# Biofilm formation under phage predation: considerations about biofilm increase

Submitted to:

*Biofouling*

January 21, 2013

ZEINAB HOSSEINIDOUST[1], NATHALIE TUFENKJI[1] and THEO G.M. VAN DE VEN[2*]

[1]*Department of Chemical Engineering, McGill University, Montreal, Quebec H3A 2B2, Canada*

[2]*Department of Chemistry, McGill University Montreal, Quebec H3A 2K6, Canada*

[*] Corresponding Author. Phone: (514) 398-6177; Fax: (514) 398-8254; E-mail: theo.vandeven@mcgill.ca

## Abstract

Bacteriophages are emerging as a strong candidate for combating bacterial biofilms. However, reports indicating that host populations can in some cases respond to phage predation by an increase in biofilm formation are of concern. In this work, we aimed to investigate whether phage predation can enhance biofilm formation and if so, is this phenomenon governed by the emergence of phage-resistance or non-evolutionary mechanisms (*e.g.* spatial refuge). Single-species biofilms of three bacterial pathogens (*Pseudomonas aeruginosa*, *Salmonella enterica* serotype Typhimurium and *Staphylococcus aureus*) were pre-treated and post-treated with species-specific phages. Some of the phage treatments studied showed an increase in the biofilm levels of their host. We propose the phenotypic change brought about by acquiring phage resistance as the main reason for increase in the level of *P. aeruginosa* biofilms. For *S. aureus* and *S.* Typhimurium biofilms, although resistance formation was detected, increased biofilm formation appeared to be a result of non-evolutionary mechanisms.

**Keywords:** biofilm, spatial refuge, host-parasite, bacteriophage therapy, coevolution, spatial heterogeneity

## 1. Introduction

Biofilms are complex microbial communities established on a wide range of surfaces and are generally encapsulated by an extracellular protective matrix composed of various types of biopolymers (Abee, Kovács et al. 2011). Microscopic investigations have revealed that biofilms develop not only on inert surfaces of medical devices or on dead tissues, but that they can also form on living tissues, as in the case of endocarditis (Kirketerp-Møller, Jensen et al. 2008). Biofilm formation can have important clinical implications particularly in chronic infections (Davies 2003). Biofilms are a cell's survival mechanism, thus cells in biofilm form are more resistant to environmental threats in comparison to their planktonic counterparts (Kirketerp-Møller, Jensen et al. 2008, Donlan 2009). Antibiotic doses used for planktonic cells are usually not enough to tackle biofilms, leading to more resistant subpopulations remaining in the biofilm and recurring infections (Bedi, Verma et al. 2009).

Bacteriophages, bacteria's obligate parasites, have been claimed to be effective in both inhibiting biofilm formation and dispersing mature biofilms. The ability of phage to inhibit and/or eradicate biofilms has been demonstrated for biofilms of *Pseudomonas aeruginosa* (Hanlon, Denyer et al. 2001, Knezevic and Petrovic 2008, Fu, Forster et al. 2010, Ahiwale, Tamboli et al. 2011, Knezevic, Obreht et al. 2011, Pires, Sillankorva et al. 2011), *Klebsiella pneumonia* (Bedi, Verma et al. 2009), *Escherichia coli* (Carson, Gorman et al. 2010), *Proteus mirabilis* (Carson, Gorman et al. 2010), and *Staphylococcus epidermidis* (Curtin and Donlan 2006) amongst others (Lévesque, Duplessis et al. 2005, Cornelissen, Ceyssens et al. 2011, Kelly, McAuliffe et al. 2012). Despite the positive results, there is evidence in the literature that phage predation can potentially lead to an increase in bacterial biofilm levels under certain conditions (Schrag and Mittler 1996, Lacqua, Wanner et al. 2006, Gödeke, Paul et al. 2011, Heilmann, Sneppen et al. 2012), a phenomenon already reported for some antibiotics (Hoffman, D'Argenio et al. 2005).

Different mechanisms are proposed for this observed increase in biofilm formation. The strong selective pressure that virulent phages exert on the host community will eventually lead to the emergence of resistant mutants. These mutants may have altered phenotypes, increasing their

tendency to form biofilms (Lacqua, Wanner et al. 2006). Therefore, whether or not emergence of phage resistance (evolutionary mechanisms) will result in biofilm increase is context-dependent and governed by the phenotypic change resulting from resistance mutations in the host bacteria against a specific phage. Non-evolutionary mechanisms are also believed to play a role in increasing tendency of bacteria to form biofilms. The establishment of coexistence by the presence of a permanent wall population, or a wall effect, is a well-known phenomenon for the co-existence of bacterial species with un-equal fitness (Chao and Ramsdell 1985). Although scarce, there are reports in the literature indicating that forming a wall population stabilizes populations of phage-sensitive bacteria with their virulent phage (Schrag and Mittler 1996, Weiss, Denou et al. 2009, Heilmann, Sneppen et al. 2012); thus lytic phage predation can result in elevated levels of biofilm formation in a bacterial community. Heilmann et al. developed a rigorous numerical model which predicts the wall effect to be a governing factor in stabilizing communities of resistant and sensitive host bacteria with their virulent phage (Heilmann, Sneppen et al. 2012); this suggests the wall effect will affect biofilm formation in any phage-host system. Therefore, we hypothesize that the biofilm controlling ability of phage is governed by the additive, synergistic, antagonistic or suppressive interaction of evolutionary and non-evolutionary mechanisms and virulent phages cannot be deemed suitable for biofilm control solely based on their lytic ability towards the host.

To investigate this hypothesis, single-species biofilms of three bacterial pathogens (Gram - and Gram +) were established and challenged with species-specific phage (administered in two different doses) under two settings: (i) during biofilm growth (pre-treatment) and (ii) after the development of a mature biofilm (post-treatment). To assess the effect of phage-resistance on the population dynamics of bacterial biofilms, we used two different single phage treatments and a mixture of the two phages; the phages were chosen from different families so that their mixture would decrease the frequency of emergence of phage resistance, as confirmed empirically. The biofilm was sampled at various time points to verify the presence of phage-resistant mutants.

## 2. Experimental Procedures

### 2.1. Bacteria culture and phage propagation

Strains used in the study are listed in Table 1. *P.aeruginosa* PAO1 was provided by M. Elimelech (Yale, USA), *S. aureus* was purchased from ATCC (CEDARLANE Laboratories Ltd, Canada), *Salmonella* and all the phages were purchased from Félix d'Hérelle Reference Center for Bacterial Viruses (Universite Laval, Canada).

For bacterial culture, an inoculum from frozen glycerol stock was streaked onto a Trypticase soy agar (TSA) plate and incubated overnight at 37°C. A single colony from the plate was inoculated into 10 mL of Trypticase soy broth (TSB), and incubated overnight (37°C, 120 rpm) from which a 200 µL aliquot was diluted 1:100 in fresh TSB and grown to an $OD_{600}$ of 0.2-0.3. Soft agar overlay technique was then used to propagate all phages in this study as described elsewhere (Kropinski, Mazzocco et al. 2009). Quantifying the frequency of emergence of phage resistance
The decrease in emergence of resistant mutants by using a phage mixture was confirmed by quantifying frequency of formation of resistant bacteria for pure phage and phage mixture treatments using the method described by Carlson (Carlson 2004). Briefly, phage was mixed with its host (phage to bacteria ratio of $10^3$-$10^4$) and spread on an LB-agar plate. The number of bacterial colonies that grew after 24 hr incubation at 37°C were divided by the number of colonies of the control (bacteria not mixed with phage) to calculate the frequency of resistance.

### 2.2. *In vitro* model for biofilm development

The microtiter plate assay (Merritt, Kadouri et al. 2011) was used to grow and study the biofilms. Each bacterial species was grown overnight in TSB (37°C, 120 rpm) from a single colony on agar plates of no more than three days old. This culture was diluted 1:100 in TSB and loaded into the wells of five untreated 96-well polystyrene flat-bottomed microtiter plates (Costar; Corning Inc., Corning, NY, USA) in two rows. The plates were then incubated at 37°C and shaken gently to limit the effect of chemical and phage gradients on the system. The biofilms were pre-treated or post-treated with phage as will be explained in the following sections. At each of the five specific time points throughout the 72 hr course of the experiment, one multiwell plate was destructively treated

for biofilm and phage quantification and phage resistance testing. First, the planktonic bacteria in the wells were transferred to a fresh plate and their optical density at 600 nm was measured before being discarded. Next, the wells were washed twice with phosphate buffered saline (PBS) and either sampled for resistance testing or stained with 1% crystal violet for quantification of the total attached biomass. The $OD_{570}$ nm value of the anhydrous ethanol subsequently used to dissolve the crystal violet was used as a quantitative measure of the biofilm level. The titer of phage in the supernatant was determined at each time point by using the soft agar overlay technique. The experiments described below were all performed in triplicates. To limit the effect of resource limitation, every 24 hrs, half of the media in each well was replaced with fresh TSB without disturbing or washing the biofilm.

