Multi-strain bacterial biofilms reduce killing by phage

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# Introduction

As we struggle to find solutions to tackle the emergence of antibiotic resistance, phage therapy has experienced renewed interest. This treatment approach involves using bacterial viruses called bacteriophage or phage to infect and kill bacterial pathogens, and therefore represents a possible replacement or complementary treatment to antibiotics. Phage treatments are typically prepared by isolating phages that can infect a target pathogen *in vitro* in mono-culture. This approach favors phage infection: first, while bacteria are in exponential growth phase, their replication machinery can be co-opted by the phage; second, planktonic growth means that at sufficiently high concentrations, phage are more likely to encounter bacteria than if they were stuck together in aggregates, facilitating infection. Perhaps because of these reasons, phage treatments *in vivo*do not always match the efficacy of the *in vitro* tests. This highlights a need to understand how bacterial pathogens can escape from phages in their natural environment.

Here we focus on two key features of the natural environments of a patient that may be key to the success of phage therapy. First, a pathogen causing an infection will rarely colonize an empty patch, but will instead live alongside different microbial species, including a patient’s resident microbial flora [4,5]. Second, bacteria - pathogens as well as commensals - tend to live in dense, surface-attached cell groups called biofilms. Biofilm-associated bacteria have a higher survival rate compared to their planktonic counterparts [6], particularly when exposed to antibiotics and importantly, also to phage (Eriksen et al. 2018).

Both of these factors - in addition to others that we are not considering here such as the host immune system - can be expected to greatly affect phage therapy. Phages tend to be quite host-specific, killing only a narrow range of bacterial strains. Nevertheless, the presence of resistant strains may alter treatment outcomes by affecting pathogen survival. Indeed, Harcombe & Bull (Harcombe and Bull 2005) have shown that competition with a co-inhabiting species can reduce the ability of the targeted pathogen to survive phage attack. Their study considered liquid cultures, however. The role of resistant strains in affecting treatment outcomes for a sensitive pathogen has been addressed in the context of antibiotic treatment in biofilms, with the opposite result: the presence of resistant cells can protect sensitive ones from antibiotics (Sorg et al. 2016; Frost et al. 2018). This is because a resistant strain that is in close proximity to the pathogen can break down antibiotics and “detoxify” its local environment. Whether a similar protective effect is expected with phage treatment in biofilms is unknown.

We also know that phage population dynamics change radically between liquid bacterial cultures and bacteria growing in or on solid surfaces (Abedon 2008). Much of phage research and clinical work relies on amplifying phages in bacteria growing in semi-solid agar, resulting in the formation of plaques. Decades of experimental and theoretical work has therefore mapped out differences in the ecology and population dynamics of phage in liquid cultures and solid agar. However, these assays were developed to assess bacterial susceptibility to phages rather than to capture features of the natural environment.

In this paper, we explore the fate of a target pathogen, *Pseudomonas aeruginosa* PAO1, when infected with a phage in the presence of a second non-target strain, *Pseudomonas aeruginosa* PA14, that is resistant to the phage. We compare the outcome for the pathogen in a well-mixed liquid environment and a structured biofilm (colony) growing on a solid agar surface. In liquid, competition between the two strains can reduce the population size of the pathogen, giving a competitive advantage to the phage and eliminating the pathogen without the emergence of resistance. In contrast, in a solid environment, the presence of the phage-resistant strain - even if competing with the pathogen - allowed the pathogen to grow despite the presence of the phage. This occurred through two mechanisms: first, competition between the strains slowed down the growth of the pathogen, which reduces the replication rate of the phage, and second, the high density of cells - sensitive or resistant - as well as biofilm matrix create a barrier for phage diffusion. Indeed, a key parameter of the ability of phage to infect is their ability to diffuse through biofilm matrix.

We need to understand how bacteria can escape from phages.

