Ear swab | + | + | + |
| PA6 | Brussels (Belgium) | Urine | + | + | + |
| PA7 | Buenos Aires (Argentina) | Unknown | + | - | - |
| UCBPP-PA14 | Boston (USA) | Wound | + | + | + |
| PA35554 | Novi Sad (Republic of Serbia) | Wound | + | - | + |
| PA36994-1 | Novi Sad (Republic of Serbia) | Wound | + | + | + |
| PA37304 | Novi Sad (Republic of Serbia) | Wound | + | + | + |
| PA40014 | Novi Sad (Republic of Serbia) | Wound | + | + | + |
| PA-M1 | Unknown | Surface water - river | + | + | + |
| PA-M2 | Unknown | Surface water - river | + | + | + |
| PA-XX | Novi Sad (Republic of Serbia) | Cosmetic product | + | - | + |
| PA-Z1 | Novi Sad (Republic of Serbia) | Land | + | - | + |
| PA-4U | Unknown | Mud | + | + | + |
| PAO1 | Melbourne (Australia) | Wound | + | + | + |
| PAO23 | Karachi (Pakistan) | River | + | + | + |
| PAO29 | Karachi (Pakistan) | River | - | - | - |
| PHDW6 | Tacloban (Philippines) | Wound | + | - | + |
| PKS685 | Leuven (Belgium) | Hospital | + | - | - |
| PER5 | Unknown | Unknown | + | - | + |
| PER9 | Unknown | Unknown | + | + | - |

| | | | | | |
|---|---|---|---|---|---|
| Pr335 | Prague (Czech Republic) | Hospital | + | - | + |
| PT31M | Mülheim (Germany) | Drinking water | + | + | - |
| PUE4ZM | Unknown | Unknown | - | - | - |
| RB2915 | Novi Sad (Republic of Serbia) | Wound | + | - | + |
| Ro124 | Rotterdam (Netherlands) | Burn wound | + | - | + |
| QEBXI | Leuven (Belgium) | Hospital | - | - | - |
| RGWNEQ | Leuven (Belgium) | Hospital | - | - | + |
| RNZ4JM | Leuven (Belgium) | Hospital | + | + | + |
| SG-17-M | Ruhr (Germany) | Sputum | + | + | + |
| SG-50-M | Mülheim (Germany) | Pool water | + | + | + |
| SHK26U | Leuven (Belgium) | Hospital | + | - | + |
| SIS3740 | Athens (Greece) | Sputum | + | + | + |
| So092 | Sofia (Bulgaria) | Burn wound | + | - | + |
| So095 | Sofia (Bulgaria) | Burn wound | + | - | - |
| So098 | Sofia (Bulgaria) | Wound | + | - | - |
| So099 | Sofia (Bulgaria) | Burn wound | + | + | - |
| Sp1709-21 | Unknown | Unknown | + | - | - |
| SV1783 | Subotica (Republic of Serbia) | Water network | + | - | + |
| SV1784 | Subotica (Republic of Serbia) | Water network | + | - | + |
| SV1787 | Subotica (Republic of Serbia) | Water network | + | - | - |
| T10JUK | Leuven (Belgium) | Hospital | + | + | + |
| TuD43 | Tunis (Tunisia) | Unknown | + | + | + |
| TuD47 | Tunis (Tunisia) | Ascites | + | + | + |
| TuD199 | Tunis (Tunisia) | Sputum | + | + | + |
| U24XK3 | Leuven (Belgium) | Hospital | - | - | + |
| UB1 | Novi Sad (Republic of Serbia) | Urine | + | - | - |
| UB3577 | Novi Sad | Urine | + | - | - |

| | | | | | |
|---|---|---|---|---|---|
| | (Republic of Serbia) | | | | |
| UB5283 | Novi Sad (Republic of Serbia) | Urine | + | + | + |
| UB5296 | Novi Sad (Republic of Serbia) | Urine | + | + | + |
| UB7027 | Novi Sad (Republic of Serbia) | Urine | + | + | + |
| UB7084 | Novi Sad (Republic of Serbia) | Urine | + | - | + |
| UB7114 | Novi Sad (Republic of Serbia) | Urine | + | - | - |
| UB7156 | Novi Sad (Republic of Serbia) | Urine | - | - | - |
| US447 | San Antonio (USA) | Urine | + | - | - |
| US448 | San Antonio (USA) | Urine | + | + | + |
| US449 | San Antonio (USA) | Sputum | + | - | - |
| US450 | San Antonio (USA) | Burn wound | + | + | + |
| UD2CIF | Leuven (Belgium) | Hospital | - | + | + |
| V2KB6Q | Leuven (Belgium) | Hospital | + | + | + |
| V9BI2K | Leuven (Belgium) | Hospital | + | + | + |
| VC80KS | Leuven (Belgium) | Hospital | + | - | + |
| VNKYUI | Leuven (Belgium) | Hospital | + | + | + |
| NCTC 12951 | Unknown | Unknown | + | + | - |
| NCTC 6749 | London (United Kingdom) | Urine | + | + | + |
| XUG8JK | Leuven (Belgium) | Hospital | + | - | + |
| W68047-1 | Unknown | Unknown | + | + | + |
| JU1 | Kula (Republic of Serbia) | Ear swab | + | + | + |
| JU2 | Zrenjanin (Republic of Serbia) | Urine | + | - | - |
| JU4 | Novi Sad (Republic of Serbia) | Wound | + | - | - |
| JU5 | Subotica (Republic of Serbia) | Wound | + | - | - |
| JU6 | Subotica (Republic of Serbia) | Urine | + | - | - |
| JU7 | Subotica (Republic of Serbia) | Urine | + | - | - |
| JU8 | Kula (Republic of Serbia) | Urine | + | + | + |

| | | | | | |
|---|---|---|---|---|---|
| JU9 | Novi Sad (Republic of Serbia) | Urine | + | + | - |
| JU10 | Novi Sad (Republic of Serbia) | Urine | + | + | + |
| JU11 | Novi Sad (Republic of Serbia) | Urine | + | - | - |
| JU12 | Stara Pazova (Republic of Serbia) | Vulva | + | - | - |
| JU13 | Stara Pazova (Republic of Serbia) | Nail | + | + | + |
| JU14 | Novi Sad (Republic of Serbia) | Wound | + | - | - |
| JU15 | Sombor (Republic of Serbia) | Wound | + | + | + |
| JU17 | Becej (Republic of Serbia) | Wound | + | + | - |
| JU18 | Novi Sad (Republic of Serbia) | Urine | + | + | + |
| JU19 | Bac (Republic of Serbia) | Wound | - | - | - |
| JU20 | Novi Sad (Republic of Serbia) | Urine | - | - | + |
| JU21 | Sombor (Republic of Serbia) | Wound | + | - | + |
| JU23 | Becej (Republic of Serbia) | Urine | - | - | - |
| JU24 | Novi Sad (Republic of Serbia) | Urine | + | + | + |
| JU25 | Becej (Republic of Serbia) | Ear swab | + | + | - |
| JU26 | Novi Sad (Republic of Serbia) | Wound | + | + | + |
| JU27 | Novi Sad (Republic of Serbia) | Urine | + | + | + |
| JU28 | Novi Sad (Republic of Serbia) | Urine | + | - | - |
| NE-PA-1 | Unknown | Unknown | + | - | - |
| NE-PA-2 | Unknown | Unknown | + | - | + |
| KCV-1 | Novi Sad (Republic of Serbia) | Wound | + | + | + |
| W7 | Unknown | Unknown | + | + | + |
| *Pseudomonas fluorescens* JR-16 | Vrsac (Republic of Serbia) | Production line | - | - | - |
| *Pseudomonas putida* JR-17 | Vrsac (Republic of Serbia) | Production line | - | - | - |
| *Escherichia coli ATCC 13706* | Unknown | Unknown | - | - | - |
| *Staphylococcus aureus* ATCC 11632 | Unknown | Unknown | - | - | - |