**Choosing phage to bacteria ratios**-To select the appropriate phage to bacteria ratios (multiplicity of infection: MOI), phage was added to a bacterial inoculum with MOIs ranging from $10^{-7}$ to $10^{5}$ (Figure S1a) and the biofilm was grown for 12 hrs. The MOI at which 50% or more biofilm inhibition was observed after 12 hrs was chosen as the 50% inhibitory phage dose. For comparison, an MOI 1/100 of this 50% inhibitory dose was also tested.

**Inhibition of biofilm formation using phage (pre-treatment)**-To investigate the ability of phage to inhibit biofilm formation, phage was added to the wells of the multiwell plate simultaneously with the bacterial inoculum at time zero with two MOIs (chosen as explained above). Three replicate wells were used for each phage treatment. Phage buffer (saline-magnesium-gelatin buffer: NaCl 5.8 g/L, $MgSO_4$ 0.96 g/L, 1 M Tris 50 ml, gelatin 0.1 g/L, pH 7.5) was added to the control wells (three replicate wells). For each bacterial species under study, three phage treatments were used, two single phage treatments (phage 1 and phage 2 in Table 1) and one mixed phage treatment (1:1 mixture of both phages 1 and 2 in Table 1). Each treatment was applied to three wells in each row. The biofilm was allowed to develop in the presence of phage for a specified time period (8, 12, 14, 48 or 72 hrs), after which the biofilm was quantified as explained above.

**Challenging mature biofilm (post-treatment)**-The efficacy of phage to eradicate a mature biofilm was investigated by challenging a 48 hr biofilm with single and mixed phage treatments. The biofilm

was prepared as described before and allowed to develop for 48 hrs, after which the planktonic bacteria were removed, the wells were washed 3 times with PBS and phage was added to the wells with two MOIs (chosen as explained above); three wells were used for each phage treatment. Addition of phage was chosen as time zero for phage post-treatment. Phage buffer was added to the three control wells in each row. The biofilm was challenged with the phages for a specified time period (8, 12, 14, 48 and 72 hrs), after which the biofilm level was quantified. To calculate the amount of phage needed to add to each well for obtaining a target MOI for biofilm post-treatment, the amount of biofilm development for each bacterial species after 48 hrs was quantified by scraping the biomass off the wells and performing CFU counts.

### 2.3. Characterization of phage-resistant biofilm isolates and verifying for the presence phage host range mutants

Several colonies of biofilm bacteria were recovered from biofilms at each specific time point(8, 12, 14, 48 and 72 hrs) during the course of the study. Biofilm samples were collected before the crystal violet quantification step (after the washing step) using an inoculating needle. The samples were streaked on an LB-agar plate and 10 single isolated colonies were chosen from each biofilm. The susceptibility of each isolate to the phage by which it was challenged was determined by the spot test. A 1 µL aliquot from the liquid culture of each variant ($OD_{600}$ 0.2-0.3) was mixed with 2 mL of 0.5% LB-agar and spread on a 5 cm 1.5% LB-agar plate. The phage ($10^9$ pfu/mL) was spotted (1 µL) on the soft agar layer, incubated at 37 °C overnight and inspected for plaque formation. Colonies that were found to be resistant to the ancestral form of their corresponding phage were further verified for contamination via PCR with species primer pairs directed against 16S rDNA (Table 1). The primers were designed according to the conserved regions reported in the literature for each species (Gürtler and Barrie 1995, Lin and Tsen 1996, Spilker, Coenye et al. 2004). Furthermore, at each time point, 100 µL of cell-free (filter sterilized) supernatant from the wells was collected. This supernatant was examined for its ability to form plaques on the resistant bacteria recovered at the same time point using the spot test assay explained above. Plaque formation indicated the presence of phage host range mutants in the supernatant.

### 2.4. Statistical analysis

All assays were repeated a minimum of three times independently with triplicates for each sample in each experiment. Results are reported in terms of means + 95% confidence interval. Significance of the difference between biofilm levels was analyzed using Student's t-test, (Statistica 8.0, Stat Soft. Inc., San Jose, CA) and p-values < 0.05 were considered significant.

## 3. Results

Appropriate MOIs for biofilm treatment were chosen via the protocol described before to be 10 (50% inhibitory MOI) and 0.1. Figure S1b is representative of the data obtained for the MOI selection experiment. Furthermore, the frequency of resistance for the phages used in this work were determined and compared to the frequency of resistance for the phage mixture. Using a phage mixture decreased the resistance by 1/100 for *P. aeruginosa* and *S.* Typhimurium and 1/10 for *S. aureus* thus confirming that the phage mixtures chosen can decrease the frequency of resistance.

### 3.1. Effect of phage and phage mixtures on biofilm inhibition (biofilm pre-treatment)

The effect of phage on the load of planktonic bacteria (as indicated by $OD_{600}$) and total sessile biomass (as indicated by $OD_{570}$) is presented in Figure 1 for MOI of 10 and Figure S2 for MOI=0.1. The $OD_{570}$ values for all biofilms were divided by the planktonic growth level ($OD_{600}$) to compensate for differences in growth rate as described in the literature (Dunne Jr 1990, Deighton and Borland 1993, Kristich, Li et al. 2004, Pérez-Giraldo, Gonzalez-Velasco et al. 2004, Frank, Reichert et al. 2007, Ma and Wood 2009, Yang, Frey et al. 2010, Pompilio, Pomponio et al. 2011, Sayem, Manzo et al. 2011). Furthermore, all the values for planktonic and biofilm levels were presented as fold change relative to the level of the control. For reference, the un-normalized values for $OD_{570}$ and $OD_{600}$ are presented in Figures S3 and S4 for phage pretreatment and Figure S5 for phage post-treatment. Samples from the biofilm were taken at each time point and tested for phage susceptibility using spot test. The percentage of resistant biofilm samples are compiled in Table 2. The resistant cells were further verified for contamination using PCR and spot tested with an aliquot of the bacteria-free supernatants from the planktonic culture in the wells to check for the presence of

phage host range mutants that could infect the resistant cells. Phage titer counts were performed at each time point for planktonic bacteria in the wells. Representative phage titers are presented for phage mixture treatments at MOI=10 in Figure S6. Other phage treatments follow the same trend and are therefore not included.

*P. aeruginosa* biofilms pre-treated with phage E79 maintained levels significantly lower than the control for up to 24 hrs (MOI=10) and 12 hrs (MOI=0.1) but there was no statistically significant difference from the control at other time points (Figure 1-a and S2-a). The planktonic growth for this system remained significantly below the planktonic growth of the control for up to 48 hrs (MOI=10) and 24 hrs (MOI=0.1) (Figure 1-b and S2-b). Phage PP7 did not slow planktonic growth at both high and low MOIs for more than 8 hrs (Figures 1-b and S1-b), however biofilms pre-treated with this phage maintained levels significantly below the control at most time points (Figure 1-a and S2-a). The phage mixture maintained the biofilm levels lower than the control for up to 24 hrs for both MOIs but the planktonic growth in the same system was maintained significantly below the control levels for a longer time period of up to 72 hrs for MOI=10. On average, 90% of the colonies isolated from *P. aeruginosa* biofilms pre-treated with E79 and 70% of those isolated from biofilms pre-treated with phage mixture were resistant to the ancestral forms of phages they were treated with (Table 2). This figure dropped to less than half for biofilms treated with PP7. The phage titer decreased initially for all systems but did not decrease significantly throughout the experiment (Figure S6-a). Cell-free supernatants formed plaques on lawns of all resistant colonies indicating the presence of phage host range mutants capable of lysing host cells resistant to the ancestral form of phage. For the objectives of this study we did not isolate or quantify these mutants.