# Results

**Inter-strain competition increases phage efficacy in liquid.**We first sought to understand how the presence of the resistant strain *P. aeruginosa*PA14 (henceforth PA14) would affect the survival of the sensitive target strain *P. aeruginosa*PAO1 (henceforth PAO1) in the presence of phage in well-mixed liquid cultures. These liquid experiments involved growing bacteria in 96-well plates containing TSB and inoculated with mixtures of bacteria and phage, depending on the condition over a period of 48 hours. In control treatments involving PAO1 growing alone, we observed that phage treatment resulted in a drop in PAO1 population size after 6 hours, after which the population size recovered somewhat (Fig. 1A). Assays testing for phage resistance revealed that after 24 hours of culture 62 out of 63 tested colonies (98.41%) were resistant to the phage, while  after 48 hours, 24 out of 24 (100%) were resistant. As a control, resistant PA14 cells growing alone did not appear to be affected by the phage (Fig. 1B, two-sample t-test at 48 hours, df=2, P=0.4). Next, we co-cultured the two strains in the absence of phage, and found that PAO1 grew worse than when it was alone, presumably due to competition with PA14 (Fig. 1C). Finally, adding the phage to this co-culture eliminated all PAO1 within 6 hours of growth (Fig. 1D). Compared to growing alone then, PAO1 resistance could not emerge when growing with a competitor. This led us to hypothesize that the presence of PA14 prevented PAO1 from increasing its population size, thereby decreasing its potential to evolve resistance to the phage and survive the attack.

To test for the effect of population size on resistance evolution, we conducted two experiments. First, we grew PAO1 in the presence of phage with different starting population sizes. In agreement with our hypothesis, we found that resistance to the phage could evolve when the initial population size was large enough (greater than 104 CFU/ml, Fig. 1E).  Second, we kept the initial population size of PAO1 constant and varied the starting population size of its competitor PA14 in the presence of phage. Again, as predicted, we observed that phage resistance could emerge when there were fewer competitors, but as the number of competitors in the starting population grew beyond 106 CFU/ml, PAO1 cells were all killed by the phage at the end of 21 hours of co-culture (Fig. 1F). In all cases, PAO1 survival depended on becoming resistant to the phage. In sum, we find that in liquid culture, competition with resident strains can prevent a target pathogen from surviving phage attack, which is consistent with previous research (Harcombe and Bull 2005).

Phage effect in liquid culture. (A) Growth of PAO1 measured in CFU/ml in liquid over 48h. In the absence of phage, PAO1 grows normally (solid green lines). When phage are added, population size of PAO1 (dashed green lines) decreases over the first few hours, but then increases again, resulting in an increasingly resistant population. The phage population measured in PFU/ml (gray lines) increases accordingly. (B) PA14, which is naturally resistant to the phage, grows similarly in the presence or absence of phage (dashed or solid red lines, respectively), while phage populations (gray) remain approximately constant.  (C) When PAO1 (green) and PA14 (red) are grown together in co-culture in the absence of phage, PAO1 grows worse than alone. (D) When the phage is added to the co-culture, PAO1 population size drop below the detection limit at 6 hours and do not recover. (E) PAO1 is grown together with phage at different initial population sizes. At the end of the experiment, bacteria are plated onto agar plates saturated with phage or PBS to count the resistant population and the total population. A starting population size greater than ~104 allows resistance to emerge. (F) Population size of PAO1 depends on the starting population of PA14. Here, initial population size of PAO1 was always 106, while initial PA14 numbers varied as one the x-axis. Once PA14 became too numerous in the population (greater than ~106), PAO1 could no longer maintain its population size high enough to evolve resistance to the phage. 

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**Phages lyse cells at edges of single-strain sensitive biofilms.** To simulate a setup where a biofilm first forms on a solid surface and is later exposed to phage, we first grew the bacteria for 12 hours in colonies growing on a membrane filter placed on agar. Our goal was to produce biofilms of comparable population sizes to those exposed to phage in the liquid experiment. This was done by transferring the filter with the 12-hour biofilm onto a new agar plate onto which we had placed a drop (approximately the diameter of the membrane filter) containing either phage or PBS as a control. The biofilm was then left to grow in the presence or absence of the phage for an additional 36 hours.