**PCR product sequences confirming the integration of Pf phage in the genomes of different *P. aeruginosa* strains**

>PAO1+PfLES58

CGACAGTTCTTCGACACTTGCCGAGACGTCCTAGATTTTTCCTTTGCATATCAATTAGTTATGAGCGCTGATTCGGCTTTGCCGA
TTTGTGCGTAAAATAGCCAGCTTTTCTCCTCGAACACCCGGATTCCAGAGCACCATGGGCACCCTTTCGGTCAATCAGAACAAA
CTGCAGAAGCGCCTGCGCCGCCTGGCCGGCGAGGCCATCACCGACTTCAACATGATCGAGGATGGCGACAAGGTCATGGTCT
GCCTGTC

| PfLES58 | tRNA 2-thiocytidine biosynthesis protein TtcA |
|---|---|

>PA14+PfLES58

CGACAGTTCTTCGACACTTGCCGAGACGTCCTAGATTTTTCCTTTGCATATCAATTAGTTATGAGCGCTGATTCGGCTTTGCCGA
TTTGTGCGTAAAATAGCCAGCTTTTCTCCTCGAACACCCGGATTCCAGAGCACCATGGGCACCCTTTCGGTCAATCAGAACAAA
CTGCAGAAGCGCCTGCGCCGCCTGGCCGGCGAGGCCATCACCGACTTCAACATGATCGAGGATGGCGACAAGGTCATGGTCT
GCCTGTC

| PfLES58 | tRNA 2-thiocytidine biosynthesis protein TtcA |
|---|---|

>LESB58+Pf4

CGGACGGTGTTCCTGCCTATGTGGTTGAGTCATATGCTGAGCGGAAGCAGCGCGATGAAGCAATTGCGCTGGTGAAGTTGCT
TGCGATTGGCTCCCGCCAGTACGCAGAAGGCAAGCATCGCTCTGTTGATGATTTGAAAGCTCGCCTTTCCAGGAGGTTCGCTC
AGCCAGAATAAGGAGGTTTAATGTCCCCGGTCGTCATTCGTTTTACTGATACCGCAGAGCAAAGCATCGAAGACCAAGTCCAC
CACTTGGCTCCATTCCAAGGTGAACAGGCTGCACTCCAGTCAGTACTGAGCCTTTTGGATGAGATTGAAGAGAAGATTTCACTT
GCACCTAAAGGTTACCCAGTCAGCCAGCAGGCGAGTCTTCTGGGGGTGCTGAGCTATCGCGAGCTTAATACCGGCCCCTATCG
TGTTTTTTACGAATTCCACGAAGAGCAAGGCGAGGTGGCAGTGATCTTGGTTTTGCGACAGAAGCAGAGCGTTGAGCAGCAA
TTGATCCGCTACTGCTTGGTGGGGCCAATCGAGTGATGGCTTTCTACTCCTGAGCATGTAGCGCTGAATGCGCCTCGACACTTC
TTCGACACCTTTCCTTCCCCCAAAAAGCAAAGCCCCCGAAACGCTAGGCATTTCAGGGGCTTGGCAGGGTGATTTGGAGCGGG
CGAAGGGAATCGAACCCTCGTCATGAGCTTGGGAAGCTCAGGTAATGCCATTATACGACGCCCGCTCGGACGGCTTTTGCGGC
CAGGGCGCCTTTTTACCAGATGCGCGGCGGCAGGTGAAGCCCGGGGCGGGGGTTTTTGTTGATTTCGCTGGGTTTTTCCGCCA
GGGGGAGCGGGGCTCCCCGTGGCGGTGGCGGTTAGCTGGCGAGGGCGGCGAGGGGGACGCTGGCGCCCGTTGGGCTGGG
GGCGTATG

| Pf4 | tRNA-Gly | LESB58 |
|---|---|---|

>PA14+Pf4

GGGTCTGCCTATGTGGTTGAGTCATATGCTGAGCGGAAGCAGCGCGATGAAGCAATTGCGCTGGTGAAGTTGCTTGCGATTG
GCTCCCGCCAGTACGCAGAAGGCAAGCATCGCTCTGTTGATGATTTGAAAGCTCGCCTTTCCAGGAGGTTCGCTCAGCCAGAA
TAAGGAGGTTTAATGTCCCCGGTCGTCATTCGTTTTACTGATACCGCAGAGCAAAGCATCGAAGACCAAGTCCACCACTTGGCT
CCATTCCAAGGTGAACAGGCTGCACTCCAGTCAGTACTGAGCCTTTTGGATGAGATTGAAGAGAAGATTTCACTTGCACCTAA
AGGTTACCCAGTCAGCCAGCAGGCGAGTCTTCTGGGGGTGCTGAGCTATCGCGAGCTTAATACCGGCCCCTATCGTGTTTTTTA
CGAATTCCACGAAGAGCAAGGCGAGGTGGCAGTGATCTTGGTTTTGCGACAGAAGCAGAGCGTTGAGCAGCAATTGATCCGC
TACTGCTTGGTGGGGCCAATCGAGTGATGGCTTTCTACTCCTGAGCATGTAGCGCTGAATGCGCCTCGACACTTCTTCGACACC
TTTCCTTCCCCCAAAAAGCAAAGCCCCCGAAACGCTAGGCATTTCAGGGGCTTGGCAGGGTGATTTGGAGCGGGCGAAGGGA
ATCGAACCCTCGTCATGAGCTTGGGAAGCTCAGGTAATGCCATTATACGACGCCCGCTCGGACGGCTTTTGCGGCCAGGGCGC
CTTTTTACCAGATGCGCGGCGGCAGGTGAAGCCCGGGGCGGGGTTTTTGTTGATTTCGCTGGGTTTTTCCGCCAGGGGGAGC
GGGGCTCCCCGTGGCGGTGGCGGTTAGCTGGCGAGGGCGGCGAGGGGACGCTGGCGCCGTTGGG