For *S. aureus* (a representative Gram+ organism), the phage mixture and bacteriophage K maintained the planktonic bacterial load below the control levels at MOI=10 (Figure 1-d). However, none of the treatments were found to be effective in decreasing the biofilm load for *S. aureus*; the biofilms pre-treated with phage reached 4-6 times the control levels between 12-48 hrs for different phage treatments (Figure 1-c). This rise was more gradual for the phage mixture treatment, and ultimately

at 72 hrs, the biofilm levels of all three systems were comparable to that of the control (Figure 1-c). The $OD_{570}$ value was relatively low for all samples at 72 hrs for MOI 10 (Figure S2-c), whereas for MOI 0.1 (Figure S2-d), the phage mixture resulted in enhanced biofilm levels at 72 hrs. Figure S2 also indicates that *S. aureus* formed less biofilms at all time points compared to *P. aeruginosa*. Biofilms pre-treated with phage at MOI=0.1 also showed significant increase above the control levels (Figure S2-c), except biofilms pre-treated with bacteriophage K (MOI=0.1), which did not attain levels significantly different from the control. In case of low phage load (MOI=0.1), the planktonic load was only significantly less than the control for 8 hrs (Figure S2-d) after which the variability of the data led to increase in p-values above the significance threshold. Less than 50% of the colonies isolated from treated *S. aureus* biofilms were resistant to the ancestral form of phages they were treated with (Table 2). Phage titer decreased 2 logs when the maximum biofilm levels were observed for each treatment and eventually increased (Figure S6-b).

Biofilms of *S.* Typhimurium pre-treated with phage at MOI=10 maintained levels significantly less than the control only for 12 hrs (treated with P22) and 24 hrs (treated with PRD1), as shown in Figure 1-e, after which the difference was not statistically significant with one exception; for P22-treated biofilm at 72 hrs the biofilm levels were significantly higher than the control (Figure 1-e and S2-e). P22 had no significant effect on the level of planktonic growth after 8 hrs (Figure 1-f) but PRD1 and the phage mixture maintained a significant difference from the control for up to 48 hrs (PRD1) and 72 hrs (phage mixture). The phage mixture did not seem to affect the biofilm levels for this system (Figure 1-e). For MOI=0.1, biofilm levels were only significantly less than the control at 12 hrs (Figure S2e). Again, less than 50% of the colonies isolated from treated *S.* Typhimurium biofilms were resistant to the ancestral forms of the phages they were treated with (Table 2). Phage titers decreased at 24 hrs for all treatments then increased at 48 hrs and eventually reached values ~1 log less than the initial values at 72 hrs (Figure S6c). Presence of phage host range mutants was confirmed by the ability of cell-free supernatants to form plaques on lawns of all the isolated colonies at all time points for both *S. aureus* and *S.* Typhimurium.

### 3.2. Effect of phage and phage mixtures on biofilm eradication (biofilm post-treatment)

To assess the effect of phage on a fully developed biofilm, 48 hr biofilms of each bacterial species were challenged with phage and the planktonic and sessile bacterial loads were monitored with time for up to 72 hrs. Changes in the biofilm level are presented in Figure 2 and the corresponding planktonic bacterial load is presented in Figure S7 for reference. *P. aeruginosa* biofilm challenged with phage PP7 exhibited significantly lower biofilm levels after 48 hrs of phage challenge (Figure 2-a and 2-b). Biofilms challenged with E79 attained values 3-4 times that of the control after 24 hrs of phage challenge after which the biofilm level remained slightly below (but not significantly different from) control levels. Biofilm challenged with the phage mixture only maintained levels significantly lower than the control for 8 hrs after which there was no statistically significant difference from the control except for the biofilm challenged with phage mixture for 24 hrs at MOI=10 which exhibited values larger than the control (Figure 2a). Interestingly, the planktonic load was kept below the control levels for up to 12 hrs by all phage treatments but did not show a significant difference with the control beyond this point (Figure S7c and d). As before, most of the colonies isolated from treated *P. aeruginosa* biofilms treated with E79 or with phage mixture were resistant to the ancestral forms of phages they were treated with. This figure dropped to less than half for biofilms treated with PP7 (Table 2).

For *S. aureus* biofilms, single phage treatments at MOI=10 resulted in biofilm levels 5-6 times higher than that of the control at 24 hrs after which the difference with the control was not statistically significant (Figure 2c). The same trend was observed for MOI=0.1 (Figure 2d); however, due to the high variability of the results, the p-values exceeded the significance threshold. The planktonic bacterial load in contact with these biofilms did not change significantly from that of the control in most cases (Figure S7c and d). It is noteworthy that from Figure S5, *S. aureus* control biofilm had an $OD_{570}$ of ~2 after 24 hrs of phage treatment(72 hrs of biofilm growth), but Figure S4 showed values close to 0.5 after 72 hrs. As explained previously, the 48 hrs biofilm was completely washed to remove waste products and planktonic cells and new media was added to the wells. This additional treatment could have resulted in the increase observed in Figure S5 compared to Figure S4 as previously reported in the literature (Jackson, Suzuki et al. 2002). The other explanation could be

the effect of phage challenge; the sensitive host population, once shocked by the addition of phage, could seek refuge in the form of biofilm to evade lysis by phage.

Level of *S.* Typhimurium biofilm was not decreased significantly lower than the control levels for any of the phage treatments at MOI=10 (Figure 2e). Biofilms challenged with P22 and PRD1 showed significantly higher biofilm levels than the control after 72 hrs of phage challenge. For MOI=0.1, biofilm levels significantly decreased below the control levels after 8 hrs of phage challenge with PRD1 and the phage mixture (Figure 2f). These levels rose above the control with increased incubation time (although not statistically significant) and subsequently decreased significantly below the control levels at 48 hrs and 72 hrs (Figure 2f). The level of planktonic bacteria did not change significantly from the control (Figure S6e and f). As was the case for phage pre-treatment, less than half of the isolated colonies from *S. aureus* and *S.* Typhimurium biofilms were resistant to their corresponding phage. Moreover, most of the spikes in biofilm levels corresponded to a decrease in phage titer, as observed for biofilm pre-treatment (Figure S6b and c).

## 4. Discussion

In this work, we investigated the effect of phage predation on bacterial biofilms for three different bacterial species. We studied the effect of single phage treatments and treatments with a phage mixture on both inhibition of biofilm formation and eradication of developed biofilms. Most of the systems studied were effective in inhibiting/removing biofilm significantly below the control levels for only up to 12-24 hrs; the PP7–*P. aeruginosa* system was effective beyond this time for up to 72 hrs. The important observation in this study was that for some systems, the biofilm level increased above that of the control (e.g., *S. aureus* pre- and post-treated) even though the level of planktonic bacteria remained less than or equal to the control level at all times. Thus, it appeared that phage predation increased the tendency of bacteria to form biofilms for certain phage-host systems. Furthermore, the use of a phage mixture generally did not prove to be superior for biofilm control. Since the phage mixture was shown to decrease the frequency of resistance in the bacterial host, this suggests that emergence of phage resistance was not the main phenomenon limiting the efficiency of phage for biofilm control in the systems under study. Moreover, phages can mutate to produce

phage strains that are lytic towards the resistant host cells (phage host-range mutants). When the bacterial host mutates, selection will favor phages that are capable of killing and replicating in these bacteria and thus evolved phages have a selective advantage (Comeau and Krisch 2005). Phage host-range mutants were present in all the systems under study (indicated by plaque formation by bacteria-free supernatant on phage-resistant biofilm isolates). This also suggests that phage-resistance may not be the main factor limiting phage efficiency.