In PAO1 mono-culture biofilms, phage treatment reduced bacterial populations to 52.8111.41% of their pre-treatment size (paired t-test CFUs at 12 and 48 hours, df=2, P=0.034). Fluorescence microscopy images taken immediately prior to infection and 36 hours later (48h total) showed that colonies treated with phage were smaller in diameter after treatment (data), with the surviving cells visible in the center of the colony. Following imaging, cells were harvested, counted and tested for phage resistance. In the colonies that had been treated with phage, resistant cells were recovered (1.36%, 1.63% and 15.74% of the population in the three replicate colonies).

To understand why so many sensitive cells survived and where in the colony resistance had occurred, before destroying the colonies, we touched an inoculation loop in the center of each colony, resuspended the cells in PBS and plated them on agar to calculate the number of resistant and sensitive cells, as well as phage. We found no resistant cells in the center of the colony, indicating that resistance arose at the colony edges where cells were growing. However, phages were detected in the center at a similar multiplicity of infection (MOI) to the initial dose (PFU/CFU of 0.990.27), suggesting that the phage could diffuse into the center of the colony, possibly infecting cells but not lysing them. This phenomenon, called “pseudolysogeny” has been observed for starved cells in stationary phase, where phage have been found to return to their lytic state once bacterial growth resumes  (Kokjohn and Sayler 1991; Abedon 2008; Ripp and Miller 1997; Los, Wegrzyn, and Neubauer 2003). In agreement with this prediction, on plating bacteria from the center for quantification, only bacteria at low dilutions could grow, indicating increased phage lytic activity (supp. Fig).

In contrast, PA14 (the phage resistant strain) mono-culture colonies were indistinguishable with and without phage treatment (Fig. for pics, t-test CFUs with and without phage, df=2.6, P=0.87). On sampling the colony centers, no phage were detected, indicating that phage could not diffuse from the agar into the colony. Indeed, total phage populations fell to 112.8% of their original size in PA14 colonies over the 36 hours, which we suspect is due to toxicity of LB to phage (Hadas et al. 1997) given that phage populations also fell to 8.15.4% in the absence of any bacteria.

Taken together, these data support a model whereby PAO1 death and the emergence of phage resistance occur mainly at the edges of the colony where cells are growing, whereas phage can diffuse into PAO1 colonies - but not PA14 - and adsorb to stationary-phase cells at the center through pseudolysogeny.

**Phage efficacy is reduced in mixed sensitive-resistant biofilms.**Knowing that phage do not diffuse into resistant PA14 colonies, we next asked how the presence of this resistant strain would impact the survival of the targeted pathogen (PAO1) within a mixed biofilm treated with phage. We repeated the previous experiment, where we grew cells - this time a mixture of both PAO1 and PA14 - on a filter for 12 hours, then transferred the biofilm onto a dried phage drop on a different agar plate and left to grow for a further 36 hours. In contrast to the decrease in population size observed in the PAO1 monoculture colony, despite phage treatment, PAO1 increased by 233.9123.4% over 36 hours (paired t-test CFUs at 12 and 48 hours, df=2, P=0.025). Curiously, the population size of the phage increased concomitantly in the three replicates, suggesting that significant bacterial infection must also have occurred (Fig. xx). Although PAO1 cells were not significantly fewer with and without phage (t-test, df=2.01, P=0.16), microscopy showed that patches of PAO1 (labelled with GFP) were absent from the edges of the colonies treated with phage, indicating cell lysis at the growing edges.

These data suggest that as in PAO1 biofilms, phage could lyse growing cells at the colony edges, while PAO1 cells in the center were not killed. In contrast to the single-strain biofilms, however, PAO1 populations continued to increase. This suggests that pockets of PAO1 may be present that are not accessible to phages. In support of this hypothesis, we found a significantly lower MOI of PAO1 in centers of mixed compared to mono-culture colonies (t-test, df=3.16, P=0.013, see Fig. xD). This led us to consider whether some sensitive bacteria might be completely escaping phage infection, particularly in the colonies where they were mixed with the resistant PA14.

