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**Two examples of exploiting bacteriophages to defeat
Pseudomonas aeruginosa: study of the viral protein
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bacteriophage cocktail to treat pneumonia in
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Mathieu De Jode

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Mathieu De Jode. Two examples of exploiting bacteriophages to defeat Pseudomonas aeruginosa : study of the viral protein Gp92 and in vivo evaluation of the efficacy of a bacteriophage cocktail to treat pneumonia in immunocompromised animals. Bacteriology. Sorbonne Université, 2021. English. NNT : 2021SORUS030 . tel-03435147

HAL Id: tel-03435147

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Sorbonne Université

Ecole doctorale 515 Complexité du vivant

Laboratoire Bactériophage Bactérie Hôte

**Two examples of exploiting bacteriophages to defeat
Pseudomonas aeruginosa: study of the viral protein Gp92
and *in vivo* evaluation of the efficacy of a bacteriophage
cocktail to treat pneumonia in immunocompromised animals.**

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Thèse de doctorat de Microbiologie

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Présentée et soutenue publiquement le 30/06/2021

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Acknowledgments/Remerciements

First, I would like to thank all the members of the jury for their time and consideration.

Je tiens à remercier particulièrement mon directeur de thèse, Laurent Debarbieux, pour m'avoir offert la possibilité de travailler sur les phages, un sujet qui me passionnait déjà des années avant ma thèse. Merci pour cette occasion, merci pour ta disponibilité, et pour la confiance et la liberté que tu m'as accordé.

Je souhaite bien sûr remercier tous les membres de notre équipe. Merci à Raphaëlle, Quentin, Baptiste, Lorenzo, Marta et Rokhaya pour toutes nos discussions scientifiques mais aussi, et surtout, pour des moments de détente bien mérité. Merci à Luisa pour tes conseils précieux que je garde en mémoire. Merci à Thierry, tu m'as beaucoup appris, et à Sophia pour ton aide sur mon projet.

J'adresse aussi mes remerciements à mes collaborateurs de l'Institut Pasteur pour leur aide précieuse : à Marc Monot pour m'avoir appris les ficelles de la qPCR, à Quentin Gaii-Gianetto pour sa patience lors de l'analyse statistique des données de spectrométrie de masse, et enfin à Ioanna Theodorou pour son assistance à l'IVIS.

Merci à tous mes amis qui m'ont soutenu, ceux de Pasteur (Emy, Flo, PhDnD, StaPa) qui partageaient mon quotidien, mais aussi ceux de la fac (team corsica), de la P1 (Gergy !) et du lycée, qui me supportent pour les plus anciens depuis plus de 10 ans...

Je tiens à remercier mes parents qui m'ont donné l'envie et les moyens de poursuivre la recherche scientifique.

Enfin, je tiens à remercier ma copine pour son incroyable patience et son soutien à tout épreuve qui furent d'un grand secours lors de cette dernière année de thèse pour le moins éprouvante.

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Introduction

Chapter I: *Pseudomonas aeruginosa* infections, a growing health problem.

1- A Versatile Pathogen

Pseudomonas aeruginosa is a Gram negative bacillus present in diverse ecological niches: aquatic environments, soil, plants and even animals such as birds and mammals have been described as reservoirs [1]. Other surprising niches like petroleum [2] or chlorinated water [3] demonstrate the adaptation capabilities of this bacterium. *P. aeruginosa* is also an opportunistic pathogen for humans, infecting mostly individuals with a compromised immune system [4]. For example, it is a major predictor of morbidity and mortality in cystic fibrosis (CF) patients as it causes severe chronic lung infections [5]. It was also shown to cause infections in humans with MyD88 deficiency, a key downstream adapter for most Toll-like and interleukin-1 receptors [6]. Over the years, numerous risk factors have been identified to influence the acquisition of *P. aeruginosa* infections (see Table 1), which can be local or systemic, benign or life-threatening. Indeed, *P. aeruginosa* is the leading cause of otitis for swimmers [7], the most frequent pathogen in burn wounds [8] and in ventilated patients in intensive care units [9], and has recently become one of the most frequent causative agents of lethal nosocomial infections [10]. Unfortunately, this pathogen is also increasingly resistant to classical antibiotic therapies and isolates resistant to carbapenems were ranked by the World Health Organization (WHO) as a critical priority pathogen for which new treatments are urgently needed [11].

Main risk factors for acquiring <i>P. aeruginosa</i> infections		
Physiological factors	Medical Procedures	Others
Neutropenia	Hospitalization	Burns
Acquired Immunodeficiency Syndrome (AIDS)	Mechanical ventilation	Severe external injuries
Innate immunodeficiency (e.g. MyD88)	Surgery	Poor living conditions
Heart diseases	Catheterization	Malnutrition
Chronic Obstructive Pulmonary Disease (COPD)	Immunosuppressive therapy	Intravenous drug use
Cystic Fibrosis (CF)	Cancer chemotherapy	
Diabetes mellitus	Radiotherapy	

Table 1 : Main risk factors for acquiring *P. aeruginosa* infections (adapted from [12])

P. aeruginosa can colonize a large range of environments and cause a wide array of infections. This versatility stands from its large genome (5.5 to 7Mbp) which includes more than 500 regulatory genes, an arsenal of virulence factors and a multitude of drug-resistance mechanisms [13].

2- The Main Weapons of *P. aeruginosa*

A) First Contacts

Motility and adhesion are amongst the first properties that *P. aeruginosa* exploit to initiate an infection. To these ends, the pathogen relies on two complex extracellular apparatus the flagellum and the type IV pili. *P. aeruginosa* possesses a single polar flagellum responsible for "swimming" and also involved in "swarming" motilities [14]. It also participates in adhesion to epithelial cells [15]. The flagellum is highly immunogenic and induces strong inflammatory response as it is recognized by Toll-like receptor (TLR) TLR5 [16]. *P. aeruginosa*'s motility also relies on the type IV pili required for "twitching" [17] and "swarming" motilities [14]. This type IV pili is also *P. aeruginosa*'s main adhesine, involved in binding to epithelial cells [18]. Adhesion was also reported to be carried by adhesins such as the lectins LecA and LecB. Both are important virulence factors as, *in vitro*, *lecA* and *lecB* mutants were associated with decreased cytotoxicity and adhesion on A549 cells compared to the ancestral strain. Moreover, *in vivo* (murine acute lung injury model), the increase in alveolar barrier permeability was reduced with both mutants, as were bacterial burden and dissemination, compared with the parental strain. Finally, coadministration of specific lectin inhibitors (α -methyl-galactoside and α -methyl-fucoside) markedly reduced lung injury and mortality [19]. However, these effects might not be due to adhesive properties of lectins as a group found that neither lectin was involved in adhesion to human tracheobronchial mucin [20]. They proposed that the contributions of lectins to *P. aeruginosa*'s virulence reported in the literature might be due to secondary effects on other systems rather than effects of the lectins themselves (*e.g.* LecB proteolytic activity and role in pilus biogenesis).

B) Type III Secretion System: Shooting Toxins in Cells

Once *P. aeruginosa* is in contact with host cells it injects toxins directly in their cytosol. This process relies mainly on the Type III Secretion System (T3SS), a major virulence factor [21]. A study looking at 108 *P. aeruginosa* clinical isolates (from respiratory tract infections) found that the relative risk of mortality was 6-fold higher when T3SS toxins were produced [22]. This T3SS allows *P. aeruginosa* to secrete 4 toxins: ExoS, ExoT, ExoU and ExoY. Both ExoS and ExoT are Rho GTPase-activating proteins (RhoGAP) and ADP-ribosyltransferases (ADPRT). These toxins are able to disrupt actin filaments [23] and block the reactive oxygen species burst in neutrophils [24]. Moreover, ExoT was shown to elicit inhibition of internalization of *P. aeruginosa* by epithelial cells and macrophages [25] and promotes the apoptosis of host cells [26]. Expression of the phospholipase A2 ExoU is associated with a cytotoxic effect on epithelial cells *in vitro*, and increases virulence in mice [27, 28]. Its toxicity relies on host cell membrane disruption [29]. Note that ExoS and ExoU are almost mutually exclusive in *P. aeruginosa*, with most strains being ExoS-positive, few strains being ExoU-positive, and barely any strains having both [30]. Finally, ExoY is an extra-cellular nucleotidyl cyclase, homologous to other virulence factors (CyaA from *Bordetella pertussis* or EF from *Bacillus anthracis*) [31] and was shown to disrupt the actin cytoskeleton of epithelial cells *in vitro* [32]. Recently, secretion of ExoY was shown to be associated with production of cUMP, higher prevalence of cell apoptosis, and a break of lung barrier integrity in mice [33], but its precise function in *P. aeruginosa* pathogenesis still needs investigation.

C) Virulence Without a T3SS

Some *P. aeruginosa* strains lack the T3SS but are still causing infections. For example, the clinical strain CLJ1 was able to cause fatal hemorrhage in a mouse model of acute pneumonia. This phenotype was associated with the expression of ExlA and ExlB. ExlA is a pore-forming toxin, secreted with the porin ExlB, via a Two-Partner Secretion System (TPSS). These two proteins are required and sufficient to provoke membrane permeabilization of human endothelial cells and macrophages (*in vitro*) and fatal hemorrhage in mice [34]. Furthermore, a strain lacking the T3SS is still able to inject toxins directly in the cytosol of cells via the Type VI Secretion System (T6SS). Indeed, the T6SS, which structurally resembles a bacteriophage tail [35], is not only able to inject toxins in other bacterial cells, but also into eukaryotic cells,

delivering what is known as “Trans-Kingdoms Toxins”: PldA, PldB and TplE. Both PldA and PldB are phospholipases D exhibiting antibacterial properties and facilitating intracellular invasion of eukaryotic cells (*in vitro*) by activation of the PI3K/Akt pathway [36]. TplE, a phospholipase A1, is able to disrupt the endoplasmic reticulum, thus promoting autophagy by the activation of the unfolded protein response [37].

D) Type II Secretion System: Shooting Toxins Next to Cells

Another toxin of interest is ToxA, the most toxic protein secreted by *P. aeruginosa* with a Lethal Dose 50 (LD50) of 0.2 mg in mice [38]. While being secreted in the extracellular milieu by the type II secretion system (T2SS), ToxA has an intracellular target. Indeed, this toxin with ADPRT activity mediates its entry into target host cells through its cell-binding domain, then ADP-ribosylates host elongation factor 2 to block protein synthesis [39, 40]. The T2SS is also known to secrete diverse proteases involved in tissue invasion including LasA, LasB, and PrpI. Both LasA and LasB are metalloproteases. LasA has low elastolytic and high staphylolytic activities [41], it also highly increases the elastolytic activity of LasB [42]. LasB has a strong elastolytic activity and allows for tissue invasion by increasing membrane permeability of epithelial cells. LasB also inactivates immunoglobulin A (IgA), IgG and complement components [43-46]. The loss of either LasB or LasA significantly decreases the ability of *P. aeruginosa* to invade epithelial cells *in vitro* and the loss of both proteases leads to an even lower invasion phenotype [47]. PrpI is another T2SS secreted protease (a lysine endoproteinase) that degrades proteins such as complement components, immunoglobulins, elastin, lactoferrin, and transferrin [48].

E) Next Target: Lipids

A different virulence strategy of *P. aeruginosa* relies on its ability to degrade host cell phospholipids. In addition to the previously mentioned phospholipases (PldA, PldB and PrpI), *P. aeruginosa* uses the hemolytic phospholipase C PlcH (secreted by the T2SS). PlcH can degrade phospholipids (phosphatidylcholine and sphingomyelin) found in eukaryotic membranes and in lung surfactant [49] and suppresses bacterium-induced neutrophil respiratory burst [50]. Furthermore, to increase the efficacy of its phospholipases, *P. aeruginosa* produces extracellular glycolipids called rhamnolipids. Indeed, these rhamnolipids act as detergents on

the phospholipids of the lung surfactant [51], disrupt mucociliary transport and ciliary beating [52], and inhibit phagocytosis [53].

F) Iron Is All I Need

Once host cells have been destroyed, *P. aeruginosa* has the ability to retrieve important nutrients, molecules and cofactors necessary to its growth, including iron. Iron is an essential cofactor of many proteins (iron–sulfur cluster-containing proteins, enzymes in mitochondrial respiration...) and is critical to *P. aeruginosa* physiology [54]. To capture this essential metal, *P. aeruginosa* uses iron chelating molecules named siderophores: pyoverdinin and pyochelin. Both siderophores have been shown to be essential virulence factors in diverse mice infection models [55-57]. *P. aeruginosa* can also get iron via the uptake of siderophores produced by other bacteria (xenosiderophores), or via the uptake of heme molecules from the host hemoproteins, or by using phenazine compounds. Phenazine-1-carboxylic acid (PCA) is the precursor of pyocyanin, the blue-green compound typical of *P. aeruginosa* (this bacterium used to be nicknamed “pyocyanic bacilli”). PCA is able to reduce Fe³⁺ bound to host proteins into Fe²⁺, allowing the uptake of iron via the Feo system [58]. If pyocyanin is less potent than PCA for iron-uptake, it nevertheless plays other roles during the infection. Indeed, *in vitro* studies have shown that pyocyanin inhibits cell respiration, ciliary function, epidermal cell growth, and also induces neutrophils apoptosis [59-61]. Moreover, it was shown to be a major virulence factor *in vivo* [62].

G) LPS: Smooth or Rough

Overall, *P. aeruginosa* infections are characterized by severe tissue damages and a strong inflammatory response. The immune system recognizes different components of the bacterial envelope via Toll like Receptors (TLRs). More specifically, the lipopolysaccharide (LPS), peptidoglycan and flagellin are recognized by TLR4, TLR2 and TLR5, respectively, driving the induction of pro-inflammatory cytokines and type I interferon (IFN) responses [63]. The LPS is composed of 3 parts: the lipid A, which can overstimulate the immune system and lead to a fatal “septic shock” [64]; the oligosaccharide core; and the O-antigen side chain, a variable part that can be present (smooth LPS) or absent (rough LPS). A *P. aeruginosa* strain with smooth LPS was shown to be significantly more virulent in mice than its rough LPS mutant

[65]. This lower virulence might be related to the involvement of LPS in *P. aeruginosa* motility. Indeed, both “swimming” and “swarming” motilities were impaired in mutants that lacked smooth LPS [14, 66]. Furthermore, *P. aeruginosa* strains with rough LPS are sensitive to human serum whereas strains with smooth LPS are not [67].

H) Hacking the Immune System

Infections by *P. aeruginosa* are characterized by a highly inflammatory immune response. Unfortunately, *P. aeruginosa* can efficiently disrupt this immune response. Indeed, numerous virulence factors previously mentioned (**Table 2**) degrade proteins of the immune system (*e.g.* immunoglobulins, complement components...), inhibit antibacterial cell functions (*e.g.* oxidative burst, phagocytosis...) but also disturb cytokine signaling. For example, the alkaline protease ArpA (secreted by the Type I Secretion System) and the elastase LasB, can inactivate human interferon (IFN)- γ , tumor necrosis factor (TNF)- α , and degrade two key pro-inflammatory cytokines, IL-6 and IL-8 as well [68-70]. These two proteases also inhibit neutrophils function by interfering with their chemotaxis [71], reduce phagocytic activities of leukocytes [72], inhibit natural killers [73] and disrupt lymphocytes via degradation of interleukin 2 [74].

Name	Product type	Secretion type	Role
ArpA	Metalloprotease	I	immune system inhibition
ExlA	Pore-forming toxin	TPSS	antiphagocytosis, cytotoxic
ExoS	RhoGAP and ADPRT	III	antiphagocytosis
ExoT	RhoGAP and ADPRT	III	antiphagocytosis, cytotoxic
ExoU	Phospholipase A2	III	cytotoxic
ExoY	Nucleotidyl cyclase	III	cytotoxic
Flagellum	Motility apparatus	-	motility, adhesion, tissue invasion
LasA	Metalloprotease	II	tissue invasion, immune system inhibition
LasB	Metalloprotease	II	tissue invasion, immune system inhibition
LecA	Lectin (binds to galactose)	-	adhesion, tissue invasion
LecB	Lectin (binds to mannose)	-	adhesion, tissue invasion
LPS	Exopolysaccharide	-	motility, inflammation
PCA	Phenazine	II	iron scavenging
PlcH	Phospholipase C	II	tissue invasion, immune system inhibition
PldA	Phospholipase D	VI	tissue invasion
PldB	Phospholipase D	VI	tissue invasion
PrpL	Lysine endoproteinase	II	tissue invasion, immune system inhibition
Pyochelin	Siderophore	-	iron scavenging
Pyocyanin	Phenazine	II	cytotoxic, immune system inhibition
Pyoverdin	Siderophore	-	iron scavenging
Rhamnolipids	Extracellular glycolipids	-	cytotoxic, antiphagocytosis
Tox A	ADPRT	II	cytotoxic
TpIE	Phospholipase A1	VI	cytotoxic
Type IV pilus	Motility apparatus	-	motility, adhesion
Alginate	Exopolysaccharide	-	resistance to immune cells killing

Table 2 : List of the main virulence factors of *P. aeruginosa*

To summarize, *P. aeruginosa* possesses a large arsenal of virulence factors allowing it to destroy cells, invade tissues, and protect itself against immune defenses. Additionally, *P. aeruginosa* primarily targets individuals with altered immunity, making self-healing unlikely. Treatment options for *P. aeruginosa* infections are relatively limited as this pathogen is intrinsically resistant to multiple drugs and was found to acquire resistance against others, strongly reducing the number highly efficient drugs available.