Self-organized bacterial refuges are believed to be one of several possible non-evolutionary mechanisms which stabilize systems of phage-sensitive and phage-resistant bacteria with their virulent phage (Schrag and Mittler 1996, Heilmann, Sneppen et al. 2012). The spatial refuge hypothesis predicts that physical heterogeneity in the environment protects some sensitive bacteria from phage infection and thus is a factor in stabilizing predator-prey communities (Van den Ende 1973, Bohannan and Lenski 2000, Brockhurst, Buckling et al. 2006). One of the implications of this theory is that the stability (co-existence without either being driven to extinction) in a bacteria-phage community will increase when opportunities for biofilm growth on solid substrates increases. Contrary to initial belief, it has been demonstrated that being in a biofilm does not form a physical barrier against phage (Adams and Park 1956, Hughes, Sutherland et al. 1998, Hughes, Sutherland et al. 1998, Hanlon, Denyer et al. 2001, Briandet, Lacroix-Gueu et al. 2008). Therefore, the spatial refuge theory could not fully explain the increase in biofilm formation especially since we kept the system otherwise homogeneous by constant shaking. However, the change in the metabolism level brought about by the change from planktonic to biofilm mode has been shown to decrease the rate of propagation of phage (Hanlon, Denyer et al. 2001, Cerca, Oliveira et al. 2007). If the cells in biofilm mode are no longer able to support phage amplification to high densities, then acquiring a biofilm phenotype could be a means for "physiological refuge" rather than just a means for spatial refuge for phage-sensitive host cells. Biofilms could also be a mode of numerical refuge for the cells; since biofilms occupy only a small fraction of the total environmental volumes, entering into a biofilm state would reduce the density of the host in the medium, thus potentially decreasing exposure to phage (Chao and Ramsdell 1985, Abedon 2012). Heilmann et al. developed a model to predict the effect of refuges on the stability of phage-host systems (Heilmann, Sneppen et al. 2012). They proposed bacterial density-dependent, or

quorum-sensing, mechanisms such as the formation of biofilm to produce refuges required for the sensitive bacteria to achieve coexistence with phage in short term, giving them time to evolve resistance which will guarantee their stability in the long term (Heilmann, Sneppen et al. 2012). A somewhat different view on this subject was recently presented by Abedon (Abedon 2012). He argued that the cost of being exposed to phage would be higher if bacterial cells are physically associated to each other since the progeny phage from infection of one host cell can immediately infect many others. He proposed a model that predicts entering into multicellular bacterial communities is only beneficial for the host if the pressure from phage predation is low (Abedon 2012). Although this model does not take into account the reduced level of metabolic activity of the cells in a biofilm, it correlates surprisingly well with some of our results and could explain situations where phages enhanced biofilm formation and then biofilms decreases significantly, as in the case with *S. aureus*. In most cases of biofilm increase above the control levels, phage titers were observed to have decreased (Figure S3). We hypothesize that this decrease in titer is a result of emergence of resistant cells which means the host population could not maintain high phage titers. According to Abedon's model, this decrease in phage titer would result in an increase in biofilm formation to stabilize host populations. As phage coevolves and phage host range mutants develop, the phage titers rise and biofilm levels decrease which is again predicted by Abedon's model under high phage pressure. In the following section, we have looked at each biofilm system separately and aimed to explain the observed response to phage predation based on one or a combination of the models presented above.

### 4.1. Interaction of *P. aeruginosa* biofilms with phage

In the case of *P. aeruginosa* biofilms, phage PP7 failed to inhibit planktonic growth but led to a significant decrease in biofilm levels. It is known that one of the main mechanisms of phage resistance is envelope modification by blocking or modifying the phage receptors on the host cell (Labrie, Samson et al. 2010). PP7 is a pili specific phage (Bradley 1972) and thus we hypothesize that cells resistant to this phage may have either lower pili synthesis levels or mutations affecting pilin protein such that it cannot bind to the phage. Twitching motility is a mode of surface translocation completely dependent on the pili (Bardy, Ng et al. 2003). PP7-resistant cells have been previously observed to have lower

twitching motility (Hosseinidoust, Tufenkji et al. 2013); although these resistant cells were not obtained from the same biofilm model, this observation nonetheless confirms our hypothesis. Pili are also principal adhesins mediating the adherence of cells to surfaces (Bardy, Ng et al. 2003) and they play an important role in the formation of *Pseudomonas* biofilms (Barken, Pamp et al. 2008). If the pili are deactivated by any mechanism, then biofilm formation is expected to decrease. Therefore, it is possible that the significant efficacy of PP7 for biofilm inhibition and eradication is a result of bacteria developing resistance to this phage. The phage resistant cells would still persist in planktonic form which explains why a reduction in $OD_{600}$ value was not observed. The observation that less than half of the isolates from PP7 treated biofilms were resistant to this phage further confirms that PP7-resistant cells do not favor biofilm formation.

Phage E79 binds to lipopolysaccharide (LPS) on the cell surface (Jarrell and Kropinski 1977). Functional LPS is required for *P. aeruginosa* swarming motility. There is evidence of a decrease in swarming motility for E79-resistant *P. aeruginosa* cells (Hosseinidoust, Tufenkji et al. 2013). Although these resistant cells were isolated from a different system, it is reasonable to hypothesize that such E79-resistant *P. aeruginosa* cells could have altered LPS. Furthermore, E79-resistant *P. aeruginosa* cells were shown to have decreased swimming motility (Hosseinidoust, Tufenkji et al. 2013). Therefore, it follows that they could become sessile more readily. This could explain the increase in biofilm formation above the control levels for biofilms challenged with E79, despite the relatively effective control of planktonic growth by this phage. Most of the isolated colonies from *P. aeruginosa* biofilms pre-treated or post-treated with E79 were resistant to this phage which further confirms our hypothesis that E79 resistant cells have a higher tendency to form biofilms. The opposing effects of selective pressures from phages PP7 and E79 on the phenotype of the resistant cells could explain why the phage mixture was not the best choice for inhibiting/eradicating *P. aeruginosa* biofilm in this study.

The other noteworthy observation for this system was that *P. aeruginosa* biofilm levels increased above control levels when challenged for 24 hrs with E79 and the phage mixture (Figure 2-a, b), but the biofilm levels decreased with further incubation at 48 hrs and 72 hrs. The observed decrease in biofilm levels could in part be attributed to the usual biofilm dispersion being reached earlier because of the

rapid increase of biofilm levels and possibly greater accumulation of waste products. It could also be attributed to phage co-evolution resulting in the emergence of host range mutants that can lyse the E79-resistant cells in biofilm mode.

### 4.2. Interaction of *S. aureus* biofilms with phage

*S. aureus* biofilms pre-treated or post-treated with phage did not exhibit biofilm levels lower than the control; in many instances, the biofilm levels were significantly higher than the control biofilm levels. However, it is important to note that *S. aureus* biofilm levels under control conditions were generally quite low which could be a result of the specific biofilm model chosen for this study. It appears that in the absence of phage, *S. aureus* did not exhibit the tendency to form biofilms, yet in the presence of phage biofilm formation was induced. The general pattern observed for phage treated biofilms was a significant increase above the biofilm levels of the control after which the biofilm level decreased eventually to the same levels as the control. The increase in biofilm levels was accompanied by a decrease in phage titers and less than 50% of the colonies isolated from the biofilms were resistant to the ancestral form of the phages they were treated with. This suggests that biofilm formation was not solely a result of phage resistance but rather a refuge for phage-sensitive cells. Based on the fact that the system is kept homogeneous and that in general the biofilm matrix does not form a physical barrier against phage action, we hypothesize that the apparent accumulation of *S. aureus* cells in biofilm mode protects the sensitive cells by providing physiological and numerical refuge.

Another important observation for this system was the high efficacy of bacteriophage K and the phage mixture to decrease planktonic growth at MOI=10 (Figure 1d), whereas the same system had significantly higher biofilm levels compared to the control. Phage 44AHJD did not decrease the planktonic growth compared to the control but it had the same effect on increasing the biofilm level as the two other phage treatments. An increase in biofilm above control levels was also observed during pre-treatment with MOI=0.1; although for this dose of phage no significant effect was observed on planktonic growth. Thus, the phage-to-bacteria ratio affects the planktonic growth as expected but higher MOIs do not result in less biofilm formation for this system. Furthermore, using the phage mixture did not appear to offer any advantage for inhibiting or eradicating *S. aureus* biofilms.

### 4.3. Interaction of *S.* Typhimurium biofilms with phage

*S.* Typhimurium biofilms pre-treated or post-treated with phage generally had a biofilm level comparable to that of the control at 8 hrs. The biofilm levels then decreased (with the exception of post-treatment with MOI=0.1) to levels lower than the control after which they eventually increased (although not monotonically) to reach the same levels as the control and in some cases even increased above the control. The biofilm response to phage predation in this system could be explained by the constant arms race between the phage and its host leading to the emergence of phage host range mutants that can infect the resistant cells thus decreasing the biofilm levels. The biofilm level increased with the emergence of resistance to the evolved phage which results in another round of phage coevolution and thus a decrease in biofilm levels. This theory is further confirmed by the phage titer which exhibits a zig-zag pattern comparable to that observed for the biofilm levels (Figure S5-c).