**Resistant cells protect sensitive cells in biofilm.**To quantify the extent to which sensitive cells could escape phage infection, we simulated a scenario where cells would have a chance to reseed a new environment and begin to grow, and asked how often regrowth occurred depending on the biofilm of origin. In particular, each colony was If cells were in pseudolysogeny in the biofilm, i.e., infected but not lysed because not enough energy as availabl for bacteria and phage to replicate, we expect them to lyse on reinoculation onto fresh media.

# Discussion

In sum, we show that a targeted pathogen is more likely to survive a phage attack if growing on a solid surface, in the presence of a phage-resistant competitor. It is also most likely to develop resistance to the phage in the absence of competitors, where it can grow to a sufficiently large population size. Competing species thus reduce the likelihood of resistance evolution, but may create a haven for phage-sensitive pathogens that can spread if dispersed.

Presumably, cells that divided at the colony edges were killed by the phage, while cells that had divided prior to phage addition could not be killed by the phage because they were no longer dividing (phage require cellular division to replicate (REF)).

# Methods

**Bacterial strains, phage, media and culture conditions.**Experiments were performed with two different strains of *Pseudomonas aeruginosa*: strain PAO1 carrying a GFP-expressing plasmid, which was susceptible to a specific phage, and strains PA14 (PA14-WT) or carrying an mCherry-expressing plasmid (PA14-mCherry), which were both resistant to this same phage. Both plasmids contained a gentamicin resistance gene. All three strains were kindly provided by Kevin Foster. The phage used for this study was chosen based on a spot assay. This assay is used to investigate which phage produces phage plaques when they are in contact with our bacterial strains. Among 14 phages tested, we chose one that induces the lysis of PAO1-GFP but not the lysis of both PA14 strains, which were entirely resistant. To count the colony-forming units (CFU) of bacterial monocultures and plaque-forming units (PFU) of phages, Tryptic Soy Agar (TSA; Trypticase TM Soy Agar, Difco) plates were used. To distinguish both strains within a community, co-cultures were plated onto TSA plates to count PA14-WT CFUs and onto LB agar plates containing 10 μg/ml of gentamicin to count only PAO1-GFP CFU. Overnight cultures were grown in tryptic soy broth (TSB; Bacto TM, Detroit, MI, USA) at 37°C, shaken at 200 rpm. Before each experiment, the optical density (OD600) of the overnight cultures of PAO1-GFP, and either PA14-mCherry or PA14-WT strains (depending on the experiment) was measured with a spectrophotometer (Ultrospec 10, Amersham Biosciences). Bacterial overnight cultures were then inoculated into Erlenmeyer flasks (100 ml) containing 20 ml of TSB to obtain a standardized OD600 of 0.05. Bacterial cultures were grown in a shaking incubator at 200 rpm and 37°C for 3 hours to obtain bacteria in exponential phase with a final concentration of approximately 108 CFU/ml at the beginning of each experiment. These cultures were then diluted to the desired starting population size.

