

# The use of bacteriophages for *P. aeruginosa* biofilm control

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*Pseudomonas aeruginosa* is a relevant opportunistic pathogen frequently associated with several nosocomial infections and, worryingly, this bacterium shows a low antibiotic susceptibility. One of its virulence factors is related with the ability to adhere to surfaces and also human epithelium and form virulent biofilms. This work describes the isolation and characterization of lytic phages capable to infect antibiotic resistant *P. aeruginosa* strains. It is also described herein the potential of the new isolated phages for planktonic cells and biofilm control. According to the results of this work, the isolated phages showed different spectra of activity and most of them were efficient even against *P. aeruginosa* multidrug resistant strains. Furthermore, the biofilm infection assays revealed that phages can be a good strategy to combat virulent biofilms achieving significant reductions in the number of biofilm cells.

**Keywords:** Bacteriophages, *P. aeruginosa*, Biofilms, Control

## I. CONTEXT

*Pseudomonas aeruginosa* is an ubiquitous organism which has emerged as a major threat in the hospital environment. This bacterium is the most frequently isolated Gram-negative organism in bloodstream and wound infections, pneumonia, intra-abdominal and urogenital sepsis and is a serious problem infecting immunocompromised patients with cystic fibrosis (CF), severe burns, cancer, AIDS, etc. [1-2]. One of the most worrying characteristics of this bacterium is its low antibiotic susceptibility which can be attributed to a concerted action of multidrug efflux pumps with chromosomally-encoded antibiotic resistance genes and the low permeability of the bacterial cellular envelopes [3]. The overuse of antibiotics has also significantly increased the emergence of antimicrobial multi-resistant bacteria and consequently, the treatment of most chronic *P. aeruginosa* infections with antibiotics is notoriously difficult [3-4]. Additionally, *P. aeruginosa* has an inapt ability to adhere to surfaces and form virulent biofilms which are particularly difficult to eradicate [5-6]. Biofilm formation is an important bacterial survival strategy and, in humans, biofilms are responsible for numerous pathologies usually associated with the use of medical devices [7-8]. In this way, new alternative strategies to antibiotherapy are highly desired by the worldwide medical and scientific community

and bacteriophages (phages) appear as one attractive solution for this problem [9-11].

Phage therapy is based on the use of lytic phages to combat bacterial infections, including multi-drug resistant bacteria, and has many advantages comparatively to antibiotics: they are very specific and efficient for their target bacteria which reduces the destruction of the natural flora on the host; they are not pathogenic for man; and they need their target bacteria to grow and only persist as long as the bacteria they are targeting is present [9, 12].

## II. GOALS

The aim of this work was the isolation and characterization of novel *P. aeruginosa* phages and also the evaluation of their efficiency on planktonic cultures and mono-species biofilms.

## III. IMPLEMENTATION

### A. Isolation and characterization of phages

Bacteriophages were isolated from 2 hospital effluents provided by Hospital de São João (Porto, Portugal). These effluents were enriched with 4 *P. aeruginosa* strains (ATCC 10145, CECT 111, PAO 1 and a clinical isolate (CLIN)) in 2 × Tryptic Soy Broth (TSB) medium. This solution was incubated (37°C, 120 rpm) during 48 hours and then centrifuged to collect the supernatant for the spot tests (Fig. 1) indicative of the existence of phage(s). Each inhibition halo was further purified with toothpicks and papers, to isolate all different phages.

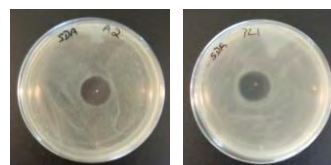


Figure 1. Examples of a spot test of enriched effluent on lawns of *P. aeruginosa* ATCC 10145 and PAO 1.

Overall, 17 phages with different characteristics were isolated and 4 of them were selected to perform planktonic

cells infection assays. This selection was done according to the results of the lytic spectra. To evaluate the lytic spectra of all isolated phages, each phage was tested against 35 different *P. aeruginosa* strains. 31 of these strains were clinical isolates provided by Hospital de São Marcos (Braga, Portugal). To perform the lytic spectra assay, one drop of each serial diluted phage suspension was placed into the different bacterial lawns and incubated overnight at 37°C. In the following day, the susceptibility of each host to the different phages was evaluated.

SDS-PAGE experiment was also performed in order to analyze the structural proteins of the 4 selected phages. After the preparation of the gels, the samples were added and the gels were run at 100 V (80 mA). After electrophoresis, the gels were stained with silver nitrate.