For the biofilms post-treated with phage at MOI=0.1 (Figure 2-f), a different pattern was observed; biofilm levels initially increased above the control levels (albeit with high variation manifested by the error bars) after which the biofilm level decreased significantly below the control levels. This decrease could be attributed to biofilm dispersal resulting from an increase in the sessile biomass and an increase in waste products. Because the phage titer did not increase significantly in this system, the biofilm decrease could not be attributed to phage action.

Biofilm levels increased significantly above control levels at 72 hrs for biofilms pre-treated with P22 and post-treated with P22 and PRD1 (single phage treatments) with MOI=10. It is not clear whether increasing the incubation time above 72 hrs would have led this biofilm level to decrease or not. The biofilm increase at 72 hrs cannot be explained by any of the previously mentioned theories. However, based on Abedon's model (Abedon 2012) a decrease in phage titers would lead the system to form multicellular structures to stabilize itself in the presence of phage. We observed the phage titer to decrease for these systems by one log at 72 hrs thus our results for these systems correlate well with the predictions of Abedon's model. Interestingly, the planktonic bacterial load increases monotonically for all phage treatments and is always less than or equal to the control levels. There are other reports suggesting the DNA released as a result of lysis could increase biofilm formation (Gödeke, Paul et al.

2011). Thus, the increase in biofilm levels could also be attributed to the accumulation of extracellular DNA as a result of phage lysis.

### 4.4. Concluding remarks

It has been proposed that lytic bacteriophages could become the new class of anti-biofilm agents (Donlan 2009). We hypothesize that the biofilm controlling ability of phage is governed by the additive, synergistic, antagonistic or suppressive interaction of evolutionary and non-evolutionary mechanisms and virulent phages cannot be deemed suitable for biofilm control solely based on their lytic ability towards the host. Based on the results of this study, the effect of phage treatment on biofilms is context-dependent and it is governed by a combination of evolutionary and non-evolutionary mechanisms. It is important to be aware that phages that show a plausible lytic effect against their host bacteria may not always be the best choice for biofilm control (*e.g.* E79-*P. aeruginosa*) since the phenotypic changes resulting from phage-resistance mutations in the host cell could result in increased biofilm formation. The same evolutionary mechanism (selection for phage-resistant mutants) could prove advantageous if the right type of phage is chosen, as was observed for the PP7-*P. aeruginosa* system; although phage-resistance developed readily, the resistant cells lacked the ability to form stable biofilms. Thus, phenotypic changes resulting from phage-resistance mutations are an important design parameter when choosing phages or phage mixtures for biofilm control.

This study provides strong evidence suggesting that resistance formation is not the main factor limiting system efficiency (and in some cases increasing the biofilm level above the control levels) for biofilms of *S. aureus* and *S.* Typhimurium. We attempted to decrease the effect of phage resistance by using phage mixtures designed for this purpose. Phage host-range mutants were also found to be present in all the systems under study, yet neither seemed to affect the efficiency of the system. This suggested that non-evolutionary mechanisms played a governing role in phage-biofilm population dynamics in the aforementioned systems. Seeking refuge in the form of a biofilm to decrease their metabolic activity (and thus ability to support phage propagation) and also decrease their volume fraction in the medium is believed to be a means of acquiring this mode of coexistence for phage-

sensitive bacteria. It is noteworthy that resource limitation as a result of using a static biofilm model will undoubtedly affect the results of this study. We sought to limit this effect by replacing half of the medium in each well with fresh TSB every 24 hrs; however, we acknowledge that the results obtained using this biofilm model may not be valid for other models and it would be interesting to confirm the effects in other biofilm models such as a continuous-culture flow cells.

This study also emphasizes that new techniques may be needed to further reduce the evolution of phage-resistant bacteria. These include using phages in combination with other antimicrobials, such as antibiotics, cycling through different phage mixtures, and engineering phages to directly target phage-resistance mechanisms (Labrie, Samson et al. 2010). Another option would be the use of phage lysins for which resistance development has not been reported (Fenton, Ross et al. 2010). One question that also deserves more study is whether the evolution of phage resistance *in vitro* is relevant to *in vivo* conditions where bacteria may be replicating more slowly and challenged with a greater set of environmental conditions (Capparelli, Parlato et al. 2007, Lu and Koeris 2011) and also whether the observed trends in this study are reproducible in other biofilm models (e.g. chemostats). Finally, our work highlights the importance of a mechanistic understanding of population and evolutionary biology of bacteria–phage interactions for the successful development of phage for biofilm control.

## Acknowledgments

This work is supported by the Natural Sciences and Engineering Research Council of Canada (NSERC Strategic Research Network on Bioactive Paper-SENTINEL) and the Canada Research Chairs (CRC) program. We thank M. Elimelech (Yale University) for providing *P. aeruginosa* PAO1, and C. O'May (McGill University) for technical advice and valuable feedback on the manuscript.

## References


Abedon, S. T. 2012. "Spatial vulnerability: Bacterial arrangements, microcolonies, and biofilms as responses to low rather than high phage densities." Viruses **4**(5): 663-687.

Abee, T., A. T. Kovács, O. P. Kuipers and S. van der Veen 2011. "Biofilm formation and dispersal in Gram-positive bacteria." Current Opinion in Biotechnology.

Adams, M. H. and B. H. Park 1956. "An enzyme produced by a phage-host cell system. II. The properties of the polysaccharide depolymerase." Virology **2**(6): 719-736.

Ahiwale, S., N. Tamboli, K. Thorat, R. Kulkarni, H. Ackermann and B. Kapadnis 2011. "In vitro management of hospital *Pseudomonas aeruginosa* biofilm using indigenous T7-like lytic phage." Current Microbiology **62**(2): 335-340.

Bardy, S. L., S. Y. M. Ng and K. F. Jarrell 2003. "Prokaryotic motility structures." Microbiology **149**(2): 295-304.

Barken, K. B., S. J. Pamp, L. Yang, M. Gjermansen, J. J. Bertrand, M. Klausen, M. Givskov, C. B. Whitchurch, J. N. Engel and T. Tolker-Nielsen 2008. "Roles of type IV pili, flagellum-mediated motility and extracellular DNA in the formation of mature multicellular structures in *Pseudomonas aeruginosa* biofilms." Environmental Microbiology **10**(9): 2331-2343.

Bedi, M. S., V. Verma and S. Chhibber 2009. "Amoxicillin and specific bacteriophage can be used together for eradication of biofilm of *Klebsiella pneumoniae* B5055." World Journal of Microbiology & Biotechnology **25**(7): 1145-1151.

Bohannan, B. J. M. and R. E. Lenski 2000. "Linking genetic change to community evolution: Insights from studies of bacteria and bacteriophage." Ecology Letters **3**(4): 362-377.

Bradley, D. E. 1972. "Shortening of *Pseudomonas aeruginosa* pili after RNA-phage adsorption." Journal of General Microbiology **72**(2): 303-319.

Briandet, R., P. Lacroix-Gueu, M. Renault, S. Lecart, T. Meylheuc, E. Bidnenko, K. Steenkeste, M. N. Bellon-Fontaine and M. P. Fontaine-Aupart 2008. "Fluorescence correlation spectroscopy to study diffusion and reaction of bacteriophages inside biofilms." Applied and Environmental Microbiology **74**(7): 2135-2143.

Brockhurst, M. A., A. Buckling and P. B. Rainey 2006. "Spatial heterogeneity and the stability of host-parasite coexistence." Journal of Evolutionary Biology **19**(2): 374-379.

Capparelli, R., M. Parlato, G. Borriello, P. Salvatore and D. Iannelli 2007. "Experimental phage therapy against *Staphylococcus aureus* in mice." Antimicrobial Agents and Chemotherapy **51**(8): 2765-2773.

Carlson, K. (2004). Working with Bacteriophages. Bacteriophages, CRC Press.

Carson, L., S. P. Gorman and B. F. Gilmore 2010. "The use of lytic bacteriophages in the prevention and eradication of biofilms of *Proteus mirabilis* and *Escherichia coli*." FEMS Immunology and Medical Microbiology **59**(3): 447-455.

Cerca, N., R. Oliveira and J. Azeredo 2007. "Susceptibility of *Staphylococcus epidermidis* planktonic cells and biofilms to the lytic action of *Staphylococcus* bacteriophage K." Letters in Applied Microbiology **45**(3): 313-317.