| Pf4 | tRNA-Gly | PA14 |
|---|---|---|

>LESB58+Pf5

GATTCTTGGGCATTCGTCGCTGAACATGACCATGCGCTATGCGCATTTGTCGCCGGAATACCTGCGGGACGCTATTCGACTCAA
CCCGCTGGCGGATTTCGACAGTTCTTCGACACTTGCCGAGACGTCCTAGATTTTTCCTTTGCATATCAATTAGTTATGAGCGCTG
ATTCGGCTTTGCCGATTTGTGCGTAGAAGTGCAGAGCCGTTTGGGGTGTTGGTCATAACTGGTCAACGCtAGGC

| LESB58 genome | Pf5 |
|---|---|

# Biography

Damir Gavrić was born on May 16, 1994 in Sarajevo, Bosnia and Herzegovina. He graduated from the "Filip Višnjić" Highschool in Bijeljina. In 2013, he enrolled in the Faculty of Sciences, University of Novi Sad. In September 2018, he finished his studies with an average of 9.64. In the same year, he enrolled in master's studies at the Faculty of Science, Novi Sad, master's degree in biology (module: Microbiologist). He finished master studies with an average of 10.0. In 2018, he enrolled in PhD program at the same faculty.

He was awarded for academic success during the 2015/2016 and 2016/17 school years. Exceptional awards were given to him in competitions for scientific creations by students in 2015 and 2016. The scholarship "Dositeja | Fund for Young Talents of the Republic of Serbia" was granted to him in 2017. He won the first place in the scientific paper competition in the field of biology at the 2018 Primatijada. Furthermore, he was awarded for the completion of studies (with an average above 9.5) at the University of Novi Sad, as well as for the Faculty of Science's promotion in 2018. He was awarded a scholarship for students of higher education institutions in the Republic of Serbia for the years 2018 and 2019.

During his doctoral studies, he participated in conducting practical classes at the Department of Microbiology in the course Basics of Microbiology and Virology for students of bachelor academic studies, as well as in the courses Bacteriophage Biology, Pathogenic Microorganisms and Biofilms for students of master academic studies. A lecture on the topic "What are viruses?" was delivered by him at the Night of Researchers 2020 festival.

He participated in the realization of two national projects. He is the first author of two scientific papers published in international journals (M21 category). He is the co-author of three papers published in top international journals (M21 category) and one paper published in a leading journal of national importance. He have had five presentations at the international conferences (M34 category).

# План третмана података

| Назив пројекта/истраживања |
|---|
| Filamentous bacteriophages of species *P. aeruginosa*<br>Филаментозни бактериофаги врсте *P. aeruginosa* |
| **Назив институције/институција у оквиру којих се спроводи истраживање** |
| а) Природно-математички факултет, Универзитет у Новом Саду |
| **Назив програма у оквиру ког се реализује истраживање** |
| Програм научноистраживачког рада Министарства науке, технолошког развоја и иновација републике Србије |
| **1. Опис података** |

*1.1* Врста студије

Студија користи *in silico* за утврђивање филогенетских анализа филаментозних бактериофага специфичних за бактеријску врсту *P. aeruginosa* као за дизајнирање прајмера за qRT-PCR и PCR. Такође, вршене су *in vitro* анализе могућности формирања плака од стране умножених филаментозних бактериофага као и промена у осетљибости на антибиотике након инфекције бактеријских сојева са филаментозним бактериофагима