## B. Phage infection

### 1. Infection of planktonic cells

The infection of planktonic cells was done in two different stages of bacterial growth – exponential and stationary phases. To perform the planktonic cell infections at the stationary stage, the 4 hosts were inoculated overnight in TSB medium at 37°C (120 rpm). The resultant cellular suspensions were diluted with TSB medium in order to have an optical density (OD<sub>600nm</sub>) approximately 0.75. To do the planktonic cells infection at exponential stage, an overnight pre-inoculum was used to inoculate fresh TSB medium which were allowed to grow until achieving an optical density (OD<sub>600nm</sub>) of 0.5. In all experiments, phages were added to the respective hosts in order to obtain a multiplicity of infection (MOI) of 1. The control experiments were performed with SM Buffer instead of phages. The infection of planktonic cell assays were performed in 96-well microtiter plates (37°C, 120 rpm) and the optical density (OD<sub>600nm</sub>) was regularly read.

### 2. Characterization of *P. aeruginosa* biofilms

Based on the phage infection experiments of the planktonic cells, only *P. aeruginosa* ATCC 10145 and PAO 1 strains were used in biofilm assays. Biofilm formation was carried out in 24-well microplates containing 1 ml of Yeast Peptone Dextrose (YPD - 10 g l<sup>-1</sup> Yeast Extract, 20 g l<sup>-1</sup> Peptone, 20 g l<sup>-1</sup> Dextrose) medium and 10 µl of cellular suspension with an optical density (OD<sub>600nm</sub>) of 1.0. Biofilms were formed during 24 hours with medium renewal every 12 hours and the plates were incubated at 37°C in an orbital shaker (120 rpm). The amounts of bacteria attached to the biofilms were determined by CFU counts using microdrop technique. Briefly, the wells of microplates were washed with saline solution (0.9% NaCl) to remove unattached bacteria and then 1 ml of fresh saline solution was added to each well and the biofilm scraped with a cell scraper prior to sonication (5 min). After this, the cellular suspension of each well was removed, centrifuged (5 min, 9,000 × g, 4°C) and the pellet resuspended in 1 ml of saline solution (0.9%). The samples were diluted in saline solution (0.9%) and one drop (10 µl) was placed into a Petri plate containing YPD solid medium and allowed to run down the plate. The plates were incubated at 37°C during 16 to 18 h and after the CFU's were counted.

## 3. Biofilm infection

The biofilm infection was done with two different phages – phiIBB-PAA2 and phiIBB-PAP21. After 24 hours of biofilm formation, all the medium and planktonic bacteria were removed from each well and washed with fresh YPD medium. Following that, 500 µl of fresh YPD medium and 500 µl of phage solution or SM Buffer, in case of control experiments, were added. The multiplicity of infection (MOI) used for the experiments was 1. The plates were incubated in an orbital shaker (120 rpm) at 37°C and samples were taken after 2, 6 and 24 hours of infection for CFU and PFU counts. The CFU counts were determined as previously described by microdrop technique and for PFU counting the Small Drop method described by Mazzoco et al. (2008) was used [13].

## IV. RESULTS

### A. Phage characterization

In the present study, 17 new phages for *P. aeruginosa* were isolated from hospital effluents using three collection strains and a clinical strain which were tested for efficiency against 35 clinical strains. Most phages showed lytic activity against several clinical isolates, even against multi-resistant strains. The least efficient isolated phages were those with the clinical *P. aeruginosa* isolate as host where the percentages of strains infected varied between 17 and 54 percent, while the most efficient phages, the ones with CECT 111 as propagation strain, were capable of lysing approximately 60 to 89 percent of the clinical strains tested (data not shown).

After lytic spectrum assays, one phage for each host was selected for further characterization and also for use in planktonic and biofilm infection experiments. The criteria used for this selection was the number of strains that each phage was able to infect. Accordingly, the selected phages were: phiIBB-PAA2, phiIBB-PAC23, phiIBB-PACL12 and phiIBB-PAP21, respectively. Phages phiIBB-PAA2 and phiIBB-PAP21 were analysed by Transmission Electron Microscopy and were found to belong to the “T7-like” genus of the *Podoviridae* family of phages (Fig. 2A). Phage structural proteins, analyzed by SDS-PAGE (Fig. 2B), showed that both phages phiIBB-PAC23 and phiIBB-PAP21 have a high degree of homology and thus belong also to the “T7-like” genus.

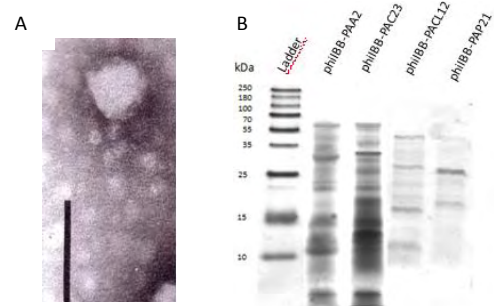


Figure 2. Characterization of phages A) Phage phiIBB-PAA2 observed by Transmission Electron Microscopy (TEM) (bar corresponds to 100 nm); B) SDS-page of structural proteins of *P. aeruginosa* phages phiIBB-PAA2, phiIBB-PAC23, phiIBB-PACL12 and phiIBB-PAP21

## B. Infection of planktonic cells

After phage selection and characterization, phage infection experiments were performed in exponential growing and stationary cells (Fig. 3). There was a clear difference in the infection patterns after infection of cells at the stationary and exponential phases with the 4 different phages. Phage phiIBB-PAA2 appeared to have a greater capacity of infecting *P. aeruginosa* host, followed by phage phiIBB-PAP21. Phage phiIBB-PACL12, although having a broad spectrum of activity in infecting of different isolates, did not cause a reduction of the optical density of neither exponentially growing nor stationary phase cells. This suggests that although phages can be selected based on their broad activity, this doesn't necessarily guarantee their effectiveness in controlling their respective host population. Nevertheless, two of the used phages, phages phiIBB-PAA2 and phiIBB-PAP21, were both efficient towards exponential as well as stationary phase cells and caused a reduction over 50% of the initial optical density ( $OD_{600nm}$ ).

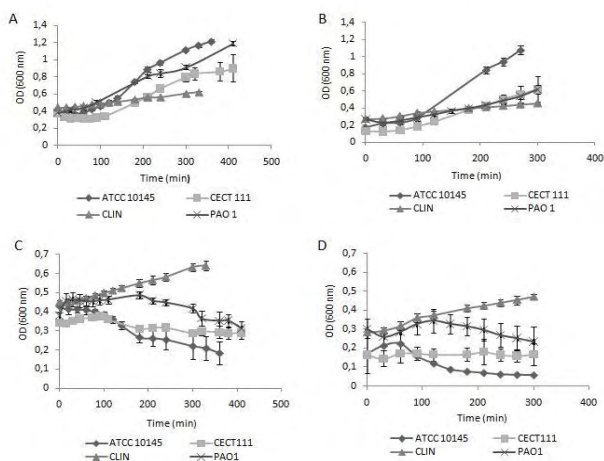


Figure 3. Infection of planktonic *P. aeruginosa* cultures with phages phiIBB-PAA2 (ATCC 10145), phiIBB-PAC23 (CECT 111), phiIBB-PACL12 (CLIN) and phiIBB-PAP21 (PAO 1). A) Control experiments with stationary phase cultures; B) Control experiments with exponentially growing cells; C) Phage infection experiments with stationary cultures; D) Phage infection experiments with exponentially growing cultures.

## C. Biofilm infection

It is expected that the phage infection in planktonic cells would be more efficient than in biofilms due to the biofilm architecture which difficult the access of phage into bacteria [14]. Due to the inefficient activity of phages phiIBB-PACL12 and phiIBB-PAC23 against planktonic cultures, only two phages, phiIBB-PAA2 and phiIBB-PAP21, were chosen for *P. aeruginosa* biofilm infection assays.

Before *P. aeruginosa* biofilm infection, the number of CFU counts of 24 hours biofilms of both strains chosen for this experiment were determined (Fig. 4) and presented similar results.

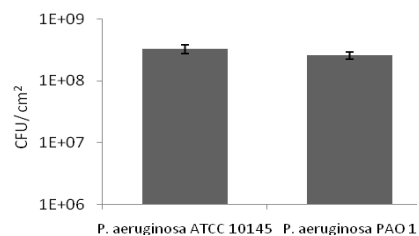


Figure 4. Enumeration of the number of viable cells present in *P. aeruginosa* ATCC and PAO 1 biofilms formed during 24 hours

As previously described, the phage infection assays of *P. aeruginosa* ATCC 10145 and PAO1 were performed in 24 hours old biofilms (Fig. 5).

Both phages, tested against 24 hours old biofilms, caused a significant reduction on the biofilm cells already after 2 hours of infection and the reduction was further enhanced after 6 hours of biofilm treatment reaching a reduction of almost 3 log with phage phiIBB-PAP21 and a 2 log reduction with phage phiIBB-PAA2 (Fig. 5A).

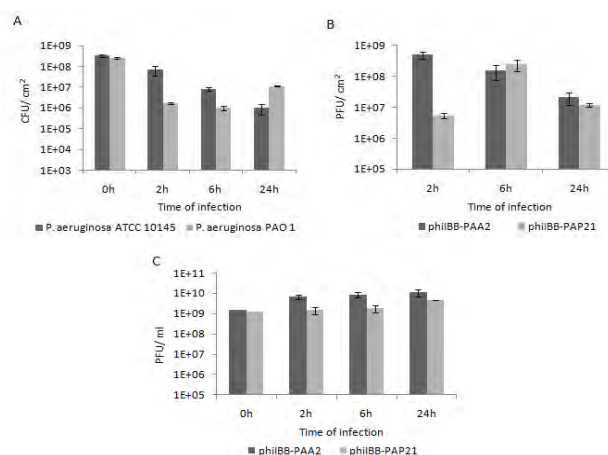


Figure 5. Infection of *P. aeruginosa* ATCC 10145 and PAO1 biofilms with phages phiIBB-PAA2 and phiIBB-PAP21, respectively. A) Number of viable cells before and after infection; B) Number of phages (PFU) attached to the biofilms; C) Number of phages (PFU) released from the infected biofilms.

Despite the lower activity against planktonic cells, phage phiIBB-PAP21 caused a higher reduction of viable cells from the *P. aeruginosa* PAO1 biofilms after 2 and 6 hours of treatment than phage phiIBB-PAA2 in *P. aeruginosa* ATCC 10145 biofilms. Similar reductions of viable cells have been observed for *P. fluorescens* phages [15] and there is only one work in literature where complete eradication of 24 hours *Enterobacter cloacae* biofilms was achieved by a combination of 3 different phages [16]. The main difference between the two phages used in this work for *P. aeruginosa* biofilm control experiments concerns the resistance of the bacteria to the phages. The interaction of phages and bacteria during long periods of time can result in the emergence of phage resistant bacteria [17-18]. Biofilm cells of *P. aeruginosa* PAO1, the host of phage phiIBB-PAP21, acquired resistance to the phage resulting in an increase on the amount of cells after 24 hours of biofilm treatment (Fig. 5A). On the other hand, phage phiIBB-PAA2 continued to destroy biofilm cells of *P. aeruginosa*

ATCC 10145 and there were no evidences of cells becoming resistant, even after 24 hours of phage infection.

One unexpected result obtained was in terms of the number of phages attached to the biofilms (and the surface of the well) measured by plaque forming units (PFU) counts (Fig. 5B). It appears that initially phage phiIBB-PAA2 adsorbed to the biofilms, however after cell lysis the number of phages present in the biofilms decreased by almost 2 logs. Unlike with phage phiIBB-PAA2, the amount of phiIBB-PAP21 phages continued to increase until 6 hours of infection, however, as in phiIBB-PAA2 experiments, there was again observed a significant decrease of the number of phages attached after 24 hours of biofilm cell infection.

Overall, this work revealed the efficacy of phages in cell lysis even against multidrug resistant bacteria. Furthermore, both novel isolated phages were well capable of controlling *P. aeruginosa* biofilms.

## V. CONCLUSIONS

In this study it was possible to conclude that most of the isolated phages have a great lytic capacity against *P. aeruginosa* clinical strains achieving high percentages of lysis.

This study also revealed that phages may be a good alternative to traditional antimicrobials for the control of *P. aeruginosa* biofilms; however, short periods of treatment seem to be a better solution avoiding the emergence of phage resistant hosts.

## VI. PLANNED DEVELOPMENTS

Some alternative approaches can be very interesting for the future work. The combination of phages with chemical agents such as detergents, disinfectants and antibiotics can result in a good option for the eradication of *P. aeruginosa* biofilms from different clinical surfaces. Another alternative strategy involves the use of a cocktail with different phages in order to obtain a solution with a greater range of action. Finally, the genetic manipulation of phages may also be an important future approach in order to obtain phages with broad lytic spectra and also with high ability to control resistant phenotypes.

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