Chao, L. and G. Ramsdell 1985. "The effects of wall populations on coexistence of bacteria in the liquid phase of chemostat cultures." Journal of General Microbiology **131**(5): 1229-1236.

Comeau, A. M. and H. M. Krisch 2005. "War is peace - dispatches from the bacterial and phage killing fields - Commentary." Current Opinion in Microbiology **8**(4): 488-494.

Cornelissen, A., P. J. Ceyssens, J. T'Syen, H. van Praet, J. P. Noben, O. V. Shaburova, V. N. Krylov, G. Volckaert and R. Lavigne 2011. "The t7-related *Pseudomonas putida* phage φ15 displays virion-associated biofilm degradation properties." PLoS ONE **6**(4).

Curtin, J. J. and R. M. Donlan 2006. "Using bacteriophages to reduce formation of catheter-associated biofilms by *Staphylococcus epidermidis*." Antimicrobial Agents and Chemotherapy **50**(4): 1268-1275.
Davies, D. 2003. "Understanding biofilm resistance to antibacterial agents." Nature Reviews Drug Discovery **2**(2): 114-122.
Deighton, M. and R. Borland 1993. "Regulation of slime production in Staphylococcus epidermidis by iron limitation." Infection and Immunity **61**(10): 4473-4479.
Donlan, R. M. 2009. "Preventing biofilms of clinically relevant organisms using bacteriophage." Trends in Microbiology **17**(2): 66-72.
Dunne Jr, W. M. 1990. "Effects of subinhibitory concentrations of vancomycin or cefamandole on biofilm production by coagulase-negative staphylococci." Antimicrobial Agents and Chemotherapy **34**(3): 390-393.
Fenton, M., P. Ross, O. McAuliffe, J. O'Mahony and A. Coffey 2010. "Recombinant bacteriophage lysins as antibacterials." Bioengineered Bugs **1**(1): 9-16.
Frank, K. L., E. J. Reichert, K. E. Piper and R. Patel 2007. "In vitro effects of antimicrobial agents on planktonic and biofilm forms of Staphylococcus lugdunensis clinical isolates." Antimicrobial Agents and Chemotherapy **51**(3): 888-895.
Fu, W., T. Forster, O. Mayer, J. J. Curtin, S. M. Lehman and R. M. Donlan 2010. "Bacteriophage cocktail for the prevention of biofilm formation by *Pseudomonas aeruginosa* on catheters in an in vitro model system." Antimicrobial Agents and Chemotherapy **54**(1): 397-404.
Gödeke, J., K. Paul, J. Lassak and K. M. Thormann 2011. "Phage-induced lysis enhances biofilm formation in *Shewanella oneidensis* MR-1." ISME Journal **5**(4): 613-626.
Gürtler, V. and H. D. Barrie 1995. "Typing of *Staphylococcus aureus* strains by PCR-amplification of variable-length 16S-23S rDNA spacer regions: characterization of spacer sequences." Microbiology **141**(5): 1255-1265.
Hanlon, G. W., S. P. Denyer, C. J. Olliff and L. J. Ibrahim 2001. "Reduction in exopolysaccharide viscosity as an aid to bacteriophage penetration through *Pseudomonas aeruginosa* biofilms." Applied and Environmental Microbiology **67**(6): 2746-2753.
Heilmann, S., K. Sneppen and S. Krishna 2012. "Coexistence of phage and bacteria on the boundary of self-organized refuges." Proceedings of the National Academy of Science of the United States of America **109**(31): 12828-12833.
Hoffman, L. R., D. A. D'Argenio, M. J. MacCoss, Z. Zhang, R. A. Jones and S. I. Miller 2005. "Aminoglycoside antibiotics induce bacterial biofilm formation." Nature **436**(7054): 1171-1175.
Hosseinidoust, Z., N. Tufenkji and T. G. van de Ven 2013. "Predation by homogeneous and heterogeneous phage environments affects virulence determinants of *Pseudomonas aeruginosa*." Applied and Environmental Microbiology **in press**.
Hughes, K. A., I. W. Sutherland, J. Clark and M. V. Jones 1998. "Bacteriophage and associated polysaccharide depolymerases - Novel tools for study of bacterial biofilms." Journal of Applied Microbiology **85**(3): 583-590.
Hughes, K. A., I. W. Sutherland and M. V. Jones 1998. "Biofilm susceptibility to bacteriophage attack: the role of phage-borne polysaccharide depolymerase." Microbiology **144**(11): 3039-3047.
Jackson, D. W., K. Suzuki, L. Oakford, J. W. Simecka, M. E. Hart and T. Romeo 2002. "Biofilm Formation and Dispersal under the Influence of the Global Regulator CsrA of Escherichia coli." Journal of Bacteriology **184**(1): 290-301.
Jarrell, K. and A. M. Kropinski 1977. "Identification of the cell wall receptor for bacteriophage E79 in *Pseudomonas aeruginosa* strain PAO." Journal of Virology **23**(3): 461-466.

Kelly, D., O. McAuliffe, R. P. Ross and A. Coffey 2012. "Prevention of *Staphylococcus aureus* biofilm formation and reduction in established biofilm density using a combination of phage K and modified derivatives." Letters in Applied Microbiology **54**(4): 286-291.
Kirketerp-Møller, K., P. Ø. Jensen, M. Fazli, K. G. Madsen, J. Pedersen, C. Moser, T. Tolker-Nielsen, N. Høiby, M. Givskov and T. Bjarnsholt 2008. "Distribution, organization, and ecology of bacteria in chronic wounds." Journal of Clinical Microbiology **46**(8): 2717-2722.
Knezevic, P., D. Obreht, S. Curcin, M. Petrusic, V. Aleksic, R. Kostanjsek and O. Petrovic 2011. "Phages of *Pseudomonas aeruginosa*: Response to environmental factors and in vitro ability to inhibit bacterial growth and biofilm formation." Journal of Applied Microbiology **111**(1): 245-254.
Knezevic, P. and O. Petrovic 2008. "A colorimetric microtiter plate method for assessment of phage effect on *Pseudomonas aeruginosa* biofilm." Journal of Microbiological Methods **74**(2-3): 114-118.
Kristich, C. J., Y. H. Li, D. G. Cvitkovitch and G. M. Dunny 2004. "Esp-Independent Biofilm Formation by Enterococcus faecalis." Journal of Bacteriology **186**(1): 154-163.
Kropinski, A. M., A. Mazzocco, T. E. Waddell, E. Lingohr and R. P. Johnson (2009). Enumeration of bacteriophages by double agar overlay plaque assay. Methods in molecular biology. M. R. J. Clokie and A. M. Kropinski, Springer. **501:** 69-76.
Labrie, S. J., J. E. Samson and S. Moineau 2010. "Bacteriophage resistance mechanisms." Nature Reviews Microbiology **8**(5): 317-327.
Lacqua, A., O. Wanner, T. Colangelo, M. G. Martinotti and P. Landini 2006. "Emergence of biofilm-forming subpopulations upon exposure of Escherichia coli to environmental bacteriophages." Applied and Environmental Microbiology **72**(1): 956-959.
Lévesque, C., M. Duplessis, J. Labonté, S. Labrie, C. Fremaux, D. Tremblay and S. Moineau 2005. "Genomic organization and molecular analysis of virulent bacteriophage 2972 infecting an exopolysaccharide-producing *Streptococcus thermophilus* strain." Applied and Environmental Microbiology **71**(7): 4057-4068.
Lin, C. K. and H. Y. Tsen 1996. "Use of two 16S DNA targeted oligonucleotides as PCR primers for the specific detection of *Salmonella* in foods." Journal of Applied Bacteriology **80**(6): 659-666.
Lu, T. K. and M. S. Koeris 2011. "The next generation of bacteriophage therapy." Current Opinion in Microbiology **14**(5): 524-531.
Ma, Q. and T. K. Wood 2009. "OmpA influences Escherichia coli biofilm formation by repressing cellulose production through the CpxRA two-component system." Environmental Microbiology **11**(10): 2735-2746.
Merritt, J. H., D. E. Kadouri and G. A. O'Toole 2011. "Growing and analyzing static biofilms." Current Protocols in Microbiology(SUPPL. 22).
Pérez-Giraldo, C., C. Gonzalez-Velasco, R. M. Sánchez-Silos, C. Hurtado, M. T. Blanco and A. C. Gómez-García 2004. "Moxifloxacin and biofilm production by coagulase-negative staphylococci." Chemotherapy **50**(2): 101-104.
Pires, D., S. Sillankorva, A. Faustino and J. Azeredo 2011. "Use of newly isolated phages for control of *Pseudomonas aeruginosa* PAO1 and ATCC 10145 biofilms." Research in Microbiology **162**(8): 798-806.
Pompilio, A., S. Pomponio, V. Crocetta, G. Gherardi, F. Verginelli, E. Fiscarelli, G. Dicuonzo, V. Savini, D. D'Antonio and G. Di Bonaventura 2011. "Phenotypic and genotypic characterization of Stenotrophomonas maltophilia isolates from patients with cystic fibrosis: Genome diversity, biofilm formation, and virulence." BMC Microbiology **11**.
Sayem, S. M. A., E. Manzo, L. Ciavatta, A. Tramice, A. Cordone, A. Zanfardino, M. De Felice and M. Varcamonti 2011. "Anti-biofilm activity of an exopolysaccharide from a sponge-associated strain of Bacillus licheniformis." Microbial Cell Factories **10**.
Schrag, S. J. and J. E. Mittler 1996. "Host-parasite coexistence: The role of spatial refuges in stabilizing bacteria-phage interactions." American Naturalist **148**(2): 348-377.