**Phage treatment in liquid cultures.**A 96-well plate was filled with 200 μl of TSB in each well, additionally containing 106 CFU/ml PAO1-GFP or 108 CFU/ml PA14-WT alone, or together with or without 106 PFU/ml of phages (MOIPAO1 = 1). Each condition (PAO1-GFP alone, PA14-WT alone, and the co-culture) was performed in triplicate. These initial population sizes were chosen since they allowed the two strains to co-exist and grow over 48 hours. The plate was then put in a Tecan i-control infinite M200 PRO plate reader at 37°C under agitation for 48 hours. After 6, 24 and 48 hours, the untreated samples were transferred into Eppendorf tubes and centrifuged at 4°C, 8000 rpm for 15 minutes, resuspended in 200 μl of PBS, serially diluted and plated on either TSA or LB Agar gentamicin 10 μg/ml plates. For the treated samples, the same protocol was followed except that after centrifugation, the supernatants containing phages were kept in the fridge at 4°C. They were further diluted in NaCl 0.9% to count the PFU/ml. The pellets containing the bacteria were then washed 3 times with 1 ml of fresh PBS at 8000 rpm, 4°C for 5 minutes to remove all the potential phages remaining in the pellet. Finally, the phage resistance rate of PAO1-GFP was calculated after 24 and 48 hours by plating them on TSA plates containing approximately 1010 PFU (plaque forming units) of pre-absorbed phages. If PAO1-GFP were growing in co-culture with PA14-WT, plates additionally containing 10 μg/ml gentamicin were used. To evaluate resistance rates, the CFU/ml of PAO1-GFP growing on plates saturated with phages was then compared to the CFU/ml growing on plates with no phage.

**Quantifying phage resistance in liquid mono- and co-cultures.** To understand the role of population size on resistance emergence, two experiments were performed (Fig. 1E, F). In the first, a 96-well plate was filled with 10 up to 108 PAO1-GFP, with 10-fold increases, together with phage to achieve an MOIPAO1 = 1 in 200 μl of TSB. We grew the bacteria for 21 hours at 37°C under agitation in the plate reader, and then assessed phage resistance rates and total population sizes. For the second experiment, a 96-well plate was filled with 106 CFU/ml of PAO1-GFP and phages at an MOIPAO1 = 1 in 200 μl of TSB, to which we added increasing amounts of PA14, starting at 102 up to 108 in 10-fold increments. Bacteria were again grown in the plate reader for 21 hours at 37°C under agitation, at the end of which we assessed phage resistance rates and total population sizes of both strains.

**Phage treatment in biofilms.**To grow bacteria in a biofilm, liquid cultures were prepared and a drop spotted onto a membrane filter (Isopore®Membran, GTTP04700, Merck) previously placed in the centre of agar plates containing 0.1x LB (1 g/L of tryptone (ThermoScientific TM Oxoid TM Tryptone), 0.5 g/L of yeast extract (ThermoScientific TM Oxoid TM Yeast Extract Powder), 10 g/L of NaCl (ACROS Organics TM , 99.5%) and 15 g/L of agar (Bacto TM Agar solidifying agent, BD Diagnostics). Liquid cultures of the two strains were prepared as described for the liquid experiments in PBS to obtain a final concentration of 106 CFU/ml of PAO1-GFP and 108 CFU/ml of PA14-WT in a drop of 2μl that was spotted onto the filter. Nine replicate plates were prepared for each condition (PAO1-GFP, PA14-WT and the mixture of both), and incubated at 37°C. After 12 hours of incubation, three replicates were removed in order to count the CFUs of both strains by removing the filters from the agar using sterile tweezers and placing them in tubes containing 3 ml of PBS. The tubes were well shaken to remove and resuspend the biofilms in the PBS, the filters removed and the bacteria plated on either TSA plates (monoculture, co-culture) or on gentamicin-containing (co-culture). Among the six remaining replicates, three were placed onto new 0.1x LB agar plates without phages and the three others were placed onto new plates pre-absorbed with a 50μl drop containing approximately 1010 phages (diameter similar to filter diameter) and incubated at 37°C. After 36 hours, the filters containing biofilms growing on agar were removed and placed in 3 ml of PBS to count the CFUs and PFUs as described above. Furthermore, for the treatments involving phage, the whole agar was also collected and put in 50 ml falcon tubes containing 10 ml of SM buffer, well-shaken, centrifuged for 20 minutes at 8000 rpm at 4°C and the supernatant containing phages further diluted in NaCl 0.9% to count the PFU/ml. Both phage concentrations (filter + agar) were added to have the final PFU/ml value.

**Image analysis.** Images of the different biofilms were acquired after 12 and 48 hours using a fluorescence microscope.

**Statistical analysis.** Each experiment was performed using three biological replicates per condition.

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