1.2 Врсте података
**а) квантитативни**
**б) квалитативни**

1.3. Начин прикупљања података
а) преузимање секвенци генома секвенцираних филаментозних бактериофага из НЦБИ базе
б) ручно претраживање секвенци генома филаментозних бактериофага у оквиру секвенцираних генома референтних бактеријских сојева из НЦБИ базе
в) визуелизација зона инхибиција након третмана са антибиотиком
г) апсорбанце спектрофотометарских мерења
д) визуелизација добијених прстена бактериофага након ултрацентрифугирања

1.3 Формат података, употребљене скале, количина података

1.3.1 Употребљени софтвер и формат датотеке:
а) **Excel фајл, датотека** _____
б) SPSS фајл, датотека _____
в) PDF фајл, датотека _____
г) **Текст фајл, датотека** _____
д) **JPG фајл, датотека** _____
ђ) Остало, датотека _____

1.3.2. Број записа (код квантитативних података)

а) број варијабли од 3 до 9 у зависности од методе
б) број мерења (испитаника, процена, снимака и сл.) од 9 до 39 у зависности од методе

1.3.3. Поновљена мерења
а) да
б) **не**

*Да ли формати и софтвер омогућавају дељење и дугорочну валидност података?*
*а) **Да***
*б) Не*

## 2. Прикупљање података

2.1 Методологија за прикупљање/генерисање података

2.1.1. У оквиру ког истраживачког нацрта су подаци прикупљени?
а) експеримент - спектрофотометријско праћење раста инфицираних и неинфицираних бактерија
б) остало – *in silico* претрага секвенци генома и филогенетске анализе у MEGA X програму

*2.1.2 Навести врсте мерних инструмената или стандарде података специфичних за одређену научну дисциплину (ако постоје).*
Спектрофотометар, ултрацентрифуга и програмски пакети за *in silico* анализе

2.2 Квалитет података и стандарди

2.2.1. Третман недостајућих података
а) Да ли матрица садржи недостајуће податке? **Не**

2.2.2. На који начин је контролисан квалитет података? Описати
Контролисано је статистичким анализама и коришћењем контролним експеримената.

2.2.3. На који начин је извршена контрола уноса података у матрицу?
Контролом са другом корисничког налога

## 3. Третман података и пратећа документација

3.1. Третман и чување података

*3.1.1. Подаци ће бити депоновани у* Google Drive *репозиторијум.*
*3.1.2. URL адреса*
*https://drive.google.com/drive/folders/1UMBPL4wyAyrDOL8MwRf0ijnQk4i0WUMf?usp=sharing*
*3.1.3. DOI nije dodeљen*
*3.1.4. Да ли ће подаци бити у отвореном приступу? Да*

3.2 Метаподаци и документација података
Нема метаподатака.

3.3 Стратегија и стандарди за чување података
3.3.1. До ког периода ће подаци бити чувани у репозиторијуму? ∞
3.3.2. Да ли ће подаци бити депоновани под шифром? Да
3.3.3. Да ли ће шифра бити доступна одређеном кругу истраживача? Да
3.3.4. Да ли се подаци морају уклонити из отвореног приступа после извесног времена? Не

| 4. Безбедност података и заштита поверљивих информација |
| --- |
| 4.1.2. Да ли је истраживање одобрено од стране етичке комисије? Не<br>4.1.2. Да ли подаци укључују личне податке учесника у истраживању? Не |
| **5. Доступност података** |
| *5.1. Подаци ће бити  јавно доступни*<br>*5.4. Навести лиценцу под којом ће прикупљени подаци бити архивирани.*<br>*ауторство – некомерцијално – делити под истим условима* |
| **6. Улоге и одговорност** |
| *6.1. Навести име и презиме и мејл адресу власника (аутора) података*<br>Дамир Гаврић, damir.gavric@dbe.uns.ac.rs<br><br>*6.2. Навести име и презиме и мејл адресу особе која одржава матрицу с подацима*<br>Дамир Гаврић, damir.gavric@dbe.uns.ac.rs<br><br>*6.3. Навести име и презиме и мејл адресу особе која омогућује приступ подацима другим истраживачима*<br>Дамир Гаврић, damir.gavric@dbe.uns.ac.rs |