Spilker, T., T. Coenye, P. Vandamme and J. J. LiPuma 2004. "PCR-Based Assay for Differentiation of *Pseudomonas aeruginosa* from Other Pseudomonas Species Recovered from Cystic Fibrosis Patients." Journal of Clinical Microbiology **42**(5): 2074-2079.
Van den Ende, P. 1973. "Predator prey interactions in continuous culture." Science **181**(4099): 562-564.
Weiss, M., E. Denou, A. Bruttin, R. Serra-Moreno, M. L. Dillmann and H. Brüssow 2009. "In vivo replication of T4 and T7 bacteriophages in germ-free mice colonized with Escherichia coli." Virology **393**(1): 16-23.
Yang, M., E. M. Frey, Z. Liu, R. Bishar and J. Zhu 2010. "The virulence transcriptional activator aphA enhances biofilm formation by Vibrio cholerae by activating expression of the biofilm regulator VpsT." Infection and Immunity **78**(2): 697-703.

## Supplementary Information

Figure S1. (a) Schematics of the experimental design for selection of phage to bacterial ratios (MOI), (b) representative results for MOIs in the range of $10^{5}$ to $10^{-7}$ for *S.* Typhimurium.

Figure S2. Effect of phage action on planktonic and biofilm bacterial load during biofilm growth (MOI= 0.1), (a) *P.aeruginosa* planktonic growth, (b) *P.aeruginosa* biofilm load, (c) *S. aureus* planktonic growth, (d) *S. aureus* biofilm load, (e) *S.* Typhimurium planktonic growth, (f) *S.* Typhimurium biofilm load; Biofilm load is presented in terms of fold change in the value of normalized biofilm level ($OD_{570}$/ $OD_{600}$) compared to the control (represented by 1). Planktonic load is presented in fold change from the control level (represented by 1). Asterisk (*) represents statistically significant difference from the control (p-value < 0.05). Time zero is chosen as the time of addition of phage to the system.

Figure S3. Effect of phage action on planktonic load for (a) *P. aeruginosa* (MOI=10), (b) *P. aeruginosa* (MOI=0.1), (c) *S. aureus* (MOI=10), (d) *S. aureus* (MOI= 0.1), (e) *S.* Typhimurium (MOI=10), (f) *S.* Typhimurium (MOI=0.1); planktonic load is presented in terms of $OD_{600}$.

Figure S4. Effect of phage action on biofilm load during biofilm growth (MOI=0.1), (a) *P.aeruginosa* (MOI=10), (b) *P.aeruginosa* (MOI=0.1), (c) *S. aureus* (MOI=10), (d) *S. aureus* (MOI=0.1), (e) *S.* Typhimurium (MOI=10), (f) *S.* Typhimurium (MOI=0.1); Biofilm load is presented in terms of $OD_{570}$.

Figure S5. Biofilm level for a 48 hrs biofilm post treated with phage, (a) *P. aeruginosa* (MOI=10), (b) *P. aeruginosa* (MOI=0.1), (c) *S. aureus* (MOI=10), (d) *S. aureus* (MOI=0.1), (e) *S.* Typhimurium (MOI= 10), (f) *S.* Typhimurium (MOI=0.1); Biofilm load is presented in terms of $OD_{570}$.

Figure S6. Change in bacteriophage titers for biofilms pre-treated or post-treated with phage mixture at MOI=10 for (a) *P. aeruginosa,* (b) *S. aureus* and (c) *S.* Typhimurium. Values presented are normalized phage titers calculated as log (pfu/mL) at each time point divided by log (pfu/mL) at time point zero.

Figure S7. Change in the level of planktonic bacterial cells for a 48 hr biofilm challenged with phage, (a) *P. aeruginosa* (MOI=10), (b) *P.aeruginosa* (MOI=0.1), (c) *S. aureus* (MOI= 10), (d) *S. aureus* (MOI=0.1), (e) *S.* Typhimurium (MOI=10), (f) *S.* Typhimurium (MOI=0.1). Asterisk (*) represents statistically significant difference from the control (p-value < 0.05).

## Figures

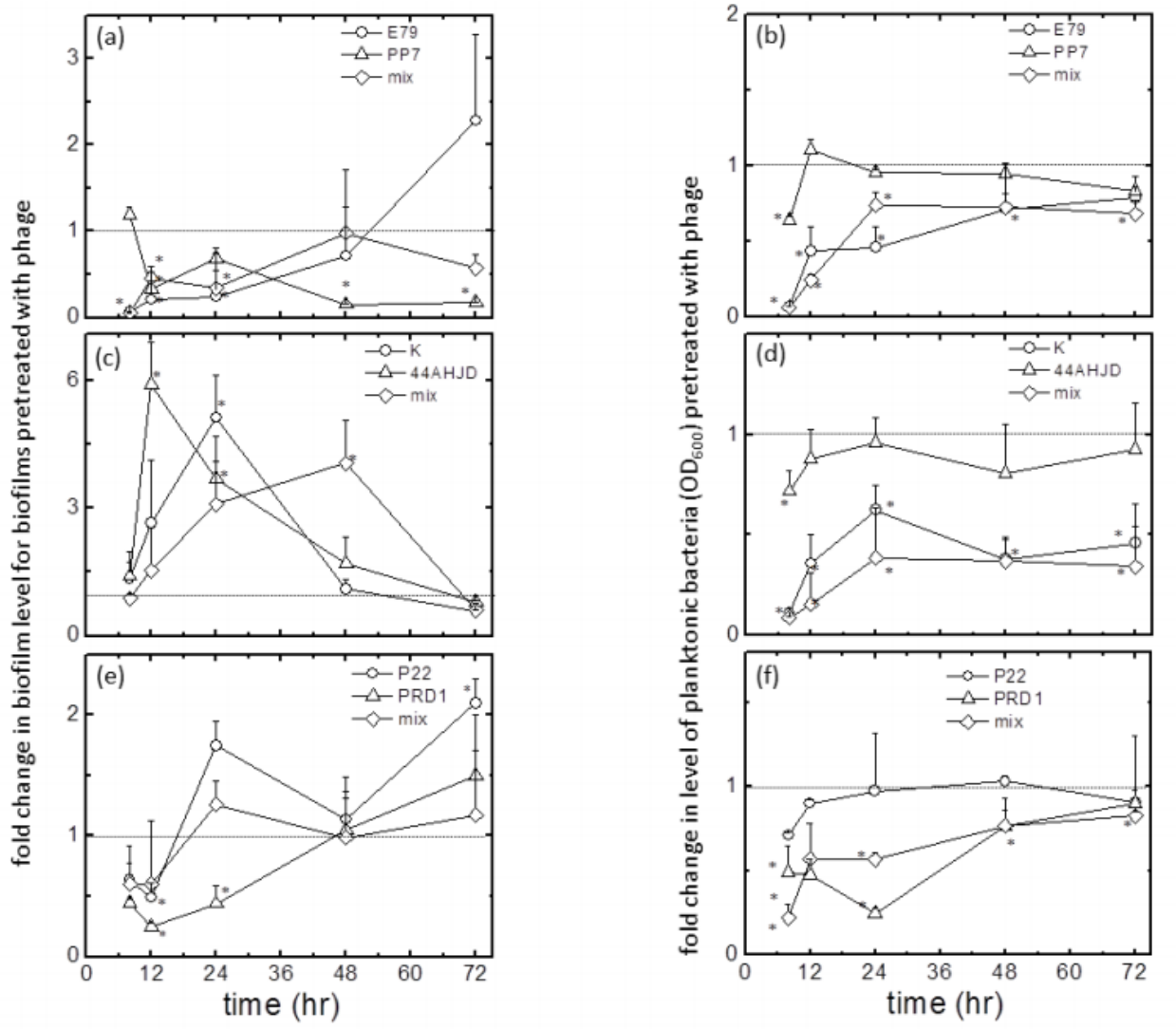


**Figure 1.** *Effect of phage action on planktonic and biofilm bacterial load during biofilm growth (MOI=10), (a) P. aeruginosa biofilm load, (b) P. aeruginosa planktonic growth, (c) S. aureus biofilm load, (d) S. aureus planktonic growth, (e) S. Typhimurium biofilm load, (f) S. Typhimurium planktonic growth. Biofilm load is presented in terms of fold change in the value of normalized biofilm level ($OD_{570}/OD_{600}$) compared to the control (represented by 1). Planktonic load is presented in fold change from the control level (represented by 1). Asterisk (*) represents statistically significant difference from the control (p-value < 0.05). Time zero is chosen as the time of addition of phage to the system.*

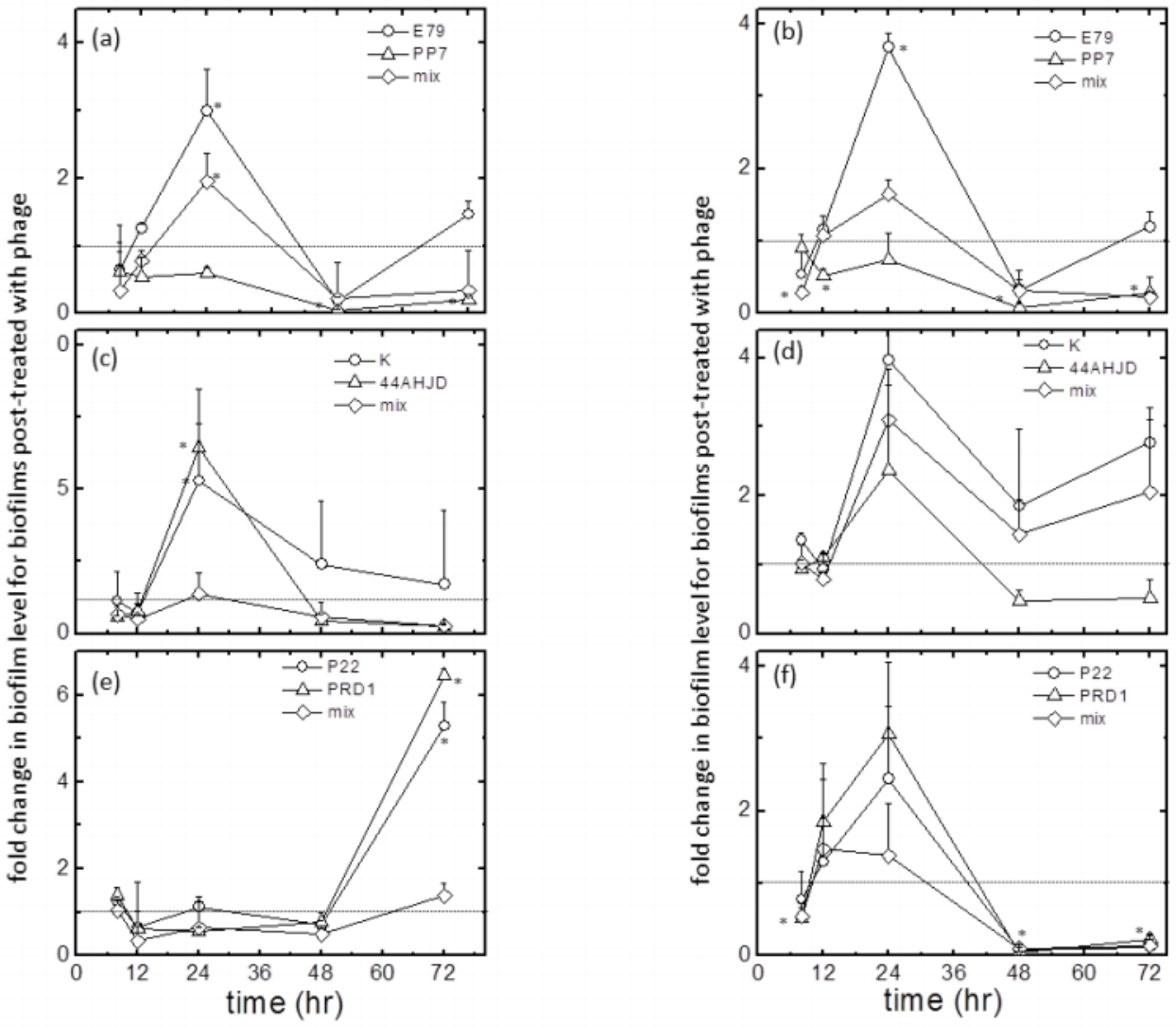


*Figure 2. Biofilm level for a 48 hrs biofilm post-treated with phage, (a) P. aeruginosa (MOI=10), (b) P. aeruginosa (MOI=0.1), (c) S. aureus (MOI=10), (d) S. aureus (MOI=0.1), (e) S. Typhimurium (MOI=10), (f) S. Typhimurium (MOI=0.1). Biofilm load is presented in terms of fold change in the value of normalized biofilm level ($OD_{570}$/ $OD_{600}$) compared to the control (represented by 1). Asterisk (*) represents statistically significant difference from the control (p-value < 0.05). Time zero is chosen as the time of addition of phage to the system.*

## Tables

*Table 1. List of bacterial species and bacteriophages used in this study along with primers used for PCR targeted against 16S rDNA*

| **Host bacteria** | ***S. enterica serotype Typhimurium) (HER1023), clinical isolate*** | | ***P. aeruginosa PAO1, clinical isolate*** | | ***S. aureus ATCC 25923, clinical isolate*** | |
|---|---|---|---|---|---|---|
| **Bacteriophage** | 1- PRD1 (HER23) | 2- P22 (HER161) | 1- E79 (HER359) | 2- PP7 (HER369) | 1- K (HER474) | 2- 44AHJD (HER101) |
| **Phage family** | *Tectiviridae* | *Podoviridae* | *Myoviridae* | *Leviviridae* | *Myoviridae* | *Podoviridae* |
| **forward primer 5′→3′** | TGTTGTGGTTAATAACCGCA | | GGGGGATCTTCGGACCTCA | | AACGGCTTACCAAGGCAACGAT | |
| **reverse primer 3′→ 5′** | CACAAATCCATCTCTGGA | | TCCTTAGAGTGCCCACCCG | | TGCGCATTTCACCGCTACACAT | |
| **Primer reference** | this study | | (Spilker, Coenye et al. 2004) | | this study | |

*Table 2.Summary of phage resistance data for biofilm samples*

| | | percentage of resistant biofilm isolates† | |
|---|---|---|---|
| **bacterial strain** | **phage treatment** | **phage pre-treatment** | **phage post-treatment** |
| | **PP7** | 23 (5) | 15 (3) |
| ***P. aeruginosa PAO1*** | **E79** | 96 (10) | 92 (5) |
| | **mix** | 75 (8) | 82 (9) |
| | **P22** | 45 (2) | 40 (6) |
| ***S. Typhimurium*** | **PRD1** | 34 (1) | 31 (7) |
| | **mix** | 28 (5) | 36 (5) |
| | **K** | 43 (5) | 35 (5) |
| ***S. aureus*** | **44AHJD** | 26 (5) | 30 (5) |
| | **mix** | 33 (5) | 27 (5) |

† The number of samples used for each data point is: 5 time points × 3 replicates × 3 biofilm samples per replicate × 10 colonies from each biofilm sample = 450. The numbers in parentheses represent the 95% confidence intervals. All numbers are rounded to the nearest digit.