3- Current antibiotic treatments and associated resistance mechanisms

A) Antibiotics Against *P. aeruginosa*: Choose Carefully

P. aeruginosa is intrinsically resistant to oxazolidinones, macrolides, lincosamides, streptogramins, daptomycin, glycopeptides, rifampin, trimethoprim-sulfamethoxazole, tetracycline, some β -lactams (aminopenicillins with or without β -lactamase-inhibitors, as well as 1st and 2nd generation cephalosporins) [12]. This impressive drug-resistance is due to a particularly low membrane permeability (12 to 100 fold lower than *Escherichia coli* permeability) associated with several efficient efflux systems and antibiotic inactivating enzymes [75]. Luckily, some antibiotics can still affect most *P. aeruginosa* isolates: fluoroquinolones, aminoglycosides, polymyxins, and some β -lactams (3rd and 4th generation cephalosporins, monobactams, carbapenems, and novel β -lactam/ β -lactamase inhibitor combinations) [12]. Unfortunately, the overuse of these drugs can lead to the selection of mutations increasing *P. aeruginosa* antibiotic resistance. Moreover, this pathogen can acquire additional resistance mechanisms through acquisition of mobile genetic elements like plasmids.

B) All the Roads Lead to Resistance

The membrane of *P. aeruginosa* is the first line of defense against antibiotics. As the efficient diffusion/uptake of antibiotics in *P. aeruginosa* require specific porin or transporters, resistance can be acquired via mutations in these components: *e.g.* β -lactam antibiotics and fluoroquinolones enter bacterial cells through porin channels, and the loss of the OprD porin leads to carbapenem resistance [76, 77]. Likewise, aminoglycosides uptake involves oxygen- or nitrogen-dependent electron transport chains, and it was shown that absence of oxygen (*e.g.* inside biofilms) or functional deficiency of ATPases contribute to resistance to these antibiotics [78, 79]; Finally, colistin (polymyxin) promotes its own uptake by interacting with the LPS, and modification of the lipid A part contributes to polymyxin resistance [80, 81].

Even when a drug successfully reaches the cytoplasm of *P. aeruginosa* cells, it can be rapidly expelled by efflux systems. *P. aeruginosa* possesses multiple efflux systems that undeniably play a critical role in its intrinsic resistance to different antibiotics including β -lactams, tetracycline, chloramphenicol, and fluoroquinolones [82, 83]. Overexpression of some

of these efflux systems (by mutations in their regulators) further contribute to the multi-drug-resistance (MDR) phenotype of *P. aeruginosa*: e.g. overexpression of MexAB-OprM leads to increased resistance to β -lactams and quinolones [84, 85].

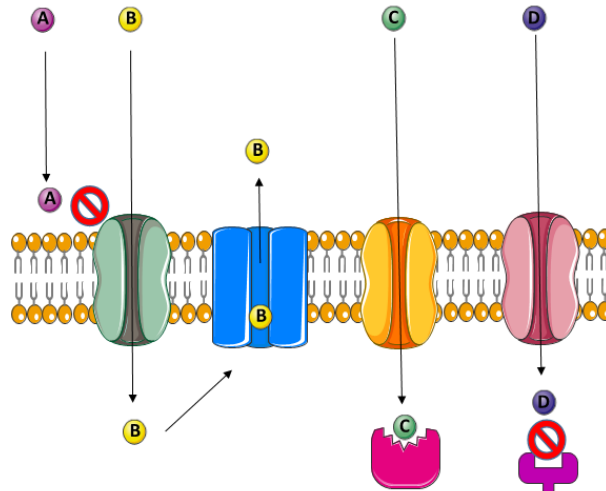


Figure 1 : The Antibiotic Resistance Mechanisms. Different antibiotics are represented by letters. A cannot diffuse through the membrane; B diffuses through the membrane but is extruded from the cell by an efflux system; C diffuses through the membrane but is inactivated by an enzyme; D diffuses through the membrane, but its target has been modified.

Moreover, drugs accumulating in *P. aeruginosa* cells can be inactivated by specific enzymes. For example, most *P. aeruginosa* strains encode an inducible AmpC β -lactamase unaffected by β -lactams inhibitors such as clavulanic acid [86]. Once again, the efficacy of this intrinsic resistance factor can be optimized by mutations leading to its overexpression, thus increasing further β -lactams resistance [87]. *P. aeruginosa* can also gain additional β -lactamases via plasmids, the most worrying being carbapenemases. The first carbapenemase (IMP-1) observed in *P. aeruginosa* was reported in a study of carbapenem-resistant clinical isolates in the 1990's in Japan [88]. This study showed that 11% of the strains harbored the *blaIMP-1* gene on a large plasmid (36 kb). Resistance via antibiotic targeting enzymes is a major mechanism for β -lactam resistance, but also for aminoglycoside resistance [89]. Indeed, aminoglycoside-modifying enzymes (AMEs) can phosphorylate, adenylate or acetylate this molecule, thus decreasing the binding affinity of the antibiotic to its bacterial target (the 30S ribosomal subunit) [90].

Ultimately, if a drug can avoid all the previously mentioned resistance mechanisms, *P. aeruginosa* might still block its toxic effect via “target modification”. This resistance mechanism is characterized by mutations in the gene coding for the antibiotic target, lowering affinity for the drug. Note that this is a major mechanism for fluoroquinolones resistance, as observed with mutations in the *gyrA* and *gyrB* genes. These mutations lead to the synthesis of modified topoisomerase II with low binding affinity to quinolone molecules. Resistance through modifications in another target of fluoroquinolones (topoisomerase IV) can also occur via point mutations in the *parC* and *parE* genes [91].

C) Biofilms: United We Stand

Another completely different strategy used by *P. aeruginosa* to resist antibiotics is the formation of biofilms. Indeed, bacteria attached to surfaces (medical devices, tissues...) can aggregate and produce a hydrated polymeric matrix (composed of exopolysaccharides, DNA and proteins) to form biofilms [92, 93]. In this biofilm form, bacteria can become 10 to a 1000 times more resistant to antimicrobials than in their planktonic form [94]. These biofilms have a complex architecture/physiology that resemble tissues of higher organisms [95]. For instance, a spatial transcriptomic study revealed that cells in the different layers of a *P. aeruginosa* biofilm have different gene expression profiles [96]. Surprisingly, when comparing free-living *P. aeruginosa* cells and those in biofilms, Whiteley *et al* [97] found that only 1% of genes showed differential expression. Additionally, both density [97] and composition [98] of *P. aeruginosa* biofilms have been showed to be important for Tobramycin resistance. Finally, *P. aeruginosa* biofilms have been observed in CF patients where they cause chronic infections [99, 100], display an increased tolerance to antibiotics, resist phagocytosis and other components of the immune system as well [101].

As *P. aeruginosa* is one of the primary causes of hospital acquired infections (isolated from 28.7% of intensive care unit acquired infections [102]), a decrease of treatment efficacy will result in a sizeable increase in mortality. Consequently, the growing prevalence of multidrug resistant *P. aeruginosa* strains has recently been declared a major health issue by the WHO [11]. On the other hand, this evolution of *P. aeruginosa* towards multi-drug resistance has been a serious concern for decades in the care of CF patients. Indeed, this pathogen infects around 35% of CF patients overall, and the prevalence is above 50% in patients over 27 years

old [103]. Since chronic airway infection is the most important cause of morbidity and mortality in CF patients, *P. aeruginosa* has been extensively studied in this environment.

4- *P. aeruginosa* in Cystic Fibrosis: From Acute to Chronic Infections.

A) Cystic Fibrosis Lungs: a Bacterial Paradise

CF is the most common life-shortening autosomal recessive disorder for Caucasian (affecting 1 in 2500 newborns), and is caused by mutations in the CF-transmembrane conductance regulator (CFTR) [104]. These mutations (over 300 have been documented by the CF Genetic Analysis Consortium) lead to a defective chloride (Cl⁻) channel, which disrupts electrolyte secretion, thus increasing the viscosity of the mucus secreted at the surface of epithelial cells and affecting osmolarity [104]. The main lung defenses against bacterial colonization (mucociliary clearance, polymorphonuclear neutrophil phagocytosis, and local production of antibacterial cationic peptides) are not performing efficiently in the CF environment, resulting in chronic bacterial infections [105, 106]. Nonetheless, in this specific environment, bacteria must survive the limitations in growth factors, dehydration, the host's immune defenses, and the yearlong antibiotic therapies. Most CF patients become permanently colonized by *P. aeruginosa*, thus the same bacterial lineage can persist in the lungs for years or even decades [107]. With the advent of cheaper genome sequencing technologies, evolution of *P. aeruginosa* in the lungs of CF patients has been extensively studied and genetic basis of several phenotypes have now been identified.

B) Mucoidy Origins

The most characterized phenotype of *P. aeruginosa* isolates from CF patients is mucoidy. In the 1960's Doggett *et al* reported “An atypical *Pseudomonas aeruginosa* associated with cystic fibrosis” [108, 109]. This “atypical” *P. aeruginosa* was defined by an uncommon aspect on agar plates and liquid cultures [108]. This mucoid phenotype corresponds to an overproduction of alginate (an exopolysaccharide). It was then reported that this mucoid phenotype was absent from recently *P. aeruginosa*-infected CF patients, whereas it was present in patients with chronic infection [110]. Later, the mechanism of mucoidy conversion was identified by Martin *et al* [111]. They discovered that a mutation in the *mucA* gene leads to a

loss of function of this inhibitor of the sigma factor AlgU, the latter regulating the transcription of genes involved in the alginate biosynthetic pathway. The couple MucA/AlgU, localized at the cytoplasmic membrane, controls the expression of more than 200 genes related to membrane homeostasis (membrane proteins, LPS, peptidoglycan synthesis...). Its activation upon membrane stress, starts with the proteolysis of MucA catalyzed by AlgW. Once free from its anti-sigma factor MucA, AlgU binds to the RNA polymerase and direct the transcription of its regulon [112]. Recently a portion of *mucA* was shown to be essential in *P. aeruginosa* [113]. Indeed, overexpression of *algU* in the absence of MucA prevents cell growth, and this phenotype can be rescued by the overproduction of RpoD (the housekeeping sigma factor).

In CF strains, mutations of *mucA* are commonly found, which implies an important role for alginate overproduction in *P. aeruginosa*'s adaptation to the CF lung environment. This role might be to protect *P. aeruginosa* from the constant inflammation in the lungs as it increases resistance to antibody-independent opsonic killing [114], confers resistance to phagocyte-generated hypochlorite [115], decreases phagocytosis of planktonic mucoid bacteria by neutrophils and macrophages [116], and reduces polymorphonuclear chemotaxis while inhibiting activation of the complement system [117]. Additionally, alginate plays a role in antibiotic resistance since biofilms formed by an alginate-overproducing strain were shown to be more resistant to tobramycin than biofilms formed by an isogenic non-mucoid strain [118]. Conversely, the use of alginate lyase proved to increase antibiotic efficacy against mucoid *P. aeruginosa* biofilms [119].

C) Evolution of *P. aeruginosa* in CF: Less Virulence, More Resistance

Longitudinal studies of *P. aeruginosa*'s genome throughout years of lung infection in CF patients, shined a light on its evolution in this environment. Such a study of the phenotypic and genotypic changes in *P. aeruginosa* isolates, was performed in a cohort of 40 CF children during the first 3 years of life [120]. A high degree of genotypic variability was found as each patient had unique genotypes. Surprisingly, the early isolates were generally non-mucoid and antibiotic susceptible, which is unusual for CF isolates. These results support the hypothesis of an initial infection by "classical" non-mucoid *P. aeruginosa* strains which then evolve during the chronic infection to become the mucoid "CF" strain.

In another study, Smith *et al.* [121] used whole-genome sequencing to investigate from the same patient genomic adaptations of *P. aeruginosa* isolates 7.5 years apart (early vs chronic). The analysis revealed 68 mutations in the late isolate, mostly single-base pair changes, and half of them predicted to result in change or loss of protein function. Remarkably, 13 out of the 68 mutated genes were coding virulence factors and regulators, including genes related to O-antigen biosynthesis, T3SS, twitching motility, regulation of exotoxin A, phenazine biosynthesis, quorum sensing, and iron acquisition. These mutations were then confirmed phenotypically, as loss of serotype-specific antigenicity, loss of twitching motility, loss of pyoverdinin production, loss of secreted protease activities, and reduced biofilm formation were observed. In addition, numerous efflux systems were mutated in the late isolate, which might explain the higher level of resistance of this strain to aminoglycosides (compare to the early strain). Moreover, by analyzing multiple samples from the same patient, at each time point, the authors found that multiple related lineages coexisted over the course of this patient's infection. For example, *mucA* mutations arose independently in three different lineages. Isolates from a single time point also had heterogeneous genotypes. Finally, they investigated whether the identified mutations were common in CF patients by genotyping paired (early and late) *P. aeruginosa* isolates from 29 other patients and found that *mexZ* (repressor of MexXY-OprM) and *lasR* (a quorum sensing regulator associated with the regulation of many virulence factors) were mutated in most CF patients. In a following study [122] analyzing the same set of strains, the authors found that the observed mutations were significantly concentrated in “mutator lineages” (defined as strain deficient for DNA mismatch repair system). Furthermore, they estimated that non-mutator lineages acquired a median of only 0.25 mutation per year of infection, while this rate was over 3 mutations per year in mutator lineages. These hypermutable *P. aeruginosa* were found to be prevalent in CF as another study reported 36% of the patients were colonized with a mutator strain [123]. This study also noted that mutator CF strains were significantly more resistant to antibiotics than their non-mutator counterpart.

To summarize, in CF patients, it seems that the initial *P. aeruginosa* infection involves a “classical/environmental” strain that will colonize the lungs thanks to its invasive virulence factors. During the following years, *P. aeruginosa* will be confronted to many challenges including the immune defenses and year-long antibiotic treatments. To survive in this stressful environment, *P. aeruginosa* will adapt by losing major virulence factors (*e.g.* loss of secreted toxins) and deploying defense systems (*e.g.* mucoid conversion and overexpression of efflux

pumps). Moreover, this adaptation seems catalyzed by the selection of mutator strains which accumulate mutations at increased rates. The adapted “CF” strains become so difficult to eradicate that current therapeutic strategies are focused on avoiding the primary infection (alcohol-based antiseptic hand rubs, use of surgical mask...) [124], and aggressive antibiotic therapies against the early infections [125]. A study found that these early antibiotic treatments can lead to a *P. aeruginosa* free-period of 18 months in average, and delays the decline of lung function, but new acquisition with different *P. aeruginosa* genotypes occurred in 73% of the studied patients [125]. Unfortunately, failure rate of treatments of exacerbation in CF patients increases when the number of active drugs decreases (three active drugs: 0% of treatment failure; two: 24%; one: 27%; zero: 43%) [126]. To summarize, if the increased prevalence of MDR *P. aeruginosa* strains is a serious threat to the general public health, it is especially worrisome for CF patients.

In conclusion, *P. aeruginosa* is a widespread bacterium, able to colonize and survive harsh environments, from chlorinated water to chronically inflamed CF lungs. This versatile pathogen possesses an arsenal of virulence factors allowing it to efficiently damage tissues and resist immune defenses. Its numerous antibiotic resistance mechanisms provide protection against most (sometimes all) current treatment options. To fight against this highly adaptable pathogen, our laboratory investigates an antimicrobial weapon that has been co-evolving with *P. aeruginosa* for billions of years: its natural viral predators, the bacteriophages.

Chapter II: Bacteriophages, the most abundant bacterial predators.

1- The Three Faces of Phage Research.

A) An Ubiquitous Predator.

Bacteriophages, literally “bacteria eaters”, are viruses infecting bacteria. This name was given upon their discovery by Félix d’Hérelle in 1917, while he was working at Institut Pasteur in Paris [127]. Bacteriophages (or phages), are ubiquitous: every place where bacteria can grow, associated phages are found, including in the human body. Indeed, phages are particularly highly abundant in the human gut microbiome and are involved in shaping this microbiome, in both healthy and diseases conditions [128]. More unexpected is the total number of phages on Earth that is estimated to be around 10^{31} , making phages the most abundant biological entity on our planet [129]. Ecological functions of these abundant phages have been investigated, particularly in marine ecosystems. There, phages play critical roles in the structure and function of aquatic food webs, and are estimated to kill almost 20% of the bacterial community daily [130, 131]. These killing rates make phages the primary predator of bacteria, which leads to a never-ending selective pressure, driving the evolution of both phages and bacteria. This relationship is a well-characterized example of the Red Queen hypothesis: predator and prey species must constantly evolve [132].

Investigations on the abundance and ecological roles of phages are actually quite recent and powered by the advances in bioinformatics and sequencing technologies (especially metagenomics). However, historically, phages were first studied for their potent bactericidal activities.

B) Phage Therapy Part 1: Chaotic Beginnings.

As soon as in its first report on phages, Félix d’Hérelle investigated their use as antimicrobials. In 1919, he successfully treated chickens infected with *Salmonella gallinarum* [133, 134], and in 1921, five humans with dysentery were cured using a phage that infects *Shigella dysenteriae* [134]. At this time, antibiotics were not yet discovered (Fleming will report

the discovery and properties of penicillin in 1929 [135]) and the antibacterial arsenal was fairly limited. In this context, d'Hérelle's successes with phages allowed him to travel the world to conduct numerous phage therapy studies. In 1925, in Egypt, d'Hérelle reported rapid healing from bubonic plague following only one injection in each of the two buboes of the patient [136]. Furthermore, in 1927, despite reluctant medical professionals, d'Hérelle conducted a study of phage treatment for cholera in India that yielded excellent results: while the mortality rate of the untreated group (124 patients) was 63%, it was only 8% in the phage-treated group [137]. Unfortunately, these encouraging results were overshadowed by contradicting results regarding the efficacy of phage therapy (probably due to the use of phages with limited host range) [138] and problems related to clinical study designs making results hard to interpret [139]. Additionally, it is now believed that phage preparations at this time were not optimized with highly variable phage titers and heavily contaminated with LPS and other bacterial debris, which could both lead to treatment failure. Moreover, the nature of phages themselves was debated for a long time as most of the scientific community (on the impulse of Nobel Prize winner Jules Bordet, involved in a personal feud with d'Hérelle) discarded d'Hérelle's theory on phages being parasites in favor of the theory of a "self-perpetuating lytic enzyme" [140]. It is only in the 1940's that this debate was put to an end as electron microscopy micrographs of phages revealed without a doubt their viral nature [141]. Overall, the lack of understanding of basic phage biology, a general distrust from part of the scientific and medical communities, and the increased use of a novel very efficient antimicrobial (the first antibiotic, penicillin) precipitated the decline of phage therapy, until its reappraisal about 100 years later.

C) Phages and the Birth of Molecular Biology.

The halt in phage therapy research around the 1940's, did not however mark the end of basic research on phages. Indeed, by the 1950's significant advances were made on the biological processes supporting the parasitic lifestyle of phages in bacteria and paved the way for the emergence of the field of molecular biology. Phages were instrumental in discovering that DNA molecules hold the genetic information [142] and that messenger RNAs are intermediate molecules carrying this genetic information to ribosomes for protein synthesis [143]. Moreover, the study of phage-encoded DNA-manipulating enzymes (DNA and RNA polymerases, ligases and endo- and exonucleases), and the discovery of bacterial restriction enzymes (used to protect bacteria against phages), provided the first molecular tools for genetic

engineering that are still frequently used today [144]. Nowadays, phage research continues to fuel molecular biology advances as witnessed by the revolution in genome editing initiated from the characterization of a bacterial phage resistance mechanism: the CRISPR–Cas system [145, 146].

The three faces of phage research (ecology, medicine and biotechnology) have emerged during the 20th century and are now fully integrated into a renewed worldwide interest on these viruses. Moreover, the lack of understanding of basic phage biology was a major downfall of phage therapy, but we have since learned much about the basic processes governing phages life cycles, their host ranges and mechanisms by which bacteria might become phage-resistant.

2- Introduction to phage biology.

A) Phage Diversity and their Classification.

Phage classification, organized by the International Committee on Taxonomy of Viruses (ICTV), relies on both genomic and morphological information [147]. Note that the ICTV continues to work on this classification and update it periodically [148] (for an up to date phage classification please consult the latest ICTV publications). This classification helps appreciate the immense diversity of phages. Indeed, their genetic information can consist of double-stranded (ds), single-stranded (ss) DNA, or RNA. Moreover, phage genomes size can vary a lot, from 3.5 kb of ssRNA for phage MS2 [149] to 670 kb of dsDNA for *Bacillus megaterium* phage G [150]. Regarding morphology, if 96% of isolated phages are tailed, other morphologies have been described (polyhedral, filamentous, or pleomorphic) [151] (**Figure 2**). Like some eukaryotic viruses, phages particles can be enveloped by a layer of lipids or lipoproteins [151].

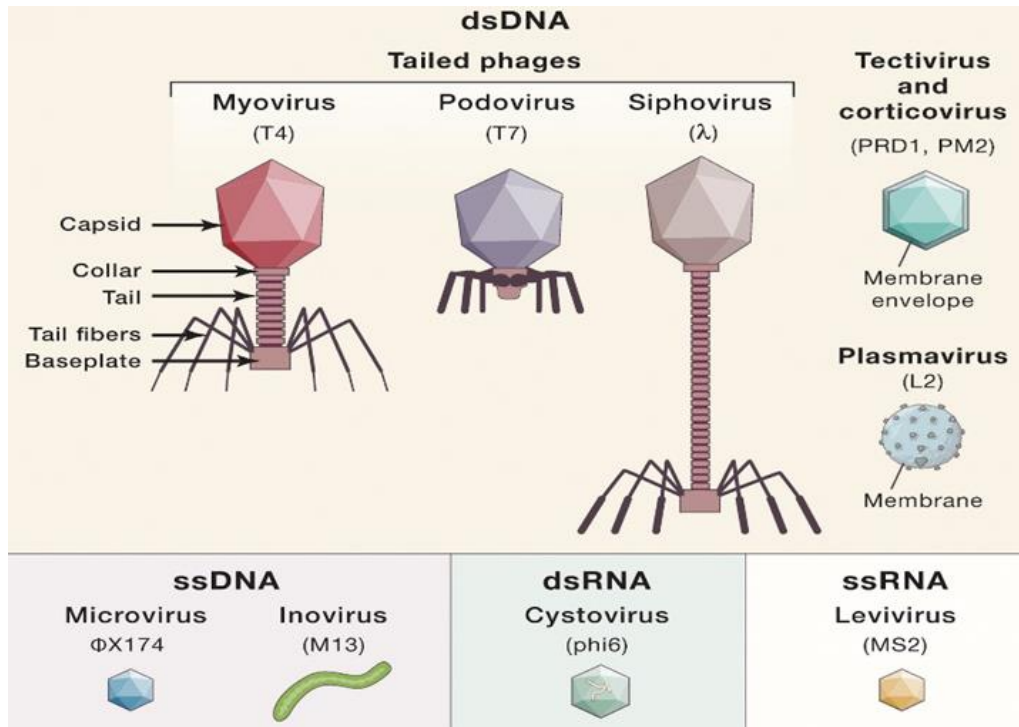


Figure 2 : Phage taxonomy based on morphology and genome composition. The name of a representative phage for each taxonomical group is indicated between brackets (from [152]).

Most studied phages belong to the *Caudovirales* order (characterized by a dsDNA genome and a tailed morphology), which is divided into three families: *Myoviridae* with contractile tails (e.g. phage T4), *Siphoviridae* with long flexible tails (e.g. phage λ) and *Podoviridae* with short stubby tails (e.g. phage T7) (Figure 2). *Escherichia coli* infecting phages remain the most studied [153, 154] and our global understanding of phage biology comes from studies with these phages. Mind that the model phages (later described in this chapter) are considered models because they were the first studied, but they do not encompass the entire phage diversity. Additionally, phages are even more diverse than bacteria, for example the single bacterial species *Escherichia coli* can be infected by 157 species of *Caudovirales* phages (grouped in 37 genera), and new phage species are still being discovered and added to this list [155]. Nonetheless, the following sections will explore the biology of *Caudovirales* phages as they represent the majority of isolated and studied phages.

Overlapping with the ICTV classification, phages can also be classified according to their lifestyle: virulent phages only able to replicate through a lytic cycle, or temperate phages able to replicate via a lytic or a lysogenic cycle.

B) Phage Life Cycles.

Phages are obligate parasites: they can only multiply within their specific host. Phage replication inside its host can follow two different infectious processes (lytic or lysogenic) summarized in **Figure 3**. The first step of these two processes is similar with the attachment of phages to their bacterial host (via an interaction with cell surface components) during the adsorption step. The second step proceeds with the injection of their genome into the host cell cytoplasm. The distinction between virulent and temperate phages occurs at the next step: a virulent phage will go into a lytic cycle, whereas a temperate phage might go into either a lytic or lysogenic cycle. During a lytic cycle, a virulent phage will proceed to replicate its genomic material, produce viral proteins, assemble new virions and finally lyse the host cell, thus releasing new viruses in the environment. This progeny will then infect other host cells. A temperate phage however, in addition to the previously described lytic cycle, can actually use a different infectious process (lysogenic cycle) in which it does not directly replicate its genomic material. Instead the temperate phage genome adopts a dormant state called “prophage”, generally integrated into the host cell genome, or sometimes in the form of a plasmid [156]. This prophage form is maintained by the expression of a phage repressor gene that inhibits the transcription of other phage genes [157]. As such, the prophage will be replicated as the host cell replicates. The prophage-containing cells are called “lysogenic” (meaning capable of lysis) as under some conditions the prophage might “awake” and enter a lytic cycle, thus lysing the host cell.

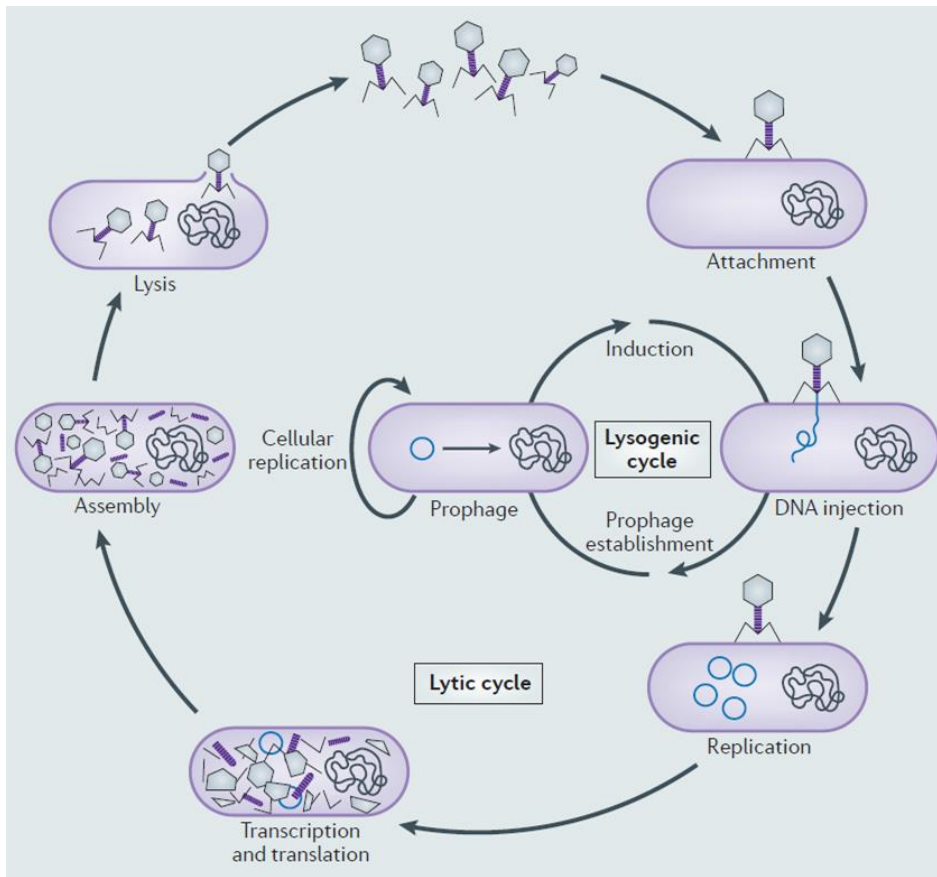


Figure 3 : Schematic representation of Lytic and Lysogenic phage life cycles (from [144]).

Temperate phages, in the prophage state, provide additional genetic material to their bacterial host. They have actually been shown to be a major contributor to genetic diversity in pathogen like *Escherichia coli* [158], and can provide virulence factors, like the cholera toxin acquired by *Vibrio cholerae* through the filamentous phage CTXΦ [159]. Temperate phages also play a role in the dissemination of antibiotic resistance genes via transduction [160, 161], a mechanism in which bacterial host DNA is encapsided in newly formed virions and delivered to another cell (the phage acting like a DNA delivery system between two bacteria). All of these properties make temperate phages ill-suited for phage therapy, and therapeutic phage genomes are routinely screened for gene coding integrases, toxins or antibiotic resistance determinants [162]. Note that this exclusion of temperate phages for phage therapy is now being questioned as recently, genetically modified temperate phages were successful in treating a *Mycobacterium abscessus* infection in a 15-year-old CF patient [163]. These temperate phages were engineered by removing the repressor genes, essential to the lysogenic pathway, making them strictly lytic.

Phage therapy relies on the antimicrobial proprieties of phages, namely their ability to lyse bacteria. Thus, knowledge on how phages perform each step of the lytic pathway is useful to guide the choice of therapeutic phages. Moreover, the bacterial mechanisms to disrupt this infectious process and gain resistance to phages also need to be known to exploit phage therapy to its maximum potential.

C) Adsorption: First Step Toward Infection.

The infection process starts with the adsorption of phage to its host. This process consists in a series of interactions between the phage binding proteins and the bacterial cell surface receptors. Doing so allows the virus to recognize a suitable host, and then positions itself to inject its DNA. The adsorption can be subdivided in three steps: the initial contact, the reversible binding and finally, the irreversible attachment.

The initial contact is a game of chance as it relies on random collisions between phage and host caused by diffusion, dispersion, Brownian motion...[164]. Subsequently, during the reversible step, phage binding to the bacterial surface components is not definitive, as phage can detach from the host [165]. This reversible interaction may help the phage in its search for a specific receptor, by keeping it close to the host cell surface [164]. Once the phage-binding domains and the specific bacterial receptor connect, conformational changes in the phage allow its genome to be ejected into the host [166].

Phage can use a wide array of cell surface receptors. Moreover, the membrane architecture of Gram positive and Gram negative bacteria being very distinctive, their associated phages use different receptors. On the one hand, phages infecting Gram positive bacteria have been shown to use element of the cell wall, including peptidoglycan and teichoic acids as receptors [167]. On the other hand, phages infecting Gram negative bacteria can use numerous receptors including proteins (like OmpC) [168], different part of the LPS [169] or capsular polysaccharides [170]. Interestingly, phages using specific bacterial appendages, such as the flagella and pili as receptors have been described for both Gram positive [171, 172] and Gram negative bacteria [173, 174].

D) Preventing Phage Adsorption: First Line of Defense.

The adsorption step is also the target of the major phage resistance mechanism identified in bacteria: cell surface modifications [175]. Indeed, studies of phage resistance revealed numerous strategies used by bacteria to block phage adsorption: modifying the receptor, blocking its access, hiding it behind extracellular matrix... (**Figure 4**).

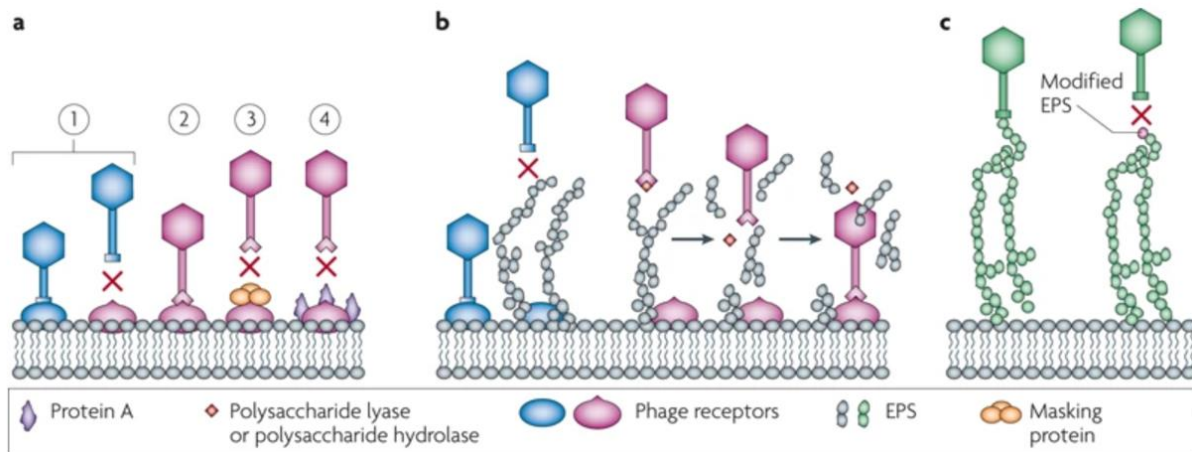


Figure 4 : Bacterial mechanisms to prevent phage adsorption (from [175]). (a) Bacteria can become phage-resistant by modifying the cell surface receptors (1); other phages can recognize these new receptors (2). Bacteria can also produce proteins masking the phage receptor (3), or impairing phage adsorption (4). (b) The production of exopolysaccharide (EPS) can also block phage adsorption, but phages can cleave this EPS layer via polysaccharide lyase or polysaccharide hydrolase. (c) The modification of EPS can also provide phage-resistance as phages recognize specific polysaccharides (*e.g.* O antigens).

Phage predation has been shown to select mutant for phage receptors expression, production or conformation. For instance, resistance to the *P. aeruginosa* infecting phage OMKO1 was achieved by mutation in the *OprM* gene, resulting in a modified protein on which the phage could not adsorb efficiently [176]. Likewise, when *P. aeruginosa* strain PA1 was cultivated in presence of phage PaP1, phage resistance occurred via a chromosomal deletion resulting in a LPS without O-antigen (necessary to the phage adsorption) [177]. Additionally, bacteria may produce proteins that disrupt the phage/receptor interaction: the outer-membrane protein TraT (encoded on the F plasmid) produced by *E. coli* was shown to alter the conformation of the outer-membrane protein OmpA, used as receptor by many T-even like phages [178]. Surprisingly, this receptor blocking strategy is also used by phages themselves! Indeed, *E. coli* phage T5, at the beginning of the infection, produces a lipoprotein (Llp) that

blocks its own receptor, the ferrichrome-iron receptor (FhuA). This, not only prevents superinfection, but also limit the binding of newly released virions to cells debris, which would inactivate them. [179]. The inactivation of phages through their adsorption on a “decoy receptor” present on outer membrane vesicles (OMVs) has also been described as a bacterial mechanism to prevent infection [180, 181]. OMVs are nanostructures composed of outer membrane lipids and proteins, and periplasmic components. They are secreted by Gram negative bacteria and play a role in diverse biological functions (stress response, nutrients acquisition...) [182]. As their surface present the same elements than the bacterial surface, phages might adsorb and get stuck on these OMVs.

Additionally, bacteria can prevent phage adsorption by masking the phage receptor behind a layer of extracellular polysaccharides. For example, bacteria might produce a capsule, thus hiding the phage-receptor and granting phage resistance. This strategy was demonstrated for different bacteria/phage couples including *Staphylococcus simulans*/phage U16 [183], *Staphylococcus aureus*/phage 84 [184] or *E. coli*/phage T7 [185]. Note that this approach can efficiently protect bacteria from multiple distinct phages as a coat of exopolysaccharide can hide different phage receptors at once. For instance, Betts *et al* [186] showed that when *P. aeruginosa* strain PA01 grew in presence of a single phage, resistant clones had mutations in genes related to the phage receptor (LPS, or pili, depending on the phage), but when the same strain was submitted to 5 different phages at once, one of the resistant clone had a single mutation in *mucA* (resulting in a mucoid phenotype). Interestingly, the selection of mucoid *P. aeruginosa* strains following phage predation has been recorded multiple times [187, 188] hinting that it is an advantageous trait in these conditions. However, mucoidy does not provide complete phage resistance [186] and some phages infecting *P. aeruginosa* encode alginate lyase [189].

E) Genome Injection or Ejection?

The first theories on phage DNA injection postulated that the virus act like an hypodermic syringe [190]. However, this representation is challenged by new insights in this phenomenon. Indeed, it is now believed that osmotic pressures and the entry of water inside of the phage particle are the main driver of the phage genome transfer in the bacterial cell. As such, many researchers refer this phenomenon as “genome ejection” instead of “genome

injection”. Briefly, when the phage genome is packaged in the capsid, most of the water that normally hydrates the DNA is removed. Moreover, most tailed phages contained within the capsid ~500 mg/mL of DNA, creating an osmotic imbalance between the capsid and the bacterial cell. Once the phage recognized its specific receptor, the tip of its tail might penetrate the peptidoglycan layer and touch or penetrate the inner membrane via enzymatic activity. According to the hydrodynamic model of phage genome ejection, once the exit channel is open, water diffuses through the capsid shell and neutralize the osmotic imbalance. It is the hydrostatic pressure gradient across the tail that pushes the phage DNA out, into the bacterial cell [166]. This general mechanism of phage genome ejection is still being investigated, and it is already known that smaller phages may eject their genome using different mechanisms. As the tail of *Podoviruses* (e.g. T7) is too short to span across the cell envelope, phage proteins (e.g. Gp14, Gp15 and Gp16) are needed to form a channel allowing phage DNA ejection into the cell [191]. Moreover, phage T7 genome ejection is a three-step process: After phage adsorption and formation of the protein channel, approximately 10% of the genome (40kb) is internalized by the cell, the transport of the next 50% is coupled to the transcription process by *E. coli* RNA polymerase, and the final 40% internalization is dependent on transcription by the T7 RNA polymerase [192, 193].

As previously mentioned for the adsorption step, this genome ejection step can be disrupted by bacteria. Interestingly these defense mechanisms are mostly conferred via integrated prophages that block genome ejection of infecting phages.

F) Superinfection Exclusion: A Prophage Protects Against Phages

Some temperate phages encode superinfection exclusion systems designed to protect the bacterial host against other phages. For instance, *E. coli* phage P1 encodes the *sim* gene conferring resistance to other P1 phages [194]. Likewise, *Salmonella* phage P22 encodes *sieA* protecting its host against phages L, MG178 and MG40 [195]. The underlying molecular mechanism of these immunity proteins is not yet fully understood, but the fact that phage adsorption is not affected by the presence of these proteins and that a bacterium can be successfully transformed with the phage genome hint that they must prevent the ejection step of the infection.

When the phage genome ejection is not prevented, bacteria might still manage to disrupt the infection process by degrading the phage genetic material.

G) Restriction-Modification Systems: Cutting Genome of Phages

Most studied bacteria encode Restriction-Modification systems (RMs). RMs main function is to protect the bacterial cell against foreign DNA, including phage DNA. Indeed, when unmethylated phage DNA is ejected in the cytoplasm, it can be rapidly degraded by restriction enzymes. Being methylated, the host DNA is protected from these restriction enzymes. Against this simple, yet effective system, phages have developed a wide array of countermeasures: use of a phage-encoded methylase [196]; absence of endonuclease recognition sites in their genomes [197]; use of modified bases, not recognized by restriction enzymes (*e.g.* phage T4 DNA has hydroxymethylcytosine instead of cytosine) [198].

Another bacterial defense mechanism based on phage DNA degradation is the now famous CRISPR-Cas system.

H) CRISPR-Cas: Adaptive Immunity against Phages

The CRISPRs (clustered regularly interspaced short palindromic repeats) Cas (CRISPR-associated proteins) system is another restriction based defense mechanism. Different bacterial species encode different CRISPR systems [199] but a general overview can be provided: CRISPR-*cas* loci are composed of direct repeats (21–48 bp) interspaced by non-repetitive spacers (26–72 bp) and, flanked by *cas* genes (**Figure 5**).

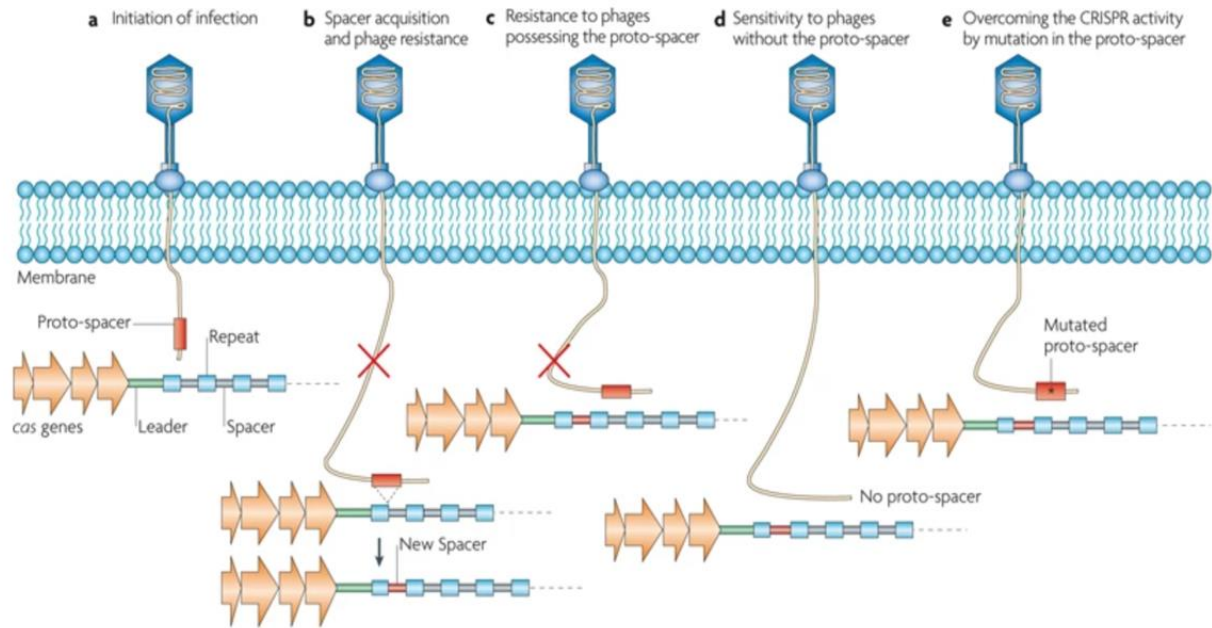


Figure 5 : Schematic overview of the CRISPR-Cas system (from [175]): (a) Phage DNA enters the bacterial cell; (b) The CRISPR acquire an additional repeat (duplicated from the CRISPR locus) and a new spacer (acquired from the infecting phage genome); (c) Incoming phages with genome carrying a proto-spacer with 100% nucleotide identity to the newly acquired spacer will be inactivated; (d) This phage resistance mechanism does not affect phages without this specific proto-spacer in their genomes; (e) Phage mutants carrying a single point mutation or a deletion in their proto-spacer are not affected by the CRISPR system and can complete their lytic cycles.

During an unsuccessful infection by a phage, a new spacer matching a part of this phage genome can be acquired. The selection of this spacer sequence from the phage DNA is guided by the recognition of proto-spacer-adjacent motifs (PAMs), usually only several nucleotides long, which vary between the different CRISPR–Cas systems [200]. On subsequent infection by the same phage, its DNA will be degraded by an endonuclease, targeting the sequence corresponding to the acquired spacer. The resistance to phages provided by this system is limited to phages with the corresponding spacer in their genome. Thus phages have been shown to escape the CRISPR-Cas system simply by acquiring mutations in either the recognized spacer or in the PAMs of their genomes [201]. Phages can also produce anti-CRISPR (Acr) protein, interfering with the CRISPR-Cas system [202]. Note that a study of the prevalence of CRISPR-Cas systems in clinical isolates of *P. aeruginosa* found it in 45 out of the 122 analyzed strains [203]. Interestingly, the spacers found in these CRISPR-Cas systems only matched

temperate pseudomonas phages. Finally, these CRISPR-Cas systems did not provide resistance against the tested phages, even for CRISPR with spacers 100% identical to a region of the tested phage. Despite this surprising results, other studies (using the PA14 lab strain) have demonstrated that *P. aeruginosa* can readily acquire phage resistance via CRISPR-Cas systems [204]. However, this mechanism of resistance seems more prevalent following mono-phage treatment as cell surface modifications were more prevalent after multi-phage treatment [205].

I) From Bacterial Cell to Virocell

To achieve a successful infection of their host cell and produce new virions, phages hijack several cell resources and shift the “bacterial cell physiology” toward a “virocell physiology” [206]. This transformation is due to efficient hijacking of different bacterial machineries, catalyzed by the expression of “early” expressed phage genes. A target of choice in this endeavor is the bacterial RNA polymerase, as disrupting the host transcription will have tremendous effects on its physiology. The *E. coli* phage T4 actually possess 11 genes dedicated to its interaction with the host RNA polymerase [207-213], which redirect its activity during the infectious process towards the transcription of specific phage genes. Other strategies exist as phage T7 inhibits the host RNA polymerase and uses its own [214]. Phages can also regulate RNA degradation as the degradosome interacting protein (Dip) of *P. aeruginosa* phage phiKZ prevents viral RNA degradation in infected cells [215]. Additionally, phages PAK_P3 and PAK_P4 (isolated in our laboratory), also infecting *P. aeruginosa*, affect RNA turnover by eliciting rapid host transcripts degradation via an unknown mechanism [216, 217]. Note that the underlying mechanism might be different for these two phages as the kinetic of host RNA degradation during PAK_P4 infection is much faster than during PAK_P3 infection [217].

Phages have also been shown to shut off ‘non-essential’ host processes presumably to preserve energy for the infectious process. For instance, *E. coli* phage Rac uses the Kil protein, interacting with the bacterial tubulin homologue FtsZ to impair cell division [218]. Another *E. coli* phage, N4, uses Gp8 to shut off host DNA replication [219].

Phages can also tune the host cell metabolism to favor viral replication. To this end, phages use acquire host-derived metabolic genes called “auxiliary metabolic genes” (AMGs). These AMGs have been found to interfere with a wide array of metabolic processes including

nucleic acid synthesis [220], phosphate [221] and nitrogen metabolism [222], the pentose phosphate pathway [223], and even photosynthesis [224]. PAK_P3 phage was shown to particularly alter the pyrimidine metabolism [216], whereas PAK_P4 manipulates the expression of iron-related host genes [217].

One of the two projects of my PhD was dedicated to decipher the molecular mechanism of an early-expressed phage gene conserved in both PAK_P3 and PAK_P4 phages that profoundly affects *P. aeruginosa* physiology.

If the first phage genes expressed (referred as “early”) are mostly dedicated to hijacking host cell machinery, the genes expressed afterwards (referred as “middle”) focus on phage genome replication.

J) Viral Genome Replication

Most phage genes involved in DNA replication are grouped in clusters (*e.g.* DNA polymerase, helicase, primase...) and expressed during the “middle” phase of the infection. The DNA replication mechanism of phage genomes does not necessarily match the one of its host. If the *E. coli* phage λ uses the same mechanism as its host (theta-type replication), the *E. coli* phage P2 uses the rolling circle-type, and the *E. coli* phage T7 uses a transcription-initiated mechanism [225].

Note that if phages encode most of the proteins they need to perform their genome replication, they still might depend on host factors to successfully achieve this process. This dependency can be exploited by bacteria as a resistance mechanism as long as the mutation of the host proteins is not detrimental. This is the case for *E. coli* phage T7 that uses the host protein thioredoxin (TrxA) as a processivity factor for its DNA polymerase [226]. A thioredoxin mutant strain was shown to be resistant to phage T7 [227], but unsurprisingly, phage T7 can escape this resistance mechanism via mutations within its DNA polymerase gene (gene 5) [227].

After the replication process, comes the “late” phase during which the newly replicated genomes are processed and packaged in freshly made capsids.

K) DNA Packaging and Phage Assembly

Generally, phage DNA replication mechanisms lead to the accumulation of concatemers in which each phage DNA genomes are joined together in a head-to-tail manner through terminal repetitions. These concatemers are processed into single genomes during the packaging process (also known as encapsidation). Phage DNA packaging relies on a packaging enzyme composed of two subunits: the large subunit has ATP-binding, prohead binding, and DNA cleavage activities, whereas the small subunit is a DNA binding protein. The packaging enzyme, thanks to ATP hydrolysis, catalyzes DNA translocation into a viral protein shell (the prohead) [228]. The DNA is then translocated through a channel formed by the portal protein. At the end of the DNA packaging, the portal channel closes and the phage tail (preassembled separately) binds to the capsid to form the mature phage particle [229].

The phage assembly process is mainly driven by phage proteins, but can also require host proteins, notably chaperones. For instance, multiple *E. coli* phages rely on the host chaperone GroEL and its cofactor GroES for their assembly. Moreover, *groE* mutants prohibit the growth of multiple phages as they inhibit the head assembly of phages λ [230] and T4 [231], prevent the tail assembly of phage T5 [232], and block the assembly of both the head and the tail of phage Mu [233].

These newly formed virions are still trapped in the host cell. They will be released from the cell after the final step of the infectious process: the host cell lysis.

L) Host Cell Lysis: Three Steps and Two Choices to Release Phages

The lysis induced by phages infecting Gram negative bacteria has been described as a three steps process. Moreover, two different pathways have been uncovered: the classical λ -like lysis pathway (involving holins, endolysins and spanins) and the pinholin-SAR endolysin lysis pathway (involving pinholins, SAR endolysins and spanins) [234]. In both pathways, while new phages are being assembled, the phage proteins involved in lysis are produced and directed to their respective compartments (Fig. 1A and 1E): both holins and pinholins accumulate in the inner membrane (IM); endolysins accumulate in the cytosol, whereas pinholins accumulates in the IM; spanins form a protein bridge that connects both IM and outer membrane (OM). These accumulations last for a genetically programmed time, depending on

the structure of the holin [235]. At a precise time, called “triggering”, the lysis begins. In the λ -like lysis pathway, the holins form micron-scale holes in the IM, releasing active endolysins into the periplasm (Fig. 1B) [236]. There, endolysins will degrade the peptidoglycan (PG) through their muralytic activity. In the pinholin-SAR endolysin lysis pathway, the accumulation of pinholins causes a depolarization of the membrane, which activates the secreted SAR endolysin, also able to degrade the PG (Fig. 1F) [237]. Finally, in both pathways, spanins are activated by the PG degradation and cause disruption of the cell envelope by inducing local fusions of the IM and the OM (Fig. 1C and 1G) [238], thus releasing phages and membrane vesicles (Fig. 1D and 1H). After the triggering, the lysis process occurs in a few seconds and can be observed via video microscopy as an explosive event [234].

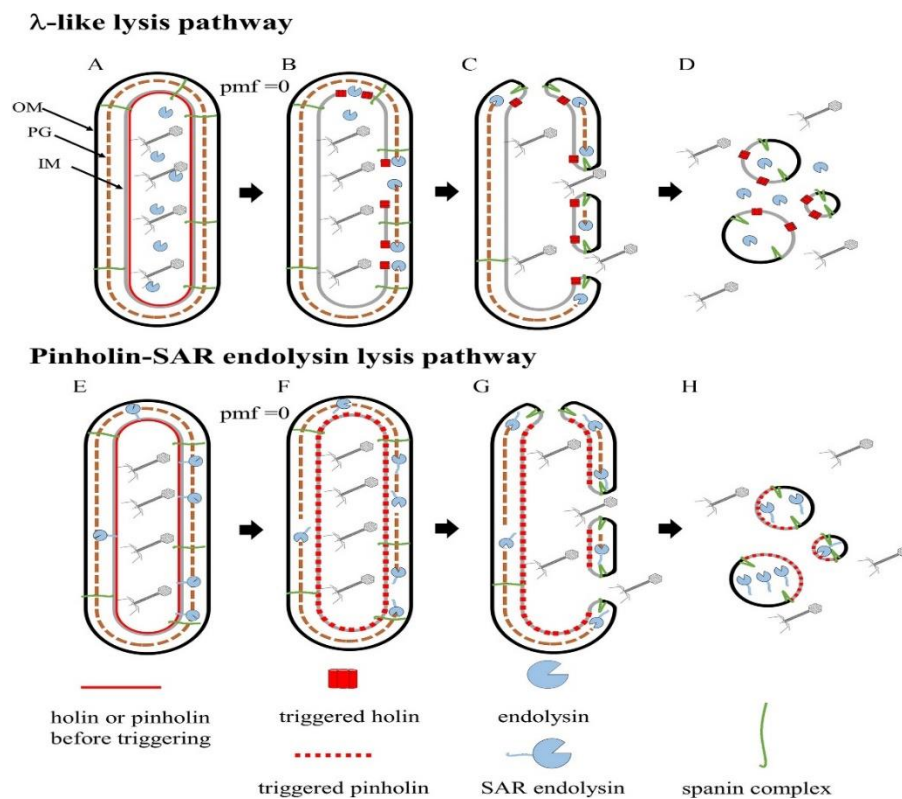


Figure 6: Schematic representation of the two lysis pathways of Gram negative infecting phages (from [234]). First, endolysins or SAR endolysins accumulate in the cytoplasm (A) or IM (E), respectively. Once a critical concentration is reached, holin triggering results in micron-scale holes (B) or small heptameric pinholes (F), which release the endolysin into the periplasm (B), or release the SAR endolysin from the IM into the periplasm; PG degradation occurs at this step for both pathways. This PG degradation leads to spanins activation, which ruins the cell envelop integrity by fusing locally the IM and the OM (C and G). Finally, phage progeny and cytoplasmic content are released (D and H).

No mechanism of lysis resistance *per se* has been described yet, but biological pathways affecting the lysis timing have been reported. First, lysis delay, sometimes referred as lysis inhibition, has been observed numerous times (starting in the 1940's [239]), and linked to superinfection (the infection by a second phage) [240]. This delay of the lysis in presence of other phages is believed to have ecological importance: the release of new virions is delayed to a time with a more advantageous ratio of uninfected/infected host cells [241]. Recently, a molecular mechanism for lysis delay in T4 was proposed: the DNA of the second phage would interact with holins, thus delaying the triggering of the lysis [242].

The opposite phenomenon can also occur as other mechanisms will induce a quicker lysis. For example, the expression of the *abiZ* gene, caused phage ϕ 31-infected *Lactococcus lactis* to lyse 15 min earlier, thus reducing a 100-fold the burst size of phage ϕ 31 [243]. Results suggest that this premature lysis might be caused through interactions between *AbiZ* proteins and holins.

This induced quicker lysis is actually part of a specific class of bacterial resistance mechanism called Abortive Infection, in which an infected bacterial cell will commit an altruistic “suicide” to protect the rest of the bacterial community.

M) Abortive Infection: Bacterial Altruistic Suicide

The term Abortive Infection (or *Abi*) does not refer to a precise resistance mechanism, but to a general strategy aiming at limiting phage propagation to the whole bacterial community. The main principle of this strategy is that a phage infected bacterial cell commits suicide before the phage can complete its replication cycle [244]. This will limit the production of virions and the spread of the phage epidemic to the neighboring cells, thus protecting the rest of the colony (**Figure 7**). *Abi* can be characterized as an altruistic mechanism, as one cell sacrifices itself to protect the other. However, considering phages specificity (only infecting one bacterial species or a few strains of a bacterial species) this altruism is restrained and primarily protects only closely related bacteria. Moreover, this protection is only efficient in a structured environment: if phages and bacteria are dispersed (*e.g.* in liquid culture), community protection is less likely to be achieved, whereas in a locally structured environment (*e.g.* a biofilm or a colony on a plate) suicide of infected cells results in an overall community protection [245].

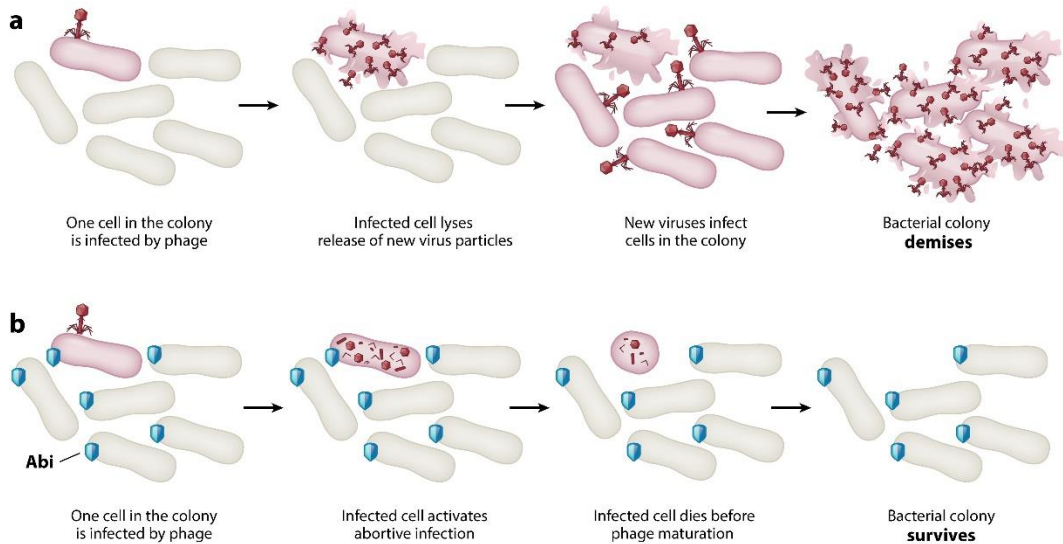


Figure 7 : Overview of the Abortive Infection (Abi) strategy (from [244]). (a) A phage infecting bacteria without Abi systems is able to propagate and decimate the bacterial colony. (b) A phage infecting bacteria with Abi systems is unable to reproduce and disseminate in the population, thus the bacterial colony survives.

Generally, Abi systems contain two functional modules: a sensing module and a killing module. The sensing module, depending on the Abi system, can recognize diverse markers of phage infection including phage proteins [246], protein-DNA complexes produced during phage genome replication [247] or even sense phage-mediated shutdown of host gene expression [248]. On the other hand, killing modules catalyze the cell suicide, via distinct mechanisms according to the different Abi system: phosphorylation of host proteins [246], host mRNA degradation [248], or the formation of a membrane ion channel that allows the passage of monovalent cations, thus collapsing the cellular membrane potential [247]. Overall, Abi systems use diverse mechanisms and can interrupt phage infection at different stages.

Interestingly, plasmids encoding Abi systems have been described [249] and even utilized to provide phage resistance in commercial *Lactococcus* starter cultures [243]. However, as bacteria adapt to phages, phages adapt to bacteria, and phage protein like Dmd of phage T4 have been shown to inhibit specific Abi system [249].

Overall, a century of research on phage biology revealed how complex a viral infection strategy can be. This process does not only rely on phage encoded proteins, but also on host cell resources. The coevolution of bacteria and phages has led to the development of numerous

bacterial resistance mechanisms, targeting every step of the phage infection process. Conversely, phages have evolved to counteract these resistance strategies. This never ending arms race is a demonstration of the plasticity of the genomic information that serves the impressive adaptation abilities of bacteria to different forms of threats.

Another example of bacterial adaptation is the development of drug resistance. Soon after the discovery of penicillin bacterial resistance was observed (in 1928) [250]. Nevertheless, penicillin and other antibiotics became widely used, and precipitated the decline of phage therapy. However, in the former Soviet Union, a limited access to western developed antibiotics actually strengthen phage therapy research and usage. The Eliava Institute, founded by Georgyi Eliava in Tbilisi (Georgia), was the main center from which phage therapy spread in former USSR countries [251]. Up to now, phage therapy has been continuously practiced in few countries like Georgia (through The Eliava Institute) or Russia, where phages are available in pharmacy over the counter [252]. In the rest of the world, the increased prevalence of antibiotic resistant bacteria and the threat of a post-antibiotic era has revived interest in phage therapy during the last two decades [253].

3- Phage Therapy Part II: A New Hope

A) Clinical Experience of Phage Therapy: Does It Work?

Phage therapy was originally dismissed partially because of inconsistent reports of efficacy. This phenomenon was attributed (post factum) to the use of ill-suited phages (*e.g.* not infecting the patient's strain) or use of poorly prepared phage products (low titer and/or contaminated with bacterial endotoxin). Since that time, microbiological diagnosis (cultured-based [254] or molecular-based [255]) and phage preparation techniques have been improved, allowing a precise diagnosis (identification of the bacterial species) and the production of high titer, endotoxin-free phage products [256, 257]. Unfortunately, this is not enough to secure a positive output during a clinical trial. For instance, in 2015, a study on the efficacy of a phage cocktail targeting the *E. coli* species was evaluated for the treatment of acute bacterial diarrhea in children, but failed to prove an improvement of the health condition [258]. It was found that the phages had a limited replication in the gut, probably because of a limited host availability, as *E. coli* represented less than 5% of the fecal bacteria in these patients. Moreover, a microbiota

analysis revealed that disease progression (increased diarrhea) was associated with the overgrowth of other bacterial species, notably *Streptococcus gallolyticus* and *Streptococcus salivarius*, that were obviously unaffected by the phage treatment. This study reasserted the importance of knowing the etiology of a disease. Indeed, phage therapy is probably one of the best example of precision medicine. In contrast to antibiotic treatments that often target multiple bacterial species, a precise identification of the bacterial species and even the strain causing the disease is a critical step for phage therapy.

Another lesson that needed to be taught again was the importance of the phage product quality. The PhagoBurn study led by the pharmaceutical company Pherecydes Pharma, partnered with military and civilian hospitals in France, Switzerland, and Belgium, consisted in a 3-year phage therapy trial on burn patients with infected wounds [259]. Due to multiple issues, notably regulatory hurdles, the trial was not only delayed but its scope was also reduced: the initial project aimed to investigate the efficacy of phage treatment in 220 patients with burn wounds infection by either *P. aeruginosa* or *E. coli*, but it finally included only 27 patients with *P. aeruginosa* infections. The phage treatment (daily topical application of phage dressing) was found to be less effective than the standard care (daily application of 1% sulfadiazine silver emulsion cream). This inefficacy was attributed to a very low phage titer, which was four orders of magnitude lower than expected (100 instead of 10^6 Plaque Forming Units (pfu)/ml per application). It seems that the phage product was unstable, as it was prepared almost 2-years ahead of its application in patients.

The two disappointing examples reported above are counter-balanced by two studies performed few years before. In 2006 Marza *et al* [260] reported successful treatments of two *P. aeruginosa* infections: a human burn wound infection and a dog otitis infection. In both cases, clinical improvements and phage replication (from 42 fold to over a million fold) were observed. No adverse effects were reported following the phage treatments and no toxicity to human keratinocytes was observed when they were cultured with the purified phage suspension.

Following the above success, Biophage Limited engaged in 2009 a small clinical trial (23 patients) for the treatment of chronic otitis caused by MDR *P. aeruginosa*. [261]. This randomized, double-blind, placebo-controlled Phase I/II clinical trial was approved by UK Medicines and Healthcare products Regulatory Agency (MHRA), First, the inclusion of

patients was well performed as four patients were excluded of the study due to the lack of susceptibility of their swab-sampled *P. aeruginosa* to the tested phage product (a cocktail of six phages). This exclusion rate was in agreement with prior *in vitro* studies where 86% of 57 isolates (from human ear infections), were susceptible to at least one of the six phages. This result highlights the role of the host range of a phage product. Presumably, a phage product including a small number of phages will likely have a narrower host range than a product with a higher number of phages, thus limiting its clinical applications. The results of this study were very promising: significant clinical improvements from baseline were observed in the phage treated group but not in the placebo group and *P. aeruginosa* counts were significantly reduced in the phage treated group whereas they were unchanged in the placebo group. Moreover, phage replication was observed in treated patients and no side effects nor evidence of local or systemic toxicity reported. Replication of the six phages was observed in patients from the phage treated group (note that up to five phages could replicate in one patient). The mean recovery of phages from swabs taken from the ears of the phage-treated group over all three post-treatment visits was 1.27×10^8 pfu. Compared to the treatment dose of 6×10^5 pfu, this suggests an average amplification in the treated ear of 200 fold. Moreover, the median duration of phage replication in the phage-treated group was 21 days, and clearance of the phages was observed after all the resolved infections. Finally, it is interesting to note that in this study (the only successful phase II clinical trial to date), a single phage application had several weeks lasting effects and manage to successfully treat three previously untreatable cases of chronic otitis. Indeed, this three cases were irresponsive to classical antibiotic therapies that required multi doses given at regular intervals over several weeks.

Overall, these three studies showed that for a phage therapy trial to be successful the inclusion of patients is critical. Only those for which the phage product show lysis activity on the patient's bacteria should be included, which *per se* introduces a bias in the analysis of the overall treatment efficacy of the trial. It also represents a hurdle for regulatory agencies that often request a new treatment to be compared to an existing treatment for which the selection of patients is not needed. Also, the production of a high titer, stable phage product has to be insured. Moreover, the lack of approved phage products on the market is slowing down the re-development of phage therapy. Facing all of these issues, a number of clinicians have nevertheless initiated tailored "compassionate" treatment for patients, which has propelled the publication of several case reports

B) Compassionate Phage Therapy around the World: Sporadic Successes

In France, a total of 15 patients with osteoarticular infections received a compassionate treatment with phages between 2006 and 2018 at the hospital of Villeneuve Saint Georges [262]. Causative agents included mostly *Staphylococcus aureus* (11/15) and *P. aeruginosa* (3/15), and 73% (11/15) of the treatments concluded with complete cure of the patient's infection. The other outcomes included clearance of the targeted pathogen but appearance of another one (2/15) or only partial disinfection nevertheless associated with clinical improvements (2/15). In addition, the local application of phages was shown to be safe. Note that phage treatments were always supplemented with antibiotics, as these patients had worrying clinical conditions, the aim was not to experiment phage therapy *per se*, but to treat these patients with all available therapeutics.

Recently, Lebaux *et al* [263] reported the first case of phage therapy for *Achromobacter xylosoxidans* lung infection in a lung-transplanted CF patient. The patient received two rounds of phage treatment (5 months apart due to regulatory and production delays). No adverse effects were reported following phage administration, but subsequent bronchoalveolar lavage (BAL) still contained *A. xylosoxidans*, including phage resistant bacteria. The patient clinical status improved slowly despite low level airway colonization by *A. xylosoxidans* that persisted for months, before samples turned negative. No re-colonisation was detected more than two years after the phage treatment, and the imipenem treatment was then stopped. This study revealed a limited short-term bactericidal effect of the phage treatment in the lungs, as it did not manage to eliminate every *A. xylosoxidans*, but was followed nonetheless by a clinical improvement of the patient.

In 2007, the Queen Astrid military hospital in Brussels was the first Belgian hospital to revive phage therapy and its use, using the article 37 (regarding unproven interventions in clinical practice) of the Declaration of Helsinki [264]. This allowed the treatment of 15 patients, including one with acute kidney injury and developing a septicemia caused by a colistin-only-sensitive *P. aeruginosa* strain [265]. Phage cocktail BFC1 [266], containing two phages with *in vitro* activity against the patient's *P. aeruginosa* isolates, was administered (50µL) as a 6-h intravenous infusion for 10 days to treat the septicemia in absence of any antibiotic treatment. Moreover, the patient infected wounds were irrigated with 50 ml of BFC1 every 8 h for 10 days.

Rapidly, blood cultures turned negative, the fever disappeared and kidney function recovered after a few days. However, the patient's wounds remained infected with several bacterial species, including *P. aeruginosa*, and caused multiple episodes of sepsis, eventually treated with antibiotics. This patient died 4 months later from a cardiac arrest due to a *Klebsiella pneumoniae* sepsis. This case report, while demonstrating a clear therapeutic effect of the phage treatment rapidly after its administration, also points out the limitation of a specific phage product in a case of a multi-species infection.

In the US, in 2017, a successful case of intravenous phage therapy to treat a disseminated MDR *Acinetobacter baumannii* infection, made the headlines [267]. Indeed, the absence of effective antibiotics pushed two laboratories to identify nine different phages with lytic activity against a bacterial isolate from the patient. An authorization to administer these phages as an emergency investigational new drug (eIND) was obtained from the Food and Drug Administration (FDA). These phages were given both intravenously and percutaneously, into the abscess cavities. *In vitro* studies of patient isolates during the treatment showed that bacteria became resistant to all phages (8 days after treatment started), leading to the design of a new phage cocktail. This treatment was finally associated with clearance of *A. baumannii* and clinical improvement of the patient. As usual for compassionate treatments, antibiotics (meropenem, colistin, minocycline, fluconazole) were administered with phages and might have played a role in the patient's recovery. Nevertheless, this case report had a significant impact on how phage therapy is perceived in the US, and this success was actually followed by the creation of the Center for Innovative Phage Applications and Therapeutics (IPATH) at the University of California, San Diego, in June 2018.

The University of California has reported 10 cases of phage therapy treatment (including 6 before IPATH) in more details. Globally, intravenous phage therapy was safe and associated with a successful outcome in 7 out of the 10 cases, and phages alone were demonstrated as a good preventive approach as *P. aeruginosa* infections were prevented in a CF patient during phage treatment and for the subsequent 3 months. Moreover, phage resistance occurred during the treatment for 3 patients, but was overcome by a personalized treatment strategy (addition of new phages active on resistant bacterial isolates). Phage treatment was also reported to fail despite *in vitro* phage susceptibility. Indeed, two cases of *P. aeruginosa* chronic and biofilm-based infections (ventricular assist device infections) were associated with clinical failure of

adjunctive phage therapy. The authors hypothesized that biofilm structure and composition might have hindered phages diffusion and efficacy. Overall, IPATH experience highlights the potential of phage therapy for several clinical indications, but also reveals the lack of reliable predictors to ensure successful phage therapy.

A recent systematic review on the safety and efficacy of phage therapy to treat superficial bacterial infections collected 6801 article since 1930's, amongst which only 27 were considered as eligible for in-depth analysis [268]. These studies showed clinical resolution or improvement in 77.5% (n = 111) of burn wound infections, in 86.1% (n = 310) of chronic wound/ulcer infections and in 94.14% (n = 734) of dermatological infections. Interestingly, on the 15 reports addressing phage therapy safety, all the reports published after 2001 expressed no safety concern, whereas 7 early reports (from 1929 to 1987), described adverse effects, which is consistent with the use of phage preparations that were not highly purified.

In conclusion, despite a lot of positive reports regarding phage therapy, an undisputable demonstration of its efficacy is still lacking. Indeed, in most of the previously described clinical cases, antibiotic treatments were not stopped (even when the bacteria were shown to be resistant), thus the phage treatment may not be declared as solely responsible for the patient recovery. Moreover, the sporadic nature of the reported cases and the lack of proper control (without phage treatment or phage treatment without antibiotics) limits our ability to conclude on phage therapy efficacy.

To design and perform a clinical study demonstrating the efficacy of phage therapy, there is a crucial need of understanding the factors influencing phage therapy success. These factors can be revealed through research focusing not only on phages and bacteria interactions but also taking into account the host. Indeed, for phage therapy to succeed we need to identify the characteristics that make one phage a better therapeutic candidate than another one, and how to formulate phage cocktails to address host range or resistance issues. We also need to understand how bacteria evolve in response to phage treatment and how the host, and in particular its immune system, reacts to both phages and bacteria during the treatment.

Since my PhD projects are related to the use of phages infecting *P. aeruginosa*, the following sections, addressing some of the challenges mentioned above, are focused on reports from studies involving phages targeting this bacterium.

C) The Importance of Dosage and Timing

First, to investigate the important factors for phage therapy success, one should establish a model in which phage therapy efficacy is well demonstrated. An early report by Vinodkumar *et al* [269] demonstrated the efficacy of phage therapy to treat MDR *P. aeruginosa* septicemia in mice. Septicemia was induced in BALB/c mice via intraperitoneal (i.p) injection of 10^7 colony forming units (cfu) of a *P. aeruginosa* clinical strain and the treatment was a single i.p. injection of phage CSV-31. The control mice died within 48h whereas, 100% of mice treated with the highest dose of phage (4×10^9 plaque forming units) 45min after infection survived. Interestingly, only 50% of mice treated with a lower dose of phage (3×10^4 pfu) survived, demonstrating a dose dependent effect of the phage treatment. This is particularly interesting as the replicative nature of phages suggests that few phages could be sufficient for therapeutic success. Phage therapy success was dependent on active phage particles as heat inactivated phages had no effect on mice survival. Moreover, a 5h delayed treatment (at the highest dose) was still efficient (100% of mice rescued) but a treatment given 24h after the infection only rescued 50% of the mice. Overall, this report not only demonstrate phage therapy efficacy, but also points out the importance of phage dosage and timing of treatment for phage therapy success.

Similar conclusions were obtained in our lab as Debarbieux *et al* [270] reported efficient treatment and prevention of *P. aeruginosa* acute lung infection in mice, using the phage PAK_P1. Indeed, this report highlighted again the importance of the phage dosage as the use of increased Multiplicity of Infection (MOI, the phage/bacteria ratio), MOI 1 or MOI 0.1, was associated with increased survival of mice 5-day post infection (0% and 100% respectively). They also demonstrated the importance of the delay between infection and treatment as mice treated 2h, 4h or 6h post infection showed decreased survival with 100%, 80%, and 30%, respectively. Using a bioluminescent strain of *P. aeruginosa* (PAK-lumi) these authors provide the first dynamic data allowing the follow-up of each animal over time (**Figure 8**). Finally, they observed that PAK_P1 particles were stable enough in the lung environment (1×10^8 pfu given, 3×10^6 pfu recovered in bronchoalveolar lavages 22h later) to protect 100% of infected mice when administered 24h before the lethal dose of the pathogen.

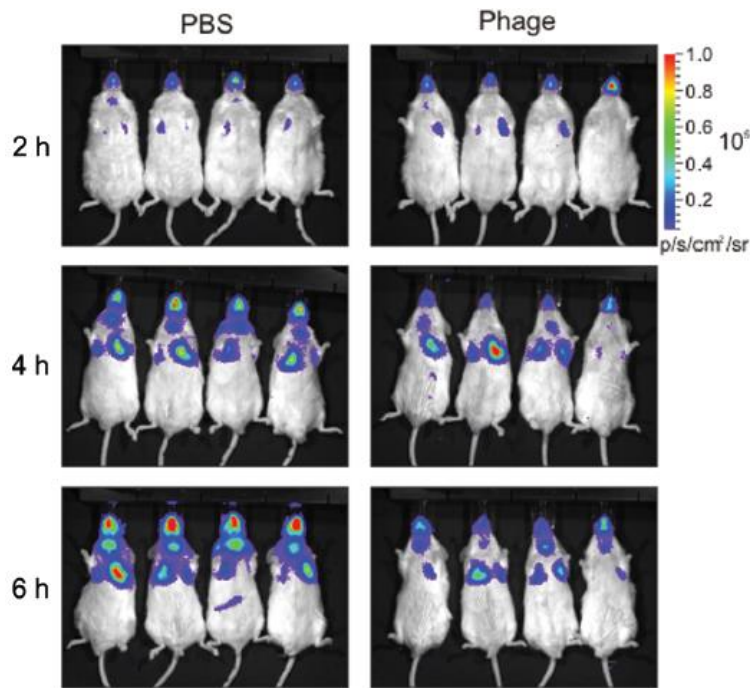


Figure 8 : Time-course images of mice infected with bioluminescent *P. aeruginosa*. (from [270]) Radiance (photons/s/cm²/steradian) of mice treated with PBS (left) or treated with the PAK_P1 bacteriophage at a bacteriophage-to-bacterium ratio of 10:1 (right) was measured in an IVIS 100 imaging system (Xenogen Biosciences).

Another study to treat mink hemorrhagic pneumonia reported on the importance of phage dosage [271]. Cao *et al* reported that phage treatment efficacy was MOI dependent: 20% survival for MOI 1, 80% survival for MOI 10 and 100% survival for MOI 100. Moreover, they also assessed phages safety in mice by intranasal administration of 10^{12} pfu for 3 consecutive days, followed by a 5-day recovery period, and observed no morbidity, mortality or significant histopathological changes in the lung of mice.

Overall, phage efficacy and safety have been demonstrated using several *P. aeruginosa* strains (clinical strains, or lab strains like PAO1, PA14, PAK...), different type of infections (septicemia, lung infections, otitis, rhinosinusitis,...) distinct phage delivery routes (ip injections, intranasal instillations, topical applications...) on a variety of animals (mice, rats, fishes, minks, dogs...) [269-274]. Once efficacy of phage therapy was proven, another question raised: Are all phages equally potent therapeutic phages?

D) Choosing the Right Phage

All virulent phages can kill bacteria, but does that mean that every phage will be a good therapeutic candidate? Are there good and bad phages regarding phage therapy? Can *in vitro* testing predict *in vivo* efficacy? These questions have been addressed (more or less directly) throughout different studies.

In 2009, Heo *et al* [275] reported antibacterial efficacy of phages against *P. aeruginosa* infections in mice and in flies. This study focused on two phages isolated from sewage using PAO1 as host, MPK1 (a *Myoviridae*) MPK6 (a *Podoviridae*). *In vitro* experiments revealed that both phages use the LPS as bacterial receptor and that MPK1 lyses faster than MPK6. Moreover, these two phages showed different pharmacodynamics as MPK1 displayed better persistence than MPK6 in blood, liver and lungs of mice, and in tissues of flies. To test the *in vivo* efficacy of these phages, they were administered in mice by either the intramuscular (i.m.) or intraperitoneal (i.p.) route with different dosage (2×10^6 pfu or 2×10^7 pfu) 6 h post peritonitis infection with PAO1. While the 2×10^7 pfu dose of each phage significantly protected the infected mice, compared to the untreated mice, the MPK6 dose of 2×10^6 pfu administered by the i.m. route failed to protect mice. Presumably, the i.p. administration displayed better efficacy than the i.m. administration for both phages because i.p. route delivers phages more directly to the infection site (in this peritonitis model). Additionally, phage efficacy was also investigated in flies, where despite large differences in the pharmacokinetics of these two phages in fly tissues, they showed similar efficacy on fly survival and no toxicity after 72h phage feeding. Overall this report clearly demonstrates that different phages might present distinct pharmacokinetics profiles, potentially impacting their efficacy (depending on the administration route and animal model).

The different efficacy of distinct phages, and the ability to extrapolate *in vivo* success from *in vitro* tests has been thoroughly investigated by our lab. Henry *et al* [276] studied 9 different phages, through *in vitro* experiments including EOPs (Efficiency Of Plating) and lysis kinetics, and *in vivo* with an acute lung infection mice model (intranasal phage treatment applied 2h post infection, at MOI 0.1). Overall, for 7 of the phages, a good correlation between *in vitro* and *in vivo* efficacy was observed. However, this was not the case for two phages: PhiKZ and CHA_P1. Despite having an EOP of 1.2 on the PAK-lumi strain and a reasonably

good lysis kinetics (*in vitro*), survival rates following treatment with phage PhiKZ (MOI 0.1) were as low as those treated with PBS (mock treatment). Interestingly, an increase of the phage dose to reach a MOI to 20 provided enough phage to obtain 100% survival. Concerning CHA_P1, despite similar *in vitro* lysis kinetics and genetic closeness to some “effective phages”, this phage was unable to cure animals infected with the PAK-lumi strain. Nonetheless, CHA_P1, isolated using strain CHA a host, was able to rescue CHA-infected mice, proving its therapeutic potential. An attempt to adapt CHA_P1 to the PAK strain by serial passages failed to increase its EOP and its *in vivo* therapeutic efficacy. Finally, this study demonstrates that *in vitro* activity (*e.g.* the ability to form plaques on an overlay of a bacterial strain) is not sufficient to ensure *in vivo* efficacy. Nevertheless, a correlation was found between *in vitro* data (high EOP, quick lysis kinetics) and potent therapeutic effects *in vivo*. It also demonstrated that *in vitro* phage adaptation is not always possible and that phages isolated using the bacterial strain of interest seem more efficient, which is a strong argument for personalized or “sur mesure” phage therapy compare to the fixed or “prêt à porter” product [277].

Another study from our laboratory included the *in vitro* efficacy of the above 9 phages on the few isolates from the sputum of CF patients [278]. Interestingly, the most active phage, PAK_P5, was genetically closely related to the least active phage tested CHA_P1. As these two phages share protein sequences with around 90% identity, it showed that phage genomic data is insufficient for predicting infectivity of clinical strains.

Overall, it seems that all phages are not equal regarding their therapeutic efficacies. Indeed, all phages do not possess the same pharmacodynamics profile or the same ability to infect a specific strain. Moreover, today, we are unable to predict phage characteristics from genomic data alone, as phages sharing high sequence identity might still present very different infection abilities. Luckily, some of these critical parameters can be assessed *in vitro* and the results might predict *in vivo* efficacy, thus helping us choosing the right phage. Unfortunately, some phage characteristics (*e.g.* EOP or lysis kinetics) are host dependent and will need to be reassessed for every clinical strain on which phage use is intended.

Once potent phages have been identified, the question of formulation rises. A therapeutic phage product with a fixed formulation will need to infect many strains, whereas a

more personal therapeutic approach might consider the synergy of different phages against a specific bacterial strain.

E) Phage Product Formulation

Phages ability to infect only a limited number of strains of a bacterial species has led to the formulation of phage combination (also known as phage cocktail). For instance, a phage product available in Russia, recommended for the treatment of *E. coli* infections, contains 18 distinct phages, including the three main families of *Caudovirales* (*Podoviridae*, *Myoviridae*, and *Siphoviridae*) [279]. The Russian strategy for formulating cocktail relies on regular update, with new phages added to adapt to the evolving epidemiology of the targeted pathogen. As the previous phages are kept and new phages are added, this historical strategy leads to very complex formulation. This formulation strategy was, and still is, also employed at the Eliava Institute in Georgia [251].

In western countries, the situation is different as current regulation regarding drugs requires to demonstrate the safety and efficacy of a defined, fixed product (no update possible). This has led phage companies to focus on host range driven strategy, as the final phage product has to be able to infect most of the clinically relevant strains of a specific pathogen. This is sometimes referred as the "prêt à porter" strategy, as patients with the same disease, but different infecting strains, will receive the same treatment [277].

However, in academic labs, focusing on specific phage/bacteria systems, another phage cocktail design strategy emerged: tailored-made or "sur mesure" [277]. This cocktail design strategy does not focus on the host range parameter, but rather on the efficacy of the cocktail on a specific strain. This strategy was motivated by observations of phage resistant bacteria emerging following phage application *in vitro*. Indeed, selection of phage-resistant bacteria was already described in details by Luria and Delbrück almost 80 years ago [153]. They observed that bacterial cultures infected by phages initially become clear (due to phage-induced lysis), but after variable amounts of time (from hours to days) the cultures became turbid because of the growth of phage-resistant bacteria. Moreover, they found that the selected phage-resistant bacteria were already present, in low abundance, in the initial bacterial culture.

Interestingly, the simple addition of a second phage can delay the growth of phage-resistant clones of *P. aeruginosa*, from 8-9 h for each single phage to more than 20 h for the combination of the two [280]. In another *in vitro* study, Hall *et al* [281] described a variability of effectiveness of single-phage treatments with frequent resistance and cross-resistance. Additionally, they showed that multi-phage treatment is more efficient than any phage alone, and that the simultaneous application of different phages was better or equal to the sequential application, in reducing bacterial population density. However, these two methods were similar (on average) regarding decreasing resistance rates. Note that phage-resistant bacteria emerged in all conditions tested and were generally characterized by a reduced growth rate in absence of phages, suggesting a fitness defect. Moreover, when investigating the therapeutic potential of these phages (in wax moth), the authors found that a phage cocktail was the most effective short-term treatment as it cured otherwise lethal infections. On the other hand, a study focusing on three *P. aeruginosa* phages from three different genera (SL1, a PB1-like virus, SL2, a phiKZ-like virus and SL3, a LUZ24-like virus), found that a cocktail with these three phages had similar effect than the best phage alone *in vivo* (*Galleria melonella*) [282]. Therefore, in this model, no synergy between the phages was detected. Finally, O’Flynn *et al* described a similar frequency (10^{-6} CFU) of resistant bacteria to either a 3 phages cocktail or each individual phages [283], demonstrating once again that a multiple phage treatment is not always more efficient than monophage treatment.

As the random combination of different phages does not seem to be the most successful strategy to design a potent phage cocktail, research groups have designed strategies relying on different rationales to build phage cocktails.

F) Phage Cocktail Design Strategies

One strategy proposed to efficiently prevent, or at least delay phage resistance was to incubate a phage and a bacterial strain to isolate phage-resistant clones, and then select additional phages for their capacity to infect the phage-resistant strain. This process could be repeated multiple times to insure that phage resistant selected against one phage would be lysed by another phage of the cocktail. Once combined this way, the cocktail should prevent the growth of at least some of the phage-resistant population. For example, this approach allowed Gu *et al* [284] to design a phage cocktail against *Klebsiella pneumoniae* that significantly delays

the growth of phage-resistant bacteria *in vitro* (26 h compared to 6 h for monophage treatment), and also reduces the frequency of phage-resistant bacteria (10^{-7} CFU, compare to 10^{-4} for monophage treatment). Moreover, the phage cocktail had a higher efficacy than monophage treatment *in vivo* as the minimal protective dose of the cocktail to protect 80% of mice from lethal infection was 3×10^4 PFU compared to 3×10^5 to 3×10^7 PFU for individual phages. This strategy is straightforward but is exceedingly time-consuming for deployment in clinics.

Another strategy to formulate phage cocktail relies on combining phages that use different receptors on the bacterial cell surface. If, the idea of combining different phages to limit cross-resistance is quite old (first report of this strategy by Smith and Huggins in 1983 [285]), the emphasis on the receptor used by the phage is more recent, as works on phage resistance mechanisms has increased by the lower cost of genome sequencing [286-288]. For example, Wright *et al* [288] investigated cross-resistance among *P. aeruginosa* resistant clones evolved against 27 different phages. The authors discovered that cross-resistance between phages using the same receptor was conferred by mutations targeting either LPS or type IV pilus biosynthesis. On the other hand, mutations affecting a general regulator (RpoN) provided cross-resistance between phages using different receptors, but were characterized by a higher fitness costs than phage receptor mutations. Overall this study supports the idea that combining phages according to their bacterial receptors could be a very potent cocktail design strategy, when the aim is to limit bacterial resistance.

However, this strategy has not yet been used as a guiding strategy by most phage laboratories or companies, probably because the identification of these receptors is not easily done: compared to quite simple *in vitro* testing such as EOPs or lysis kinetics, performed in 24 to 48h at almost no costs, the determination of a phage receptors might take a much longer time from days to weeks. Therefore, besides model phages studied by academic groups, most of phages isolated with the aim to treat patients do not have their receptors characterized [289].

Overall, different strategies have been imagined to formulate potent phage cocktails, and our growing understanding of bacterial phage resistance mechanism will surely inspire new ones. Nonetheless, despite their inability to prevent phage resistance *in vitro*, various phage treatments still manage to be efficient *in vivo*. For instance, when testing a 6 phages cocktail (including PAK_P1 and PAK_P4 from our laboratory), Forti *et al* [290] observed that this

treatment was unable to prevent the growth of phage-resistant *in vitro* but still managed to rescue mice from lethal acute lung infections. As phage resistant bacteria have been isolated *in vivo*, either from animal models [291] or from patients [267], they are not “artefacts” from *in vitro* conditions. However, their relevance during the treatment of bacterial infections *in vivo* remained imprecise, suggesting that other factors might contribute to phage therapy success. One of these factors, and perhaps the most critical, is the involvement of the immune response that is building upon exposure to a bacterial pathogen.

G) Interactions Between Phage Therapy and the Immune System

In 2017 our lab reported a new concept termed “immunophage synergy” describing the important contribution of innate immune components to the success of monophage therapy [292]. Indeed, Roach *et al* explored phage therapy efficacy in different immunocompromised mice, and demonstrated that while a treatment with PAK_P1 alone is successful to rescue immunocompetent wild-type (WT) mice from acute lung infections, it is unsuccessful in immune signaling deficient mice (*MyD88*^{-/-}). Moreover, bioluminescence monitoring of the PAK-lumi strain showed that the signal declined during the first 24 h, consistent with phage lysis, but then increased during the next 2 days until the death of animals. As the 10 clones isolated at 24 h were found to be resistant to PAK_P1, it seems that *MyD88*^{-/-} mice were permissive to the growth of phage-resistant bacteria, in contrast to WT mice. Interestingly, *MyD88* deficiency in mice [293] and children [6] leads to impaired innate immune responses and increased susceptibility to *P. aeruginosa* infections. This is because of the major role of *MyD88* as an adaptor protein required for signal transduction of inflammation via Toll-like receptors (TLRs) and receptors of the IL-1 family [294].

As *MyD88* deficiency prevent immune cell activation and recruitment to the site of infection [294, 295], the authors asked which effector cells actually contribute to phage therapy success. First they evaluated the contribution of Innate lymphoid cells (ILCs) by performing phage therapy in *Rag2*^{-/-}*Il2rg*^{-/-} mice unable to produce ILCs (lymphocytes NK, B and T) [296]. They found that phage therapy was successful in the absence of ILCs as phage treatment rescued more than 90% of the infected animals. Moreover, the bioluminescence profile of phage-treated *Rag2*^{-/-}*Il2rg*^{-/-} mice was overall similar to the profile of phage-treated WT mice. Thus, ILCs do not seem to contribute to phage therapy success in this acute lung infection

model. Next, the authors investigated the potential role of neutrophils in phage therapy efficacy, using an antibody to deplete these cells in mice. In these neutropenic mice, phage treatment did not improve their survival, thus demonstrating the importance of neutrophils in phage therapy success (in this experimental model).

Additionally, the authors also established that the half-life of phage PAK_P1 in the lungs of an immunocompromised mice (*MyD88*^{-/-} mice) is longer than in a WT mice, pointing to a clearing of the phages by immune components (a phenomenon already described in other studies [297, 298]).

These results allowed a better understanding of the interactions between phages, bacteria, and the immune system, represented in the **Figure 9**.

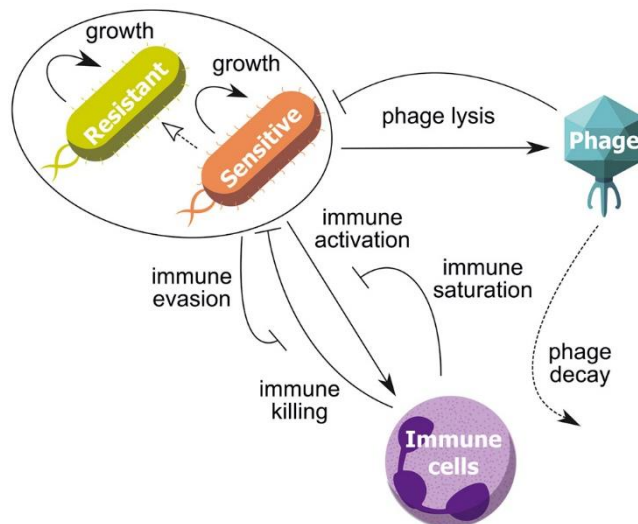


Figure 9 : Schematic representation of the interactions between bacteria, phages and the immune system (from [292]): Phage can inhibit the growth of phage-sensitive bacteria through phage lysis, whereas the immune cells can kill both phage-sensitive and phage resistant bacteria. This immune killing can be activated by the recognition of pathogen components, but also inhibited by bacteria through immune evasions mechanisms. Additionally, immune regulation can also limit the inflammation process (to limit tissue damages) and immune cells contribute to phage elimination.

To summarize, in an immunocompetent mouse, a bacterial infection might be uncontrolled by the immune system, thus causing the death of the animal. A phage treatment given to this animal can efficiently reduce the bacterial population by lysing the phage

susceptible bacteria, while the immune system (especially neutrophils) can eliminate both phage susceptible and phage-resistant bacteria: the synergistic action of phages and neutrophils allows a successful treatment. However, in the absence of neutrophils (*e.g.* in *MyD88*^{-/-} mice) phages can lyse the phage-susceptible bacteria, but phage-resistant clones will grow uncontrolled leading to the animal death.

The second project of my PhD was directly linked to the above results with the objective to restore phage therapy efficacy in immunocompromised mice (*MyD88*^{-/-}). The strategy that I tested aimed to limit phage resistance through the design of a tailored phage cocktail.

Results

Overview

During my PhD I worked on two different project both related to the study of phages infecting *P. aeruginosa*. Hereafter, I briefly summarized the main results obtained for each of these two projects, which are then developed under the form of two drafted papers. For the first project on a phage protein, we are currently looking for a journal that will send it out for review. For the second project, on phage therapy and phage resistance in immunocompromised animals, a last piece of data from genome sequencing will be obtained in the next few weeks and the draft will be submitted soon after.

1- A bacteriophage protein favors bacterial host takeover by impairing stress response.

Our laboratory has isolated several phages infecting the *P. aeruginosa* strain PAK and initiated the characterization of the original molecular mechanisms they deploy to hijack bacterial resources. More precisely, two publications focused on phages PAK_P3 [216] and PAK_P4 [217].

The transcriptomic analysis of strain PAK infected by either phage revealed rapid degradation of the host mRNA, although with different kinetics as PAK_P4 was faster than PAK_P3. Moreover, these two phages showed different pattern of expression of their core genes. To better understand the underlying molecular mechanisms of the infection by these phages, we followed these studies by focusing on the only early-expressed gene conserved between these two phages: *gp92* in PAK_P3 and *gp109* in PAK_P4.

Since the function of the proteins coded by these two genes could not be predicted and since they do not share sequence homology with any other proteins except few homologs present in closely related *P. aeruginosa* phages, we decided to combined both global and focal approaches to identify the role of these proteins during phage infection. For this we cloned these phage genes and inserted them on the bacterial chromosome, under an IPTG inducible promoter. Overall, we found that expression of *gp92* alters bacterial motility, morphology and proteome, without affecting bacterial growth. Surprisingly, despite sequence homology and similar localization (in the cytoplasmic membrane), expression of *gp109* had none of these effects. Moreover, we linked the morphological changes induced by Gp92 with its interaction with the anti-sigma factor MucA, a major regulator of membrane-associated stress response in *P. aeruginosa*. Finally, we found that Gp92 impairs this stress response, which results in an increased susceptibility of *P. aeruginosa* to antibiotics, including Imipenem.

This work demonstrates that the study of phage proteins of unknown functions can reveal new bacterial weaknesses and could guide the development of new drugs.

Title

A bacteriophage protein favors bacterial host takeover by impairing stress response.

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Conceptualization, MDJ, AC and LD; Methodology, MDJ, AC, GK, EB, MM, RL and LD; Investigation, MDJ, AC, EB, GK; Data Analyses, MDJ, AC and LD; Writing – Original Draft, MDJ, AC and LD; Writing – Review & Editing, MDJ, AC and LD with contributions from RL and GK; Supervision, AC and LD; Funding Acquisition, LD and RL

Competing interest statement

The authors declared no competing interest.

Abstract

Bacteriophages have developed unique and diverse strategies to hijack bacterial functions. However, the underlying molecular mechanisms remain largely understudied. We characterized *gp92*, an early-expressed gene from the virulent bacteriophage PAK_P3 infecting the opportunistic pathogen *Pseudomonas aeruginosa*. The ectopic expression of *gp92* alters bacterial morphology and motility and provokes a major shift of the cell proteome, including several sigma factors, without affecting bacterial growth. Through its interaction with the anti-sigma factor MucA, Gp92 impairs the membrane-associated stress response mediated by the sigma factor AlgU. This in turn revealed an unexpected link between AlgU-mediated stress response and phage lysis timing. In addition, expression of *gp92* increased the susceptibility of *P. aeruginosa* to antibiotics, including Imipenem, attesting that targeting the membrane stress response can increase bacterial susceptibility to carbapenems. Investigations of unknown bacteriophage functions not only identify original molecular virus-host interactions but also provide opportunities to uncover new bacterial targets for drugs development.

Introduction

Bacteriophages (phages) are the viral enemies of bacteria. Through evolution they have built an arsenal of mechanisms to disrupt the bacterial physiology, including genes that are involved in the subversion of the host metabolism, a process which occurs at the early stages of infection and consists in the implementation of specific hijacking tasks (1). Some of these early tasks have been well characterized, such as the redirection of the bacterial RNA polymerase towards phage genes (2) or blocking host defense systems (3, 4), but could as well highlight putative targets for developing new antibacterial drugs (5, 6). This is particularly needed in the current context of antimicrobial resistance. Metagenomics studies of environmental and clinical samples provide a large resource to be exploited (7, 8). However, most of the viral genetic information has not yet been associated with defined functions and is referred to as viral “dark matter” (9).

To identify the functions of early-expressed phage genes, several approaches have been proposed, ranging from targeted searches for a given phenotype (*e.g.* growth inhibition) to more general strategies relying on mass spectrometry (5, 10-12). Despite the potential for discovery of novel biological functions and biotechnological development (5), systematic studies of phage early gene products remain scarce.

We previously uncovered two related genera of virulent phages infecting the opportunistic pathogen *Pseudomonas aeruginosa*, namely *Nankokuvirus* and *Pakpunavirus*, from which one representative (PAK_P3 and PAK_P4, respectively) was studied in depth (13-15). Despite the overall genetic divergence between PAK_P3 and PAK_P4, we found that the transcriptional landscapes of cells infected by these phages displayed strong similarities, notably with the activation of a common host response, suggesting that the host subversion strategies of PAK_P3 and PAK_P4 may rely on conserved functions that we aim to uncover in the present study.

We identified and functionally characterized the only early-expressed gene family conserved across *Nankokuvirus* and *Pakpunavirus* genera (15). The representative genes from PAK_P3 and PAK_P4 genomes, *gp92* and *gp109*, respectively, both encode small proteins that localize at the cytoplasmic membrane. Surprisingly, only the ectopic expression of *gp92* altered the morphology and swimming motility of *P. aeruginosa* cells. By combining global and targeted approaches, we showed that expression of *gp92* reduced the ability of the host cell to respond

to membrane-associated stress and proposed that this is required for the control of optimal phage lysis timing. In addition, we uncovered that cells expressing *gp92* were more susceptible to carbapenems, one of the last resort antibiotics.

Results

Phages PAK_P3 and PAK_P4 share a common set of early expressed genes

To identify early-expressed phage genes, we first analyzed transcriptomic data collected at an early time point (3.5 min) during the infection of *P. aeruginosa* strain PAK with either the virulent phage PAK_P3 or PAK_P4 (13, 14) and identified 50 and 49 genes, respectively (Table S1). Next, by comparing this information with the list of core genes of *Nankokuvirus* and *Pakpunavirus* genus members (15), we found that the gene encoding Gp92 from PAK_P3 (Prot ID: YP_008857727.1) and its homolog in PAK_P4 encoding Gp109 (Prot ID: YP_008859320.1) were the only early-expressed genes that were conserved across the two phage genera (Table S2) and were therefore selected for further characterization. In addition, the analysis of the host transcriptome at the onset of PAK_P3 and PAK_P4 infections (3.5 min) revealed similar variations, with 44 host genes being commonly upregulated during both infections (Table S3). This indicates that both phages may trigger the same responses as soon as they infect their host cell, which might rely on the conserved early phage proteins Gp92 and Gp109.

The phage protein Gp92, but not Gp109, alters bacterial morphology and motility

To explore the functions of Gp92 and Gp109, the corresponding genes were inserted into the chromosome of PAK, downstream an IPTG-inducible promoter, yielding the isogenic strains PAK-*gp92* and PAK-*gp109*. The control strain, denoted PAK(-), carried an “empty” expression cassette (*i.e.* devoid of a gene product). Upon induction, we found that the colonies of strain PAK-*gp92* were less expanded compared to the control strain (Figure 1A). This phenotype was not associated with significant alteration of cell viability nor growth rates (Figure S1A,B). By contrast, the colony expansion of strain PAK-*gp109* remained unchanged (Figure 1A),

suggesting that despite the similarity of their protein sequences (47% identity over 90% of the length), Gp92 and Gp109 may not have identical functions. We also observed that expression of *gp92* had the same effect in *P. aeruginosa* strains CHA (cystic fibrosis isolate) (16) and PAO1, which are respectively susceptible and resistant to both phages PAK_P3 and PAK_P4 (Figure 1A). Interestingly, the mucoid phenotype (resulting from overproduction of alginate) of strain CHA was also lost upon expression of *gp92* (Figure 1A). Finally, we found that expression of *gp92* (but not *gp109*) had the same impact on the expansion of *Escherichia coli* colonies, suggesting that Gp92 interferes with a process that is conserved across *P. aeruginosa* and *E. coli* species (Figure S1C).

Next, we investigated the first stages of colony formation using time-lapse microscopy, by monitoring the growth of PAK-*gp92* and control strains on acrylamide pad soaked in LB with IPTG. After 4.5 h of growth we observed morphological changes of cells (Figure 1B). The circularity index of PAK-*gp92* cells (0.66 ± 0.1 ; mean \pm standard deviation (sd), $n=70$) was significantly higher than that of the control cells (0.38 ± 0.1 ; mean \pm sd, $n=30$; $p<0.0001$, Mann Whitney test). Another factor that can affect colony expansion is bacterial migration (17), so we measured bacterial motility and found that it was significantly reduced upon the induction of *gp92* (Figure 1C). Altogether, these results showed that the expression of *gp92* modified both the morphology and the motility of cells, the latter leading to the reduction of the size of colonies.

Gp92 massively alters the composition of the bacterial proteome

Following these population-level and single-cell analyses, we evaluated the impact of Gp92 at the molecular level by non-targeted mass spectrometry. We analyzed and compared the whole proteomes of induced and non-induced PAK-*gp92* cells (Table S4). A total of 1,004 proteins were detected in non-induced cells, amongst which 83 (8.3%) were significantly more abundant than in induced cells. Among the 1,018 proteins detected in induced cells, 113 (11.1%) had significantly higher levels than in non-induced cells (proteins detected in only one or the other condition could not be statistically compared nor displayed in the plot; see methods; Figure 2; Table S4). Therefore, the expression of *gp92* had a large impact on the measured proteome of *P. aeruginosa*. In particular, we observed in induced cells an increased abundance of proteins

involved in the maintenance of membrane integrity (yellow dots in Figure 2, Table S4), including Tol-Pal and Lol systems. We also observed the presence of the sigma factor RpoH (not detected in absence of induction), a global transcriptional regulator playing a role in protein folding, particularly during the heat-shock response (18) (Table S4). The sigma factor FliA, involved in flagellum synthesis (18) and the motor brake FlgZ, involved in swimming inhibition (19), were also more abundant upon *gp92* expression (Table S4). As an excess of FliA was shown to negatively impact swimming motility (20), this observation is consistent with the reduced motility of induced PAK-*gp92* cells (Figure 1C). Interestingly, proteins related to antibiotic resistance such as the beta-lactamase AmpC and CpxR, which is an activator of the MexAB-OprM efflux system, were more abundant in non-induced compared to induced cells (orange dots in Figure 2, Table S4) (21). Finally, we observed an impact of *gp92* expression on proteins related to Type 6 secretion system (T6SS), and R2-type pyocins, which were found more abundant in non-induced cells, whereas proteins related to Type 3 secretion system (T3SS) were more abundant in induced cells (dark blue dots in Figure 2, Table S4). Altogether, the phenotypical and molecular data indicate that the expression of *gp92* has a broad effect on the bacterial envelope homeostasis and membrane-related processes (*e.g.* motility, exopolysaccharide synthesis, protein secretion, antibiotic resistance).

Gp92 is likely anchored to the cytoplasmic membrane

To understand how Gp92 mediates such broad effects on *P. aeruginosa* cells, we investigated the characteristics of this short protein (78 amino acid) in more details. Alignment of its amino acid sequence with homologous proteins (encoded by related *P. aeruginosa* phages) revealed a modular structure composed of two domains (hereafter referred to as “Nt” for amino-terminal and “Ct” for carboxy-terminal) with high levels of identity, which are separated by a non-conserved stretch of seven to nine amino acids (Figure 3A). The size and composition of the Nt domain (21 to 23 aa, half of which being hydrophobic) suggested that Gp92 may be localized at the membrane or secreted in the periplasm, despite *in silico* analyses predicting no transmembrane segment nor signal peptide (SP) (see methods). Fusions of different sections of Gp92 (full length, Ct or Nt domains) with a PhoA-LacZ reporter were introduced in *E. coli* and their activities were evaluated using chromogenic media (Figure 3B). These assays showed that

the Nt domain supports the periplasmic localization of the reporter, while the Ct domain remains in the cytoplasm.

Next, we evaluated if and how the localization of Gp92 would affect colony expansion. We observed a reduction of the size of colonies only with the full-length Gp92, but not with the Ct or the Nt domains on their own, nor when the Ct domain was artificially exported into the periplasm using PhoA SP (Figure 3C). Therefore, we concluded that Gp92 is likely anchored to the membrane and this localization is crucial to mediate its function. Importantly, since both Gp92 and Gp109 displayed the same localization (Figure S2) but did not cause the same effects on colony expansion, we concluded that the production of such a likely-anchored membrane protein *per se* was not responsible for the observed phenotypes. It also suggested that the differences between Gp92 and Gp109 activities were likely caused by the divergence in the sequences of their Ct domains. Finally, homology searches (Phyre2) (22) with the full length or with the Ct domain of Gp92 did not reveal further predictions about the function of this protein.

The membrane stress response regulator MucA is targeted by Gp92

To unravel the function of Gp92, we searched for putative bacterial protein partners by performing two independent bacterial adenylate cyclase two-hybrid (BACTH) screens (23) using either an *E. coli* or a *Salmonella typhi* peptide library. The choice of these libraries was guided by the fact that the effect on colony expansion was observed in both *P. aeruginosa* and *E. coli* (Figure 1 and S1), therefore we aimed at identifying an interacting partner that is conserved across diverse bacterial species. The screens identified 13 inner-membrane protein candidates (Tables S5), amongst which the anti-sigma factor RseA was most frequently identified and the only one present in the two independent screens (Table S5). Anti-sigma factors are regulatory proteins that sequester sigma factors thereby preventing their binding to the RNA polymerase core and the transcriptional activation of genes they control (known as regulon). The homolog of RseA in *P. aeruginosa* is the transmembrane protein MucA that sequesters the sigma factor AlgU, which is involved in mediating the response to membrane-associated stresses (24). Using pairwise interaction tests, we confirmed interactions between Gp92 and both RseA and MucA (Figure 4A). To confirm the involvement of MucA, we used

an isogenic *P. aeruginosa* strain carrying the *mucA22* allele, which encodes a truncated non-functional version of MucA. We found no reduction of the size of colonies upon *gp92* expression in this strain (Figure 4B). Importantly, in the *mucA22* background, AlgU is no longer sequestered and consequently, the membrane-associated stress response is constitutively activated (25). Therefore, the loss of the phenotype may be the direct consequence of the absence of MucA or indirectly due to the constitutively activated membrane-associated stress response.

Gp92 impairs cell response to membrane-associated stress

Because Gp92 has a broad effect on the bacterial proteome related to envelope homeostasis (Figure 2) and directly interacts with MucA (Figure 4), we next investigated whether Gp92 impacted the ability of bacteria to respond to membrane-associated stress. We tested the transcriptional activation of AlgU regulon by quantifying the transcripts level of *algD*, commonly used as a reporter of the activation of AlgU-response (25). In absence of stress, the expression of *gp92* by itself did not induce the transcription of *algD* (Figure S3A). By contrast, in presence of D-cycloserine, an analogue of D-alanine interfering with cell-wall synthesis, we observed a strong increase of *algD* transcript levels, as previously reported (Figure S3A) (25). Under this stress, the induction of *gp92* expression significantly reduced the level of *algD* transcripts compared to cells that do not express *gp92* (Figure S3A), indicating that Gp92 limits the transcriptional activation of the AlgU-stress response.

Next, we evaluated with strain PAK-*gp92* how the expression of *gp92* affected the proteome during a membrane-associated stress. A total of 1,005 proteins were detected in non-induced stressed cells, amongst which 99 (9.9%) were significantly more abundant than in *gp92*-induced stressed cells, suggesting that *gp92* expression lowers the abundance of these proteins (Figure S3B and Table S6). Among the 1,007 proteins detected in *gp92*-induced stressed cells, 95 (9.4%) had significantly higher levels than in non-induced stressed cells (Table S6). These results indicated that the induction of *gp92* had a broad impact on the proteome of stressed cells with variations detected across several pathways, including 20 transcriptional regulators. Nevertheless, proteins related to stress and/or adaptation (black dots in Figure S3B and Table S6) and proteins regulated by AlgU (purple dots in Figure S3B and Table S6) were detected in

both conditions, but with different abundance, hinting that the AlgU stress response is altered in *gp92* expressing cells. Notably, we found that AlgW, the protease that cleaves MucA to release AlgU and activate the stress response (26), was more abundant in *gp92*-induced stressed cells, which suggests that Gp92/MucA interaction may affect AlgW/MucA interaction. Consistent with previous results (Figure 2), we found that RpoH was over-represented in *gp92*-induced stressed cells, as well as the proteins it regulates (green dots in Figure S3B and Table S6). The impact of Gp92 on protein secretion systems (TSS3 and TSS6) previously observed (Figure 2, Table S4) was maintained upon membrane-associated stress (dark blue dots in Figure S3B and Table S6). We also noticed the significant increased abundance of several proteins related to antibiotic resistance (a beta-lactamase in non-induced stressed cells and two components of efflux systems, OprM and MexV, in induced stressed cells) (orange dots in Figure S3B and Table S6).

Altogether, these data suggest that Gp92 affects the capacity of bacteria to activate a stress response, both at the transcriptional and protein levels. Strengthening this hypothesis, we found that bacteria expressing *gp92* became more susceptible to sub-lethal concentrations of D-cycloserine as their growth rate decreased by 20% compared to wild type bacteria (Figure S3C,D).

Expression of *gp92* is lethal in cells lacking *algU*

Two observations: (i) the lack of Gp92-associated phenotype in the *mucA22* strain and (ii) the alteration of the AlgU-mediated stress response by Gp92, prompted us to investigate the effect of *gp92* expression in a strain lacking *algU* ($\Delta algU$ strain). Surprisingly, we observed that the viability of these bacteria was dramatically reduced (Figure 5A), with an effect on the growth of the bacterial population detectable as soon as 4 h after induction of *gp92* expression (Figure 5B). A *trans*-complementation with a plasmid encoding AlgU restored cell viability ($2.6 \cdot 10^6 \pm 1.8 \cdot 10^6$ CFU/mL without IPTG compared to $8.3 \cdot 10^5 \pm 7.2 \cdot 10^5$ CFU/mL with 1 mM IPTG; $n=3$). Strikingly, time lapse microscopy observations revealed that $\Delta algU$ cells expressing *gp92* underwent extensive morphological changes and eventually burst (Figure 5C). Interestingly, the severity of Gp92 effects on the physiology of $\Delta algU$, WT and *mucA22* strains (respectively, lethal, mild, none) was inversely correlated with the level of free AlgU available in the cell

(null, intermediate, high), strengthening the link between the activity of Gp92 and the AlgU-mediated stress response. Therefore, we speculated that cells with a functional AlgU-response may be protected from a lethal effect caused by Gp92.

Alginate biosynthesis rescues $\Delta algU$ cells from the lethal expression of *gp92*

Within the large AlgU regulon (293 genes),(27) the *alg* operon is the most well-known and is responsible for the biosynthesis of alginate, an exopolysaccharide that protects *P. aeruginosa* cells against adverse conditions (including oxidative stress, antibiotics and phages) (28). The promoter of the *alg* operon (*PalgD*, located upstream of the first gene, *algD*) is dependent of AlgU, therefore a strain lacking AlgU does not produce alginate. To test if alginate biosynthesis could protect cells against Gp92 activity, we studied a strain with an in-frame deletion of *algD*. We found that the expression of *gp92* reduced the viability of $\Delta algD$ cells by approximately two orders of magnitude (Figure 5D). The optical density of a liquid culture of the $\Delta algD$ strain remained unaffected upon *gp92* induction, which may be explained by the filamentous morphology that these cells adopted upon *gp92* induction (Figure 5E,F). To demonstrate further the protective role of alginate biosynthesis, we restored the transcription of the *alg* operon in a $\Delta algU$ strain, by replacing the native *PalgD* promoter by an arabinose-inducible promoter. The viability of these cells was largely recovered upon arabinose induction (Figure 5G) demonstrating that the biosynthesis of alginate plays a protective role against Gp92.

Nevertheless, the mechanisms by which Gp92 exerts its toxicity and by which alginate rescue cells will require further investigations. Next, we sought to understand the role of Gp92 during phage infection.

Gp92 reveals a novel link between phage lysis timing and membrane stress response

Given that Gp92 primarily interacts with MucA, we tested the performance of phage PAK_P3 on the *mucA22* strain. First, we found that the phage efficiency of plaquing (EOP) was significantly increased in the *mucA22* strain compared to the PAK(-) strain (Figure 6A). This effect was not caused by differences in phage adsorption (Figure S4A). Second, in conditions

of synchronized infection, we determined the time necessary to observe the first sign of lysis in the bacterial population (measured as the time needed to observe a 20% reduction from the maximum optical density). We found that lysis of *mucA22* cells was significantly delayed compared to PAK(-) cells (Figure S4B,C). Altogether, these data showed that the constitutive activity of the AlgU-mediated stress response delayed phage lysis and increased the EOP. We propose that, through its interaction with MucA, Gp92 participates to the control of optimal phage lysis timing, which is a critical determinant of phage fitness (29). Several mechanisms have been reported for the control of the lysis timing, but to our knowledge, none relied on the manipulation of a host sigma/anti-sigma system.

Gp92 increases the susceptibility of *P. aeruginosa* to carbapenems

Interestingly, the AlgU membrane-associated stress response is induced by several antibiotics (30), although it remains unclear to which extent this response contributes to the antibiotic-resistance of *P. aeruginosa*. We first defined the resistome of strain PAK using CARD (31) and ResFinder (32) and identified 52 genes predicted to be involved in antibiotic resistance, amongst which 43 (83.7%) are involved in efflux systems (Table S7). We also found that 15.4% of these genes are regulated by AlgU, including *mexAB-oprM* and *mexCD-oprJ*, two major efflux systems (33, 34). The mass spectrometry analyses reported above (Figure 2 and S3) have highlighted that the abundance of several proteins involved in antibiotic resistance were affected by the expression of *gp92*, leading us to measure the susceptibility of several strains of *P. aeruginosa* to antibiotics.

We found that the level of resistance to drugs targeting peptidoglycan synthesis (Penicillin, Cefoxitin, Ceftriaxone) was not affected in PAK cells expressing *gp92*, with the exception of Imipenem, one of the last resort antibiotics (Table 1). The synergy between Gp92 and Imipenem was also observed in the cystic fibrosis isolate CHA, with a tenfold reduction of the minimum inhibitory concentration (MIC). Moreover, we found that the MIC of Imipenem for strain PAK Δ *algU* was reduced while it was increased for strain PAK*mucA22* (Table 1). This suggests that the susceptibility to Imipenem can be modulated by the AlgU-stress response. Consequently, this stress response is a candidate target to raise the susceptibility of *P. aeruginosa* to carbapenems. We hypothesize that the increased susceptibility to Imipenem but

not to other β -lactams is due to the presence of two β -lactamases (*ampC* and OXA-50), which are not AlgU-dependent in their expression (35).

On the other hand, the susceptibility of *gp92*-expressing cells to drugs targeting protein synthesis gave mixed results. Indeed, MIC values of Kanamycin, Tetracycline and Chloramphenicol were reduced (making the resistant strain sensitive again) while it was unchanged for Erythromycin (Table 1). The presence of 18 AlgU-independent macrolide resistance genes (Table S7) provide an explanation for the unchanged resistance to Erythromycin. Interestingly, the susceptibility to Chloramphenicol and Tetracycline was restored, but a strict link to AlgU regulon was not confirmed when testing PAK*mucA22* and PAK Δ *algU* strains (Table 1). Finally, the lower MIC value of Kanamycin was unrelated to AlgU regulon as both PAK*mucA22* and PAK Δ *algU* strains also displayed a reduced MIC value.

To conclude, this study of gene *gp92* revealed that phage PAK_P3 targets the membrane-associated stress response of *P. aeruginosa* to control phage lysis timing, but also unveiled that impairing this stress response provide a novel strategy to lower *P. aeruginosa* resistance to carbapenems.

Discussion

Metagenomics sequences have revealed a huge diversity of phage coding sequences. Aside from a handful of conserved functions (*e.g.* polymerase, major capsid protein and helicase) that are sufficient to re-organize viral classification (36), the function of most phage genes remain elusive. While the identification of bacterial phage resistance systems is racing ahead (37), the identification of phage genes that interfere with bacterial physiology is still lagging behind. Notably, the lack of genetic tools allowing an easy manipulation of virulent phage genomes is slowing down the identification of phage protein functions. Here we studied *gp92*, an early-expressed phage gene, which has a subtle effect on colony expansion. Our investigations demonstrated that Gp92 has a broad impact on bacterial physiology and notably impairs the AlgU-mediated stress response, which is involved in the susceptibility to Imipenem. We hypothesize that Gp92 disrupts this stress response to control the phage lysis timing, as activation of this response was associated with delayed lysis.

Following the initial observation of the modest effect that Gp92 has on the expansion of bacterial colonies, we uncovered unexpectedly broad consequences linked to the expression of *gp92*. First, the composition of the cell proteome was largely affected, with important variations in the levels of proteins involved in membrane homeostasis, which was in agreement with the drastic modifications of cell morphology that we observed. Strikingly, a large number of transcriptional regulators were affected by the expression of *gp92*, including two sigma factors (RpoH and FliA). A role of RpoH in phage infection has been reported previously, as it regulates chaperon proteins necessary for the assembly of phage capsids (38-40). We noticed that out of the 53-upregulated genes during early infection by phage PAK_P3, eleven (20.8%) were upregulated by RpoH (in bold in Table S3). This suggests that Gp92, might contribute to the massive transcriptomic alteration elicited by phage PAK_P3 described previously (14). Looking for specific targets of Gp92, we identified the anti-sigma factor MucA. The Gp92/MucA interaction impaired the AlgU membrane-associated stress response. Replacing this finding in the context of phage lifecycle revealed a novel link between this stress response and lysis timing.

Another surprising result came from the lethality of *gp92* expression in the absence of AlgU or alginate biosynthesis as it is unclear how the interaction of Gp92 with MucA would lead to cell death. Based on BACTH and mass spectrometry results, we hypothesize that the affinity of Gp92 for multiple membrane proteins may make cells more fragile. Further studies are now required to identify the molecular mechanism(s), underlying the pleiotropic effects of this small protein, as well as how it controls phage lysis timing.

Finally, our study uncovered a link between the AlgU regulon and the susceptibility to Imipenem. We hypothesize that peptides or drugs mimicking the activity that Gp92 has on the membrane stress response could be proposed as adjuvants to carbapenems, one of the last resort class of antibiotics.

Materials and methods

Bacterial strains and plasmids

Bacterial strains and plasmids are listed in Table S8. Strains were grown in LB medium at 37°C supplemented with appropriate antibiotics when required (kanamycin 50 µg.mL⁻¹, carbenicillin 100 µg.mL⁻¹ for *E. coli*, gentamycin 30 µg.mL⁻¹ for *P. aeruginosa*).

Genetic manipulation of bacteria and phage genes

All primers used for this study are listed in Table S8. Insertion of *gp92* and *gp109* coding sequences (CDS) on the chromosome of *P. aeruginosa* were performed as previously described (10). Briefly, phage CDS were PCR amplified from phage lysates and cloned in *E. coli* – *P. aeruginosa* shuttle expression vector pUC18-mini-Tn7T-Lac and verified by DNA sequencing. Expression vector was co-transformed with pTNS2 in *P. aeruginosa* cells allowing the integration of the expression cassette (inducible by IPTG) at the unique transposon site Tn7 on the bacterial chromosome.

The PAKΔ*algU*-*gp92*-P_{ara}(*algD*) strain was generated by replacing a 386bp-region containing the native *PalgD* promoter by a 1236bp-cassette including the *araC* regulator and the arabinose-inducible *araBAD* promoter, following a method previously described (41). Briefly, a 429bp-region upstream and a 446bp-region downstream *PalgD* were amplified from strain PAKΔ*algU*-*gp92*. The 1236bp cassette was amplified from the pJN105 plasmid. These three PCR amplicons were assembled together and flanked with *attB1* and *attB2* sites to form a recombination cassette, which was then inserted into the donor allelic exchange vector pDONRpEX18Gm using BP Clonase II (Invitrogen). The resulting vector was introduced into the strain PAKΔ*algU*-*gp92* through conjugation with *E. coli* S17.1 λ*pir*. Recombinant clones were isolated upon sucrose-mediated counter-selection and verified by amplifying and sequencing the recombinant region using primers AC_82F, AC_82R, AC_83F and AC_84R (Table S1).

Growth, motility and survival assays

Overnight cultures at 37°C in LB medium under agitation of strains carrying phage ORFs and their corresponding controls were diluted 100-fold in fresh LB medium and incubated 2 h until OD_{600nm} reached 0.15-0.4. To monitor growth in presence or absence of IPTG (1 mM), 100 µL of bacteria were introduced in 96-well plates and OD_{600nm} was recorded overtime (OD measured every 15 min after 30 sec of shaking, Infinite M200 Pro, Tecan). To evaluate the survival of strains upon induction of phage genes, ten-fold serial dilutions of refreshed overnight cultures were plated on LB agar supplemented with appropriate antibiotics with or without 1 mM IPTG. Plates were incubated at 37°C for 24 h to 48 h and scanned with Epson perfection V700 Pro scanner. Colonies areas were measured using ImageJ software. Motility assays were performed by spotting 3 µL of overnight cultures at the centre of a LB soft agar (0.3%) plate containing 1 mM IPTG and the swimming diameters were measured after 20 h of incubation at 37°C. To monitor the growth of bacteria in presence or absence of D-cycloserine (10 µM), refreshed overnight cultures were first cultivated in presence or absence of IPTG (1 mM) during 1 h before the addition of D-cycloserine or buffer and OD recording in the microplate reader. Growth rates correspond to the slopes (OD.h⁻¹) of linear regressions calculated between 1 and 3 h. For each strain, growth rate ratios between induced and non-induced conditions were calculated.

Time Lapse Microscopy

Exponentially growing bacteria were diluted to reach the OD_{600nm} value of 0.1 and then 2 µL were spotted on an acrylamide pad (previously soaked in LB + 1 mM IPTG for 4 h). Samples were eventually sealed under a coverslip with solvent-free sealant “Valap” (1:1:1, w/w/w vaseline, lanolin, paraffin) and placed under the microscope equipped with an incubation chamber set at 37°C. Successive divisions of single cells were monitored by taking pictures every 20 min during 8 h. Images were compiled and analysed using NIS-Elements Viewer 4.2 (Nikon) software. The circularity index is defined as $Circ = 4\pi \times [Area]/[Perimeter]^2$ and ranges from 0 (infinitely elongated polygon) to 1 (perfect circle) and was assessed with ImageJ software.

Sequence analysis tools

Homologs of *gp92* and *gp109* belonging to family of core ORFs #21 (15) from the corresponding phage genomes available from Genbank were aligned with ClustalO. Prediction of protein localization were performed with the online tools Topcons (<http://topcons.cbr.su.se/>) and TMHMM (<http://www.cbs.dtu.dk/services/TMHMM/>). The search for structural homologs using Phyre 2 returned for Gp92 a stretch of 17 amino acids with 11% identity to TGF- β with a confident score of 52% and a stretch of 32 amino acids with 25% identity to LuxS/MPP-like metallohydrolase for Gp109 with a confident score of 48%

Topological analysis of Gp92 and Gp109

Both *gp92* and *gp109* ORFs were PCR-amplified from phage lysates and cloned in frame in the pKTop plasmid encoding the dual PhoA-LacZ reporter (42). Specific primers (Table S8) were used to amplify both Nt (from nucleotide 1 to 78) and Ct (from nucleotide 69 to 234) parts of Gp92. Plasmids were verified by DNA sequencing and *E. coli* DH5 α strains carrying the resulting plasmids were plