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Design of a broad-range bacteriophage cocktail that reduces *Pseudomonas aeruginosa* biofilms and treats acute infections in two animal models.

Francesca Forti[1#], Dwayne R. Roach[2], Marco Cafora[1], Maria E. Pasini[1], David S. Horner[1], Ersilia V. Fiscarelli[3], Martina Rossitto[3], Lisa Cariani[4], Federica Briani[1], Laurent Debarbieux[2], Daniela Ghisotti[1#]

[1]Department of Biosciences, University of Milan, 20133 Milan, Italy
[2]Department of Microbiology, Institut Pasteur, 75015 Paris, France
[3]Children's Hospital Bambino Gesù IRCCS, 00165 Rome, Italy
[4]Fondazione IRCCS Ca' Granda, Ospedale Maggiore Policlinico, University of Milan, 20122 Milan, Italy

Running title: Phage therapy of *Pseudomonas aeruginosa*

#Co-corresponding authors:
francesca.forti@unimi.it
daniela.ghisotti@unimi.it

ABSTRACT

The alarming diffusion of multidrug resistant (MDR) bacterial strains requires investigations on non-antibiotic therapies. Amongst them, the use of bacteriophages (phages) as antimicrobial agents, namely phage therapy, is a promising treatment strategy with support by recent successful compassionate treatments in Europe and the U.S.A. In this work, we combined host range and genomic information to design a 6-phage cocktail killing several clinical strains of *P. aeruginosa*, including those collected from Italian cystic fibrosis (CF) patients, and analyzed the cocktail performance. We demonstrated that the cocktail composed of four novel (PYO2, DEV, E215 and E217) and two previously characterized (PAK_P1 and PAK_P4) phages was able to lyse *P. aeruginosa* both in planktonic liquid cultures and in biofilm. In addition, we showed that the phage cocktail could cure acute respiratory infection in mouse and treat bacteremia in the wax moth *Galleria mellonella* larvae. Furthermore, administration of the cocktail to larvae prior to bacterial infection provided prophylaxis. In this regard, efficiency of the phage cocktail was found to be unaffected by the MDR or mucoid phenotype of the pseudomonal strain. The cocktail was found to be superior to individual phages in destroying biofilms and providing a faster treatment in mice. We also found the *Galleria* larvae model to be cost-effective for testing clinical strains susceptibility to phages, suggesting that it could be implemented in the frame of developing personalized phage therapies.

INTRODUCTION

The opportunistic pathogen *Pseudomonas aeruginosa* infects principally the airways of immunocompromised patients, and is one of the principal bacteria isolated from adults with cystic fibrosis (CF). The appearance and diffusion of multidrug resistant (MDR) isolates of *P. aeruginosa* is responsible for the increasingly unsuccessful use of antibiotics. Thus, alternative therapies are urgently needed, and the use of bacteriophages (phages), the natural viral enemies of bacteria, has received renewed attention (1,2). Phage therapy has been proposed 100 years ago before the discovery of antibiotics (3). Following an initial worldwide expansion, the use of this therapy declined being replaced by the more successful use of antibiotics. Beyond efficacy itself, the lack of precise knowledge in the complex interaction between phages and bacteria has played a major role in the shift from using phages to antibiotics. For instance, while today genome sequencing and experiment can determine whether a phage is virulent or temperate, such information was not available in early 20th century. Temperate phages are known to serve as vehicles for bacterial sequence exchanges between strains, eventually leading to the dissemination of genes coding for toxins or antibiotics resistance. Therefore, for therapeutic applications, only virulent (strictly lytic) phages are advised. Compared to antibiotics, phages have several advantages. First, as obligate bacterial viruses, they tend to be specific to their bacterial hosts, confined to killing a narrow range of pathogenic strains. This avoids collateral damage to human and animal healthy commensal microbiota, contrary to broad-spectrum antibiotics (4,5). Another advantage of phage therapy over conventional antibiotics is the dynamic dosing provided by phages that multiply when the target bacterial host strains are present and decrease in number as the target bacteria are eliminated (6). Thus, phages provide an infection site specific augmentation of dose that cannot be achieved through the standard repeated dosing of antibiotics. Lastly, phages are often able to kill bacteria independent of their MDR phenotype (7,8).

Several reports have demonstrated that the growth of a pathogenic bacterium can be controlled *in vitro* and *in vivo* with specific phages. These range from the experimental treatment of *Escherichia coli* diarrhea (9), *Klebsiella pneumoniae* pulmonary infection (10), *Acinetobacter baumannii* pneumonia (11) to *P. aeruginosa* keratitis (12). In addition, the therapeutic effect of phage administration to *P. aeruginosa* infected mice (13,14,15) or *Galleria* larvae have also been reported (16). However, no consensual and validated guidelines for the selection of individual or multiple therapeutic phages that target a specific pathogen have been adopted (17). One strategy is to isolate the bacterial infection causative agent from the patient and then identify *in vitro* one or more phages that lyse that strain(s). This approach is laborious and time consuming, as well as requires a large pool of phages to be on hand. An alternative strategy would be to preemptively combine a mixture of phages (cocktail) that together are able to efficiently kill a broad range of clinical strains of a disease-specific pathogen (18).

In this study, we investigate the preemptive tactic, using *in vitro* and *in silico* criteria, to combine a mixture of phages to efficiently treat a broad range of *P. aeruginosa* clinical strains isolated from Italian patients with CF. We show that a cocktail of six *Pseudomonas* virulent phages can kill with 77% CF *P. aeruginosa* clinical strain coverage, while as being effective at reducing pseudomonal biofilms *in vitro*. Furthermore, the phage cocktail resolved pseudomonal acute pneumonia in mice and treated bacteraemia in wax moth *Galleria* larvae.

RESULTS

Isolation and electron microscopy imaging of broad host range phages infecting Italian *P. aeruginosa* strains isolated from cystic fibrosis patients.

We collected 40 *P. aeruginosa* strains from CF patients at several Italian Medical Centers (see Materials and Methods). In addition, we added 2 Italian isolates from COPD, 9 non-Italian clinical strains isolated previously from CF and non-CF patients, 5 environmental isolates, and 2 laboratory strains (PAO1 and PAO1 *pilA*) to increase the genetic diversity of bacterial hosts tested (Table S1).

Then, we isolated 23 novel phages as described in the Materials and Methods (Table S2). Next, we measured the efficiency of plating (EOP) of each phage on the panel of 58 *P. aeruginosa* strains (Table S3). As expected, each phage had a distinct host range with no individual phage being able to lyse all strains in the aforementioned collection, and some only lysed less than 30% of strains. Intriguingly, none of our tested phages was able to lyse *P. aeruginosa* strains PaPh23 and PaPh30. However, about half of isolated phages exhibited a much broader host range by lysing more than half of the strain collection.

From the pool of moderately broad host range phages, we selected six phages (PYO2, E215, E220, S218, E217, and DEV) that when combined was theoretically predicted to infect 97% of the strain collection (Table S3). Transmission electron microscopy images of these phages are shown in Fig. S1. All belong to the order Caudovirales: PYO2, DEV and E220 are members of the Podoviridae family, and share highly similar morphology (regular icosahedral head of 72 nm diameter and a short tail of 18 nm); E215 and E217 belong to the Myoviridae family and both share a very similar morphology with regular icosahedral head of 80 nm diameter and tail 185 nm long; S218 is a member of the Siphoviridae, and possesses an icosahedral head of 100 nm in length and 60 nm wide with a flexible 210 nm long tail.

One step growth experiments in PAO1 were performed in order to characterize PYO2,

DEV, E215 and E217 phages (Fig. S2). Phages PYO2 and DEV have a similar latent period (20 min) and burst size (100 and 200 PFU/ml respectively), whereas E215 and E217 show a longer latent period (30-40 min) and both a burst size over 200 PFU/ml.

Genome characterization of the selected phages.

We sequenced phages PYO2, E215, E220, S218, E217, DEV and their genomic characteristics are reported in Table S4. The genomes of phages PYO2 and DEV were both 72,697 bp in length and 99% similar. Alignments show PYO2 and DEV were related to Podoviridae Lit1virus group and share similar levels of conservation with the publically available sequences of *Pseudomonas* phages PEV2 and RWG. Genomes of phages E215 (66,789 bp) and E217 (66,291 bp) also had a high level of similarity (97% identity over 98% of their length) and are related to members of the P1virus subfamily of the Myoviridae, and closely resemble *Pseudomonas* phage vB_PaeM_CEB_DP1.

In order to discard temperate phages and more broadly phages that could serve as vehicles for undesirable functions, we searched for similarities between all putative viral open reading frames and a custom database of genes expressing bacterial virulence factors, antibiotic resistance and integrases/excisionases/recombinases. We found that the genomes of the phages PYO2, DEV, E215 and E217 do not encode proteins with similarity to “undesirable functions” leading us to classify them as virulent. On the contrary, as reported in Table S4, the genome of phages E220 and S218 contain open reading frames with high levels of similarity to genes annotated as integrases in the ACLAME database (19). This suggests phages E220 and S218 have a temperate lifecycle not conducive of a desirable antimicrobial agent and thus were not studied further.

Evaluation of _in vitro_ efficiency of transduction of the selected phages.

We tested the capacity of PYO2, DEV, E215 and E217 phages to transduce genetic markers. We found that the frequency of transduction was less than 10^{-9} per infecting phage, a frequency that is about 100 fold lower than the typical general transduction rate for virulent phages leading us to conclude that these phages would unlike serve as vehicles to carry on antibiotic resistance or virulence genes (20).

Definition of a genetically diverse phage cocktail.

With the aim to assemble a phage cocktail that displays a broad host range and genetic diversity, we selected 6 virulent phages PYO2, DEV, E215, E217, PAK_P1 and PAK_P4. The latter two, are previously characterized *P. aeruginosa* Myoviridae virulent phages isolated in France (13,21,22), with 93,198 bp and 93,147 bp, respectively and display 93% identity over 98% of their genome length. 'PYO2/DEV, 'E215/E217', and 'PAK_P1/PAK_P4' constitute three groups of genomically similar phages and represent three viral genera. The six phages do not show significant sequence similarity to each other, other than between pairs of closely related phages (Fig. S4 and Table S4). Hereafter, we refer to the mixture of all six phages as the 'phage cocktail'.

***In vitro* characterization of the cocktail and its individual components.**

The EOP of the cocktail was compared to that of each individual phage on 58 *P. aeruginosa* strains and is reported in Table 1. As to be expected, the phage cocktail was able to lyse a broader range of bacterial strains than any of the individual phages that make up the cocktail. That is, the broadest host range of a single phage only lysed 36 of the 58 strain collection (62%) and 22 of the 40 Italian CF clinical strains (55%). Whereas, combining phages in a cocktail expanded the full strain collection host range by 15% (45 out of 58 strains) and Italian CF clinical strain collection by 20% (30 out of 40 strains). Of note, the phage cocktail *in vitro* host range was lower than that predicted theoretically from

summing the host ranges of each individual phage within the cocktail. This phenomenon could be due to host infection competition between phages (23,24).

The lysis kinetics of *P. aeruginosa* strains PAO1 and PAK-lumi cultures infected with each phage and with the cocktail were followed by monitoring the optical density (OD_{600}) over time (Fig. 1A, B and C). For PAO1, with a multiplicity of infection (MOI) of 2.5, PYO2 and DEV caused a decrease in OD_{600} at 1-1.5 h post-infection (PI), and E215 a relatively smaller decrease around 2 h. For PAK-lumi, the effect was less pronounced both after DEV and E215 infection. Interestingly, phage E217 did not cause lysis but clearly stopped the growth of both PAO1 and PAK-lumi. PAK_P1 and PAK_P4 did not alter PAO1 growth, whereas the OD_{600} of PAK-lumi started to decrease at 2 h PI.

After infection of both PAO1 and PAK-lumi cultures with the phage cocktail (Fig. 2C), OD_{600} decreased about 70 min PI. This indicates that phages are able to kill sensitive bacteria *in vitro* in a relatively short time after infection. After overnight incubation, however, the OD_{600} reached high values, due to growth of resistant bacteria, as confirmed by testing several bacteria (10/10 resistant clones), indicating that the cocktail failed to prevent the outgrowth of phage-resistant variants.

Phages disrupt *P. aeruginosa* biofilm.

We tested the capability of phages to reduce biofilms formed by *P. aeruginosa* GFP-expressing PAO1 or PAK-lumi strains. After 48 h of biofilm formation on glass slides, phages were applied as a cocktail (Fig. 2A) or individually (Fig. S3). The biofilm biomass nearly disappeared after incubation with the phage cocktail. To quantify the biofilm reduction, we measured the biofilm biomass by crystal violet staining of 24 h biofilms and found that the cocktail caused a significant reduction in PAO1 and PAK-lumi biofilm

biomass (63% and 65%, respectively; Fig. 2B). In addition, the efficiency of the cocktail in destroying a preformed biofilm formed by various clinical *P. aeruginosa* strains was also tested. The cocktail reduced 64% (p<0.001) of a highly dense biofilm produced by the 1st infection strain PaPh5 and reduced biomass by 19% (p=0.6) and 37% (p<0.001) for the mucoid AA43 and the mucoid, MDR PaPh32 strains, respectively.

Intriguingly, PAK_P1 and PAK_P4, which are unable to replicate on PAO1, caused a significant increase in PAO1 biofilm biomass (Fig. S3). This effect was not observed when the phages were component of the cocktail. Although, the cause of this phenomenon is unclear, it could be related to host defense system to reduce phage growth (25).

Phage treatment of *P. aeruginosa* respiratory infection in mice.

We tested the capacity of the phage cocktail to cure a *P. aeruginosa* acute respiratory infection in a mouse model. Fig. 3 shows that phage treatment even at the lowest MOI (0.05 each phage) was effective at reducing respiratory bacterial burden by 48 hours and achieving 100% survival rate. Non-invasive longitudinal monitoring of *P. aeruginosa* infection showed that the phage cocktail administered at each of the MOIs tested led to reduction of bacterial burden in mouse lungs (Fig. 3B). Comparatively, the highest MOI (1.0) began to reduce significantly the bacterial density by 6 h post treatment, whereas with the two lower MOIs (0.05 and 0.1) it took up to 9 h before the reduction becomes significant.

Phage treatment of *P. aeruginosa* systemic infection of *G. mellonella* larvae

In the frame of developing personalized phage therapies, we investigated the wax moth *G. mellonella* larvae as a rapid and economical *P. aeruginosa* clinical isolate pre-treatment

phage screening method. First, we assessed that administration of the phage cocktail at the higher dose (CK25) does not cause per se adverse effects to the larvae (Fig. 4A and 4B). Then, we showed that larvae infected with a lethal dose of PAK-lumi and 1 h later treated with the phage cocktail significantly delayed death (Fig. 4A). Indeed, larvae survival after 20 h increased from about 17% to 49% at MOI 8 (CK8) and 63% at MOI 25 (CK25), respectively (Fig. 4E). Even at a later time point (40 h), survival increased from 6.6% of larvae not treated with phages to 26.6% and 30.5% (Mantel-Cox $p<0.0001$) of the groups that received the phage cocktail at different MOIs (Fig. 4A). Moreover, we showed that pretreatment with the phage cocktail 1 h before PAK-lumi challenge provided prophylaxis against lethal infection (Mantel-Cox test $p<0.0001$; Fig. 4B and E). Interestingly, the two clinical strains PaPh5 and AA43 were also both controlled by the phage cocktail (Fig. 4C, D and E; $p<0.0001$).

DISCUSSION

CF patients, who experience pulmonary infections predominantly caused by *P. aeruginosa*, are, due to the recurrent use of antibiotics, increasingly exposed to the risk of infection caused by MDR strains. In this study, we isolated and characterized new phages, assembled a 6-phage cocktail and tested its efficacy against MDR *P. aeruginosa* strains both *in vitro* and in two *in vivo* animal models.

Several studies have reported successful phage therapy treatments of experimental bacterial infections supporting its use as first line therapy, in particular for infections caused by MDR pathogens (26,27,28,29). In line with these data, two compassionate phage treatments were recently reported in Europe and U.S.A. comforting the efficacy and safety of such approach (30,31). However, for these two examples, the choice of phages

was guided first by their *in vitro* activity in patient's pathogen, without, to our knowledge, any *in vivo* validation step. In addition to such customized solution, a parallel strategy would be to design ready to use cocktails with broad host range. Here, we assembled a 6-phage cocktail taking into account host range and genomic information and assessed *in vitro* and *in vivo* efficacies.

Genome sequencing revealed that the cocktail is made up of 3 pairs of closely related phages (with divergences of less than 8% sequence identity between components of each pair). Although the pairs have highly similar genomes, the related phages differ in their host range *in vitro* – part of the rationale for construction of the cocktail. For example, phage DEV lyses strain LESB58, whereas the closely related phage PYO2 does not. These two phages, however, differ by less than 1% of nucleotides over their complete genomes. We noticed that the genomic variation is mainly confined in two genes. The 3'-end region of RNA polymerase gene (orf 71 in Fig. S4B) in which most substitutions observed were synonymous, and the orf encoding for dUTPase (orf 81 in Fig. S4B) in which 21/128 amino acids (16%) differ. dUTPase has been reported to be essential for viral replication in certain hosts (32) and implicated in host range in specific conditions (33). Our observations suggest that dUTPases can be considered attractive candidate genes for future studies of host specificity mechanisms, beyond the expected structural genes involved in host recognition (34).

Compared to individual phages our cocktail has *in vitro* a broader host spectrum on clinically isolated *P. aeruginosa* strains. In addition, the cocktail lysed all 3 MDR strains in our collection (PaPh24, PaPh25 and PaPh32), implying that phage infection was independent of cells harboring an antibiotic resistance mechanism. Moreover, the phage cocktail was able to infect and kill mucoid strains isolated from chronically infected CF

patients.

Treatment of a *P. aeruginosa* biofilm with the phage cocktail demonstrated that phages are able to enter the biofilm, destroying the biomass and reaching the bacteria embedded inside. In this respect, the use of the phage cocktail greatly increased the effect of single phage infections (compare Fig. 2 with Fig. S3). We also observed that the phage cocktail reduced to different degrees biofilms formed by different strains, which may be due to the differences in biofilm formation and composition observed with clinical *P. aeruginosa* strains (35).

Our findings indicated that in mice, lethal acute respiratory infection can be cured by a treatment with the cocktail. Compared to our previous data obtained with a single phage, the cocktail shows the advantage of a more rapid efficacy in reducing the bacterial load (13,21). This suggests a synergistic action when using multiple phages. Further investigations will be required to identify the mechanisms behind such synergy, but in the light of our recent investigations on the role of the immune system during monophage therapy, we can hypothesize that the cocktail reduces the probability of phage-resistant bacteria to grow (15). In systemic *G. mellonella* infection, in which bacteria are directly injected in the haemolymph, a significant death delay over non treated controls was observed upon phage injection. The presence of a significant difference in lethality at an early time point after infection (20 h) between untreated and phage-treated larvae suggests that this test could be introduced for *in vivo* evaluation of effectiveness of phage therapy. Moreover, the phage cocktail was able to prevent *P. aeruginosa* infection in the larvae. Prophylaxis with phages could be proposed for CF or immunocompromised patients, who are frequently hospitalized and therefore at higher risk of exposition to nosocomial infections. The use of the larvae model provided several advantages over the

murine model amongst which the flexibility to test many clinical strains was shown here. Indeed, some *P. aeruginosa* clinical strains have been found not to infect the respiratory tract of mice [L. Debarbieux, personal communication and (36)], which limits the use of this model. Other advantages are related to the cost, the easy management in a microbiology lab, and to some extend the experimental time. These characteristics of the larvae model provide a solution for the *in vivo* evaluation of phages and cocktails against a clinical isolate that could be integrated into the process of the selection of the best suited phages to formulate a cocktail.

Overall, our strategy based on 1) host range, 2) genomic information and 3) *in vitro* efficacies led to the formulation of 6-phage cocktail that was validated in two *in vivo* models. It should be noticed that *in vitro* efficacies in liquid and biofilms were the less encouraging data as the cocktail did not prevent within 24 h the growth of bacterial resistant clones and that some individual phages enhanced the density of biofilms. Therefore, to design phage cocktails, the pertinence of *in vitro* tests in irrelevant conditions relative to the treatment of human bacterial infections can be questioned despite its rationale being at the root of phage therapy (37).

316

317 **MATERIALS AND METHODS**

318

319 ***P. aeruginosa* strains.** Clinical isolates of *P. aeruginosa* were isolated from primary and
320 chronically infected patients at the Centro di Riferimento per la Fibrosi Cistica della
321 Regione Lombardia, Milan, and at the Ospedale Bambino Gesù, Rome, and kindly
322 provided by Dr. A Bragonzi of the Infection and Cystic Fibrosis Unit at San Raffaele, Milan,
323 Italy. Strain PAO1 *pilA*, in which the *pilA* gene has been deleted, was kindly provided by
324 Dr. F. Imperi (Università degli Studi di Roma-Sapienza, Rome). The other strains were
325 present in the lab collection. All the strains are listed in Table S1. Strains PAO1 and PAK-
326 lumi were transformed with plasmid tPUCP19-GFP (C. Penaranda and D. Hung personal
327 communication), which expresses high level of GFP, for making them fluorescent and
328 strain PAO1Tc^{R} (38) was used for transduction experiments.

329

330 **Bacteriophage isolation.** Our phage collection includes 25 independent isolates listed in
331 Table S2. Several isolates originated from sewage samples collected from Milano Nosedo
332 and Milano Peschiera Borromeo wastewater treatment plants. Independent samples
333 collected in different days were used to avoid the re-isolation of the same phage. Clear
334 plaques were purified with standard procedures (39). Two phages were isolated from
335 commercial preparations Phagyo (Batch # 06.06.13, JSC Biochimpharm, Tbilisi, Georgia)
336 and Intesti Bacteriophage (Batch # M2-501, Eliava Institute, Tbilisi, Georgia), by plating the
337 preparation on PAO1 and isolating a single clear plaque from each preparation. Several
338 natural derivatives of these two initial isolates with a different plaque morphology or host
339 range were added to the collection. On few occasions, evolution of some phages variants
340 was performed, as indicated in (40): an exponential culture of a specific strain was infected
341 with a phage and infection continued for 24 h; then the culture was centrifuged and the

supernatant used to re-inoculate a fresh bacterial culture. This procedure was repeated daily for six days. The EOP of the evolved phage present after six days was compared to the EOP of the ancestral phage used at the inoculum. If the EOP was improved, the phage was added to the collection. Despite several attempts, no phage able to infect strains PaPh23 and PaPh30 was isolated.

High-titer phage stock preparations. High-titer preparations of the phages were obtained by infection of 500 ml of liquid culture of PAO1 or PAK-lumi, as described in (21) with the following modifications: the lysates were filtrated with 1.2 μm diameter filters and incubated for 30 min at 37°C with DNase (1μg/ml) and RNase (1 μg/ml) before PEG precipitation. For *in vivo* experiments, phage lysates were purified by cesium chloride ultracentrifugation, as described in (41), and dialyzed against TN buffer (10 mM Tris, 150 mM NaCl, pH 7). Then, each phage preparation was passed through an endotoxin-removal column (EndoTrap HD, Hyglos, Germany) before measuring endotoxin level by the LAL Chromogenic Endotoxin Quantitation (Pierce). Levels of endotoxins of phage preparations were below the limit value recommended for intravenous administration (5.0 international units/kg body mass/hour (http://www.who.int/medicines/publications/pharmacopoeia/Bacterial-endotoxins_QAS11-452_FINAL_July12.pdf).

Plating efficiency. The plating efficiency of the isolated phages on clinical strains of *P. aeruginosa* was determined according to standard protocols: 5 μl of serial dilutions of a phage preparation were spotted on agar plates on which a specific bacterial host was spread. The number of plaques observed after overnight incubation were compared to the one obtained on strains PAO1 or PAK-lumi.

***In vitro* infection.** For determining the lysis kinetics of infected cultures, a culture of PAO1 or PAK-lumi strains in LD broth (42) at 37°C with shaking was infected at OD_{600} = 0.1 with each phage or with the 6-phage cocktail at MOI = 2.5, and the OD of the culture followed.

Sequencing and assembly of phage genome sequences and their screening for the presence of potential undesirable gene products.

Genomic DNA extracted from purified high titer phage preparations was subjected to standard Illumina library preparation protocols and sequenced on an Illumina Mi-Seq instrument at the CNR IBBIOM Institute in Bari (Italy) to generate paired end (2*250nt) sequence reads. Raw paired-end sequence data were subjected to stringent quality trimming and removal of library adapters using the Trimmomatic software (43) and assembled using SPAdes (v3.7.1) (44) using K-mer lengths of 75, 97 and 119.

Each genome was assembled as a single contig and deposited in GenBank. BLAST similarity searches of complete genome sequences against GenBank recovered high levels of identity and contiguity with previously sequenced genomes and allowed taxonomic assignment of each isolate (Table S4). An “in-house” database of undesirable genes was constructed by merging entries from the ACLAME database of mobile elements (19) whose descriptions contained any of the terms “integrase", "excisionase", "recombinase" or "repressor", with the Comprehensive Antibiotic Resistance Database (CARD) (45) and the Virulence Factor Database (VFDB) (46). All ORFs, with "ATG", "GTG" or "TTG" start codons were inferred from viral genome sequences using a custom python script and used as queries for BLASTX (47) searches (evalue cutoff 5e-04) against the undesirable gene database.

393 **Transduction assay.** Phages were tested for their ability to transduce the Tc^R marker
394 from PAO1Tc^R into wild-type PAO1. An aliquot (100 µl) of a high-titer lysate
395 (>1×10^{10} PFU/ml) obtained in PAO1Tc^R was used to infect a 10 ml overnight culture of the
396 recipient strain PAO1. After static incubation at room temperature for 30 min to allow
397 phage adsorption, the tubes were transferred to 37 °C for 20 min, the cells centrifuged and
398 the pelleted cells resuspended in 300 µl LB. Aliquots (150 µl) of the cell mixture were
399 spread onto two agar plates containing tetracycline (100 µl/ml). Frequency of transduction
400 was calculated as the ratio of Tc^R colonies transductants to the adsorbed phage.
401
402 **Composition of the phage cocktail.** Four new isolated *Pseudomonas* phages that
403 presented different and complementary host ranges were selected as constituents of the
404 phage cocktail. The four new isolated phages, were named according to a recent proposal
405 for a rational scheme for the nomenclature of viruses (48), vB_PaeP_PYO2,
406 vB_PaeP_DEV, vB_PaeM_E215, and vB_PaeM_E217, abbreviated in this paper in PYO2,
407 DEV, E215 and E217, respectively.
408
409 To these, phages PAK_P1 and PAK_P4, previously characterized for their therapeutic
410 efficacies on mice infections (13,21), were added: both are Myoviridae, with a head
411 diameter of 80 nm and a tail around 130 nm, and share no homologies with the other 4
412 phages in the cocktail. Their genome sequences (GenBank accession number KC862299
413 and KC862300, respectively) are 93,198 bp and 93,147 bp, respectively, with 93% identity.
414
415 Our final cocktail included these 6 phages: PYO2, DEV, E215, E217, PAK_P1 and
416 PAK_P4. The cocktail was composed by phages mixed at the same PFU/ml and prepared
417 immediately before each experiment to ensure accurate phage titers.
418

Biofilm disruption. Two methods were used to monitor biofilm disruption: fluorescent microscopy and crystal violet staining. For better visualization of the biofilms, we used either PAK-lumi or PAO1 strains transformed with plasmid tPUCP19-GFP that expresses high level of GFP. Biofilms of PAK-*gfp* and PAO1-*gfp* were grown in an 8-well chamber microscope slide (Nunc Lab-Tek Chamber Slide) for 48 h in 200 μl LD broth (42) at 37°C. Every 24 h the supernatant was gently removed and substituted with fresh LD broth. After 48 h, phages at $1x10^8$ PFU/ml were added and incubation at 37°C continued for 4 h. The supernatant containing the planktonic cells was removed, the slide gently washed, and examined with a Leica DMRB microscope equipped with standard fluorescence filters using a 100x objective. Images were acquired with a CCD video camera (Leica DCF 480).

For biofilm evaluation by crystal violet staining, an overnight culture of either *P. aeruginosa* PAO1 or a clinical isolate strain was diluted to OD_{600} about 0.02 in LD broth and 100 μl inoculated in 96-well polystyrene microtiter plates. The plates were incubated at 37°C for 24 h to allow biofilm formation. Broth containing planktonic cells was removed gently, the wells washed with 200 μl of LD, 120 μl of LD containing phage lysate at 10^8 PFU/ml added, and incubation continued for 4 h. After incubation the wells were carefully emptied and gently washed with H_2O. Bacteria adhering to the walls of the plate were stained with 150 μl of 0.1% crystal violet solution in H_2O for 20 min. After washing with tap water, the dye was eluted from the adherent biofilm with 150 μl 5% SDS and quantified by measuring the optical density of 10-fold dilution of the eluate at 600 nm. Each treatment was repeated in 18 wells, and the medium value and standard deviation calculated.

Animals and Ethics

Mice were housed under pathogen-free conditions with ad libitum access to food and water. Animal experiments were conducted in accordance with European directives on

444 animal protection and welfare, approved by the French Ministry of Education and
445 Research (Ref. #2015-0041) and Institut Pasteur (Ref. #10.565).
446
447 **Mouse acute respiratory infection.**
448 Female BALB/C mice between 8-12 weeks of age were anesthetized with 100 mg/kg
449 Ketamine and 10 mg/kg Xylazine. Subsequently, animals were intranasally infected with
450 $1x10^7$ CFU mid-log *P. aeruginosa* PAK-lumi suspended in 30 µl phosphate buffered saline
451 (PBS). After two hours post-infection (PI), lung infected mice were treated intranasally with
452 the 6–phage cocktail at the indicated PFU dose suspended in 30 µl PBS. The IVIS
453 Spectrum *in vivo* imaging system (PerkinElmer) was used to facilitate non-invasive
454 longitudinal monitoring of *P. aeruginosa* infection in live individual animals in real-time
455 performed as previously described (13,49,50).
456
457 ***Galleria mellonella* larvae systemic infection.**
458 A PAK-lumi culture was grown to OD_{600} = 0.5 in LD broth at 37°C with shaking, pelleted
459 and diluted to OD = 1 in physiological solution, equivalent to $1x10^9$ CFU/ml. After
460 appropriate dilution, 10 µl of inoculum, containing about 30 cells of *P. aeruginosa* PAK-
461 lumi, was delivered into the larvae haemolymph behind the last proleg. Phage suspension,
462 10 µl containing the 6-phage cocktail at 1500 or 4500 PFU, was delivered behind the last
463 proleg on the opposite site 1 h PI. For prophylaxis experiments, phages were infected 1 h
464 before bacteria. All experiments used 15 or 20 larvae. A positive control group (larvae
465 infected and treated with physiological solution) and two negative control groups (one
466 group injected with physiological solution only, and one group injected with phage
467 suspension only, assessing the toxicity of the phage cocktail) were also included. Larvae
468 were placed into Petri dishes and incubated at 37°C in the dark. Survival of larvae was
469 followed hourly after 16 h PI; larvae were recorded as dead when they did not move in

response to touch.

Phage treatment of larvae infected with clinical *P. aeruginosa* strains were performed after determination of the lethal dose of bacteria for each strain, equal to 110 or 30 CFU/larva for AA43 and PaPh5 strains, respectively. After 1 h from bacteria injection into the larvae, a fixed dose of phage cocktail (4500 PFU/larva) were injected.

Statistical analysis. The statistical analysis was performed using a Student's *t*-test or Two-way ANOVA with Tukey test or Chi square test, with Yates correction. *P* values for Kaplan-Meier curves were calculated with Mantel-Cox test. Statistical analysis was done using GraphPad software (http://www.graphpad.com/quickcalcs/).

Genome accession numbers. GenBank accession numbers: vB_PaeP_PYO2 (MF490236); vB_PaeP_DEV (MF490238); vB_PaeM_E215 (MF490241); vB_PaeM_E217 (MF490240); vB_PaeP_E220 (MF490237); vB_PaeS_S218 (MF490239).

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652

TABLES

Table 1. Efficiency of plating of single phages selected for the cocktail and of the cocktail[a].

BACTERIAL STRAIN	EFFICIENCY OF PLATING OF						
	PYO2	DEV	E215	E217	PAK_P1	PAK_P4	COCKTAIL
PAO1	+	+	+	+	-	-	+
PA14	+/-	-	-	-	-	-	-
PAK-lumi	+	+	+	+	+	+	+
PAO1 *pilA*	+	+	+	+	-	-	+
LESB58	-	+	-	-	-	-	-/+
E1	+/-	-	+	+	-	-	+
E2	+	+	-	-	-/+	-	+/-
E4	-	-	-	-	-	-	-
E5	-	-	-/+	-	-/+	-/+	-/+
E9	-	-	-	-	-/+	+/-	-/+
AG5	+	+	+	+	-/+	+/-	+/-
GS3	+	+	+	+/-	+	+	+
AA10	+	+	-	-	-	-	+/-
GJY9	-	+/-	-/+	-	-	-	-/+
CL1	-	+	+/-	-	-	-	+/-
CL2	-	-	+/-	+	-/+	-	-/+
VR8	+	+/-	+	+	+	-	+
AG6	-	-	+	-	-	-	+/-
DV4	+	+	-	-	-/+	+/-	+/-
GA7	+	-	-/+	+T	+	+	+
AA2	+/-	-	-/+	-/+	-	-/+	-/+
AA43	+	+/-	+	+	-	-	+
AA44	+	-/+	+/-	+	-	-	+/-
TR1	+	+	-	-/+	-	-	+
TR66	+	+/-	-	-	-	-	-/+
TR67	+	+	-	-	-	-	-
PaPh1	+/-T	+	-	-	-	-	-/+
PaPh2	-	-	-	+/-	-	-	-
PaPh3	+	+	-	-/+	+/-	-/+	+/-

BACTERIAL STRAIN	EFFICIENCY OF PLATING OF						
	PYO2	DEV	E215	E217	PAK_P1	PAK_P4	COCKTAIL
PaPh4	+	+	+	+	-	-	+
PaPh5	+	+	-/+	+	+	+/-	+
PaPh6	-/+	-	-	+	-	-	-
PaPh7	-	-	+	+	-	-	+
PaPh8	+T	+	-	-	+/-	+/-	+
PaPh9	-	-	+	+	-	-	+
PaPh10	-	-	-	-	-	-	-
PaPh11	-	-	+	+	-	-	+
PaPh12	+	+/-T	+	-/+T	-	-/+	+
PaPh13	-	-	-/+	-/+	-	-	-
PaPh14	+	+	+	+	-	-	+
PaPh15	-/+	+/-T	-	-	-	-	+/-
PaPh16	+	+	+	+	-	-	+
PaPh17	+	+	+	+	-	-	+
PaPh18	-	-/+	-	-	-	-	-
PaPh19	-	+T	-	-	-	-	-
PaPh20	-	-	+/-	-	-	-	+/-
PaPh21	-	-	+	-/+	-	-	+/-
PaPh23	-	-	-	-	-	-	-
PaPh24	+	+	-	+/-	+	+/-	+
PaPh25	+/-	+/-	-	+	+/-	-/+	+
PaPh26	-/+	+	-	-	-	-	-
PaPh27	-	-	+	+	-	-	+
PaPh28	-/+	-	+	+	-	-	+
PaPh29	-/+	-	-	-	-	-	-
PaPh30	-	-	-	-	-	-	-
PaPh31	-	-	-/+	-/+	-	-	-/+
PaPh32	-/+	-/+	+	+	+/-	+/-	+
PaPh33	+	+	-/+	-	+/-	-	+

[a]5 μl of ten-fold serial dilutions of the indicated single phages or of the phage cocktail were spotted on a lawn of each specific bacterial host; the plates were observed after overnight incubation at 37°C. (+) = EOP 1; (+/-) = EOP 10^{-1}-10^{-2}; (-/+) = EOP 10^{-3}; (-) = EOP <10^{-4}. T = turbid plaques.

663 **LEGEND TO FIGURES**

664 **Fig. 1. Growth kinetics of *P. aeruginosa* cells in liquid culture in presence of phages.**
665 Exponentially growing bacteria (OD_{600} = 0.1), either PAO1 **(A)** or PAK-lumi **(B)**, were
666 infected by the indicated phages, each at MOI of 2.5. For clarity, only one out of three
667 independent experiments is shown. The infection with E217 was repeated three times with
668 each strain, with superimposable results. **(C)** PAO1 and PAK-lumi infection with the 6-
669 phage cocktail (CK; total MOI=2.5). The average and SD of two independent experiments
670 are shown.

671

672 **Fig. 2. Disruption of the *P. aeruginosa* biofilm by the phage cocktail. (A)** 48 h biofilms
673 of PAO1-*gfp* and PAK-*gfp* without and after addition of the 6-phage cocktail (+ CK). **(B)** 24
674 h biofilms of indicated *P. aeruginosa* strains were exposed for 4 h to the phage cocktail.
675 Reduction of the biofilm biomass following phage treatment (+ CK) was compared with the
676 untreated strain by measuring the OD_{600} after crystal violet staining. The biofilm reduction
677 by the cocktail reached 62% for PAO1, 64% for PaPh5, 19% for AA43 (p=0.6), 37% for
678 PaPh32, and 66% for PAK-lumi. The error bars indicate standard deviations and statistical
679 significance of biofilm reduction (***p<0.001) was assessed by Student's t-test.

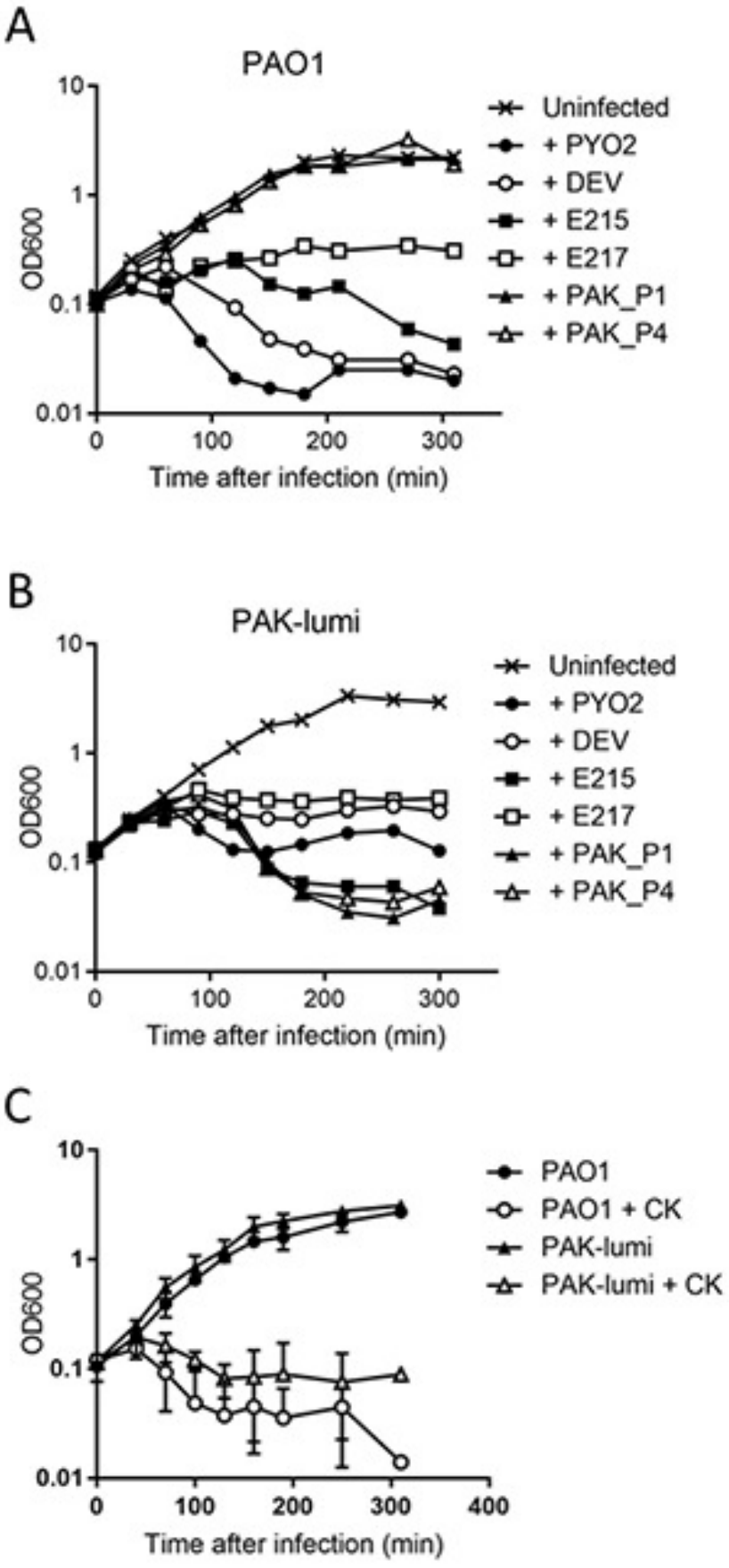
680

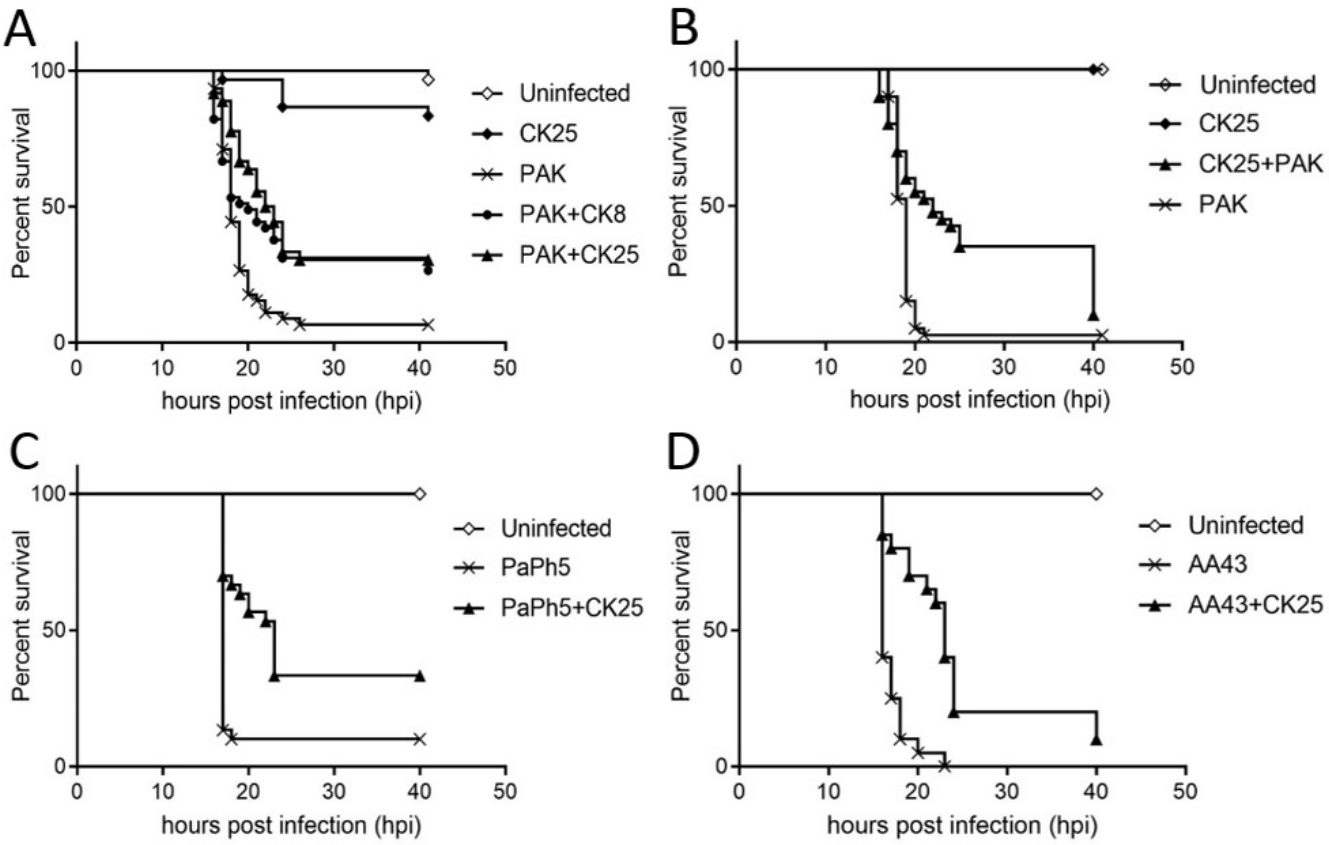
681 **Fig. 3. The phage cocktail fully cures acute respiratory infections in mice. (A)** Fatal
682 respiratory infections after mice were intranasally instilled with $1x10^7$ CFU of *P. aeruginosa*
683 strain PAK-lumi (n=5 for each treatment group and n=3 for untreated) were cured by
684 administration of the 6-phage cocktail (CK) 2 h post infection. CK consisted of a mixture of
685 six *Pseudomonas* phages each given at the MOI indicated (i.e. 0.05, 0.1, or 1.0). **(B)**
686 Photon emission of the chest area of infected mice quantified using an IVIS 100 imaging
687 system. Letters beside data points indicate 2way ANOVA significance with Tukey

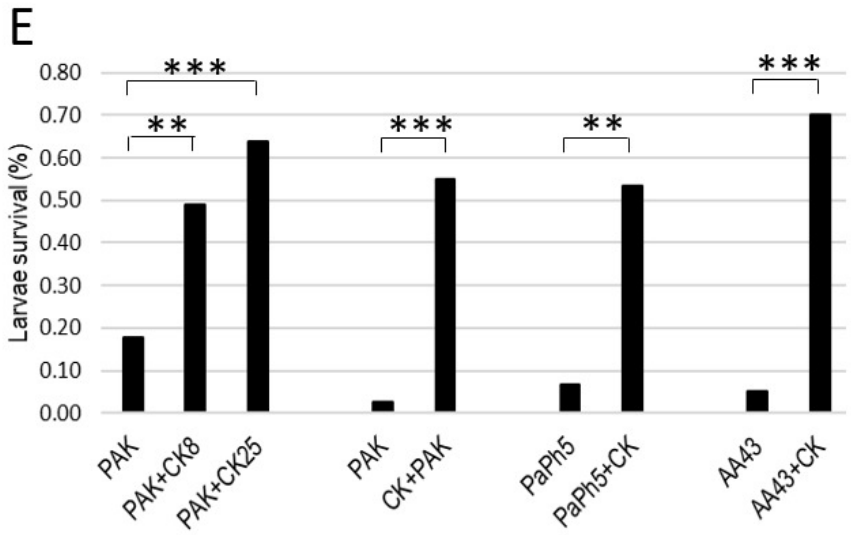
correction: a) PAK *vs.* PAK+CK1.0; p=0.0012, b) PAK *vs.* PAK+CK1.0; p<0.0001, c) PAK+CK0.05 *vs.* PAK+CK1.0; p=0.0406, d) PAK+CK0.1 *vs.* PAK+CK1.0; p=0.0365, e) PAK *vs.* PAK+CK0.05; p=0.0003, f) PAK *vs.* PAK+CK0.1; p=0.0008, g) PAK *vs.* PAK+CK1.0; p<0.0001, h) PAK *vs.* PAK+CK0.05; p<0.0001, i) PAK *vs.* PAK+CK0.1; p<0.0001, and j) PAK *vs.* PAK+CK1.0; p<0.0001.

Fig. 4. The phage cocktail prolongs survival of systemically infected *G. mellonella* larvae. (A) Kaplan-Meier survival curves of larvae infected with PAK-lumi (30 CFU/larva, n=45 per group) and treated 1 h after with PBS (PAK) or the 6-phage cocktail (PAK+CK) at two different MOI of 8 (PAK+CK8) and 25 (PAK+CK25). In addition, uninfected larvae received the MOI of 25 (CK25) dose. Pairwise comparisons between larvae and phage-treated infected larvae using Mantel-Cox test, indicated a significant difference (p<0.0001 for both CK8 and CK25). **(B)** Kaplan-Meier survival curves of MOI of 25 prophylactically treated larvae 1 h before PAK-lumi challenge (CK25+PAK) or PBS challenge (CK25) (n=20 per group). Pairwise comparisons between larvae and phage-treated infected larvae using Mantel-Cox test indicated a significant difference (p<0.0001). **(C)** and **(D)** Kaplan-Meier survival curves of larvae infected with PaPh5 (30 CFU/larva) or AA43 (110 CFU/larva) (n=35 per group) and treated 1 h after with PBS (PaPh5 and AA43) or the 6-phage cocktail (PaPh5+CK25 and AA43+CK25). Pairwise comparisons between larvae and phage-treated infected larvae using Mantel-Cox test indicated a significant difference (PaPh5 p<0.0001; AA43 p<0.0001). **(E)** Survival comparison of phage cocktail (CK) treatments efficacies at 20 h on larvae infected by the indicated strains. Statistical significance was assessed by Chi square test, with Yates correction when needed: PAK vs PAK+CK8, p <0.01; PAK vs PAK+CK25, p <0.0001; PAK vs CK+PAK, p < 0.0001; PaPh5 vs PaPh5+CK, p <0.01; AA43 vs AA43+CK, p <0.0001.

713







BACTERIAL STRAIN	EFFICIENCY OF PLATING OF						
	PYO2	DEV	E215	E217	PAK_P1	PAK_P4	COCKTAIL
PAO1	+	+	+	+	-	-	+
PA14	+/-	-	-	-	-	-	-
PAK-lumi	+	+	+	+	+	+	+
PAO1 *pilA*	+	+	+	+	-	-	+
LESB58	-	+	-	-	-	-	-/+
E1	+/-	-	+	+	-	-	+
E2	+	+	-	-	-/+	-	+/-
E4	-	-	-	-	-	-	-
E5	-	-	-/+	-	-/+	-/+	-/+
E9	-	-	-	-	-/+	+/-	-/+
AG5	+	+	+	+	-/+	+/-	+/-
GS3	+	+	+	+/-	+	+	+
AA10	+	+	-	-	-	-	+/-
GJY9	-	+/-	-/+	-	-	-	-/+
CL1	-	+	+/-	-	-	-	+/-
CL2	-	-	+/-	+	-/+	-	-/+
VR8	+	+/-	+	+	+	-	+
AG6	-	-	+	-	-	-	+/-
DV4	+	+	-	-	-/+	+/-	+/-
GA7	+	-	-/+	+T	+	+	+
AA2	+/-	-	-/+	-/+	-	-/+	-/+
AA43	+	+/-	+	+	-	-	+
AA44	+	-/+	+/-	+	-	-	+/-
TR1	+	+	-	-/+	-	-	+
TR66	+	+/-	-	-	-	-	-/+
TR67	+	+	-	-	-	-	-
PaPh1	+/-T	+	-	-	-	-	-/+
PaPh2	-	-	-	+/-	-	-	-
PaPh3	+	+	-	-/+	+/-	-/+	+/-

BACTERIAL STRAIN	EFFICIENCY OF PLATING OF						
	PYO2	DEV	E215	E217	PAK_P1	PAK_P4	COCKTAIL
PaPh4	+	+	+	+	-	-	+
PaPh5	+	+	-/+	+	+	+/-	+
PaPh6	-/+	-	-	+	-	-	-
PaPh7	-	-	+	+	-	-	+
PaPh8	+T	+	-	-	+/-	+/-	+
PaPh9	-	-	+	+	-	-	+
PaPh10	-	-	-	-	-	-	-
PaPh11	-	-	+	+	-	-	+
PaPh12	+	+/-T	+	-/+T	-	-/+	+
PaPh13	-	-	-/+	-/+	-	-	-
PaPh14	+	+	+	+	-	-	+
PaPh15	-/+	+/-T	-	-	-	-	+/-
PaPh16	+	+	+	+	-	-	+
PaPh17	+	+	+	+	-	-	+
PaPh18	-	-/+	-	-	-	-	-
PaPh19	-	+T	-	-	-	-	-
PaPh20	-	-	+/-	-	-	-	+/-
PaPh21	-	-	+	-/+	-	-	+/-
PaPh23	-	-	-	-	-	-	-
PaPh24	+	+	-	+/-	+	+/-	+
PaPh25	+/-	+/-	-	+	+/-	-/+	+
PaPh26	-/+	+	-	-	-	-	-
PaPh27	-	-	+	+	-	-	+
PaPh28	-/+	-	+	+	-	-	+
PaPh29	-/+	-	-	-	-	-	-
PaPh30	-	-	-	-	-	-	-
PaPh31	-	-	-/+	-/+	-	-	-/+
PaPh32	-/+	-/+	+	+	+/-	+/-	+
PaPh33	+	+	-/+	-	+/-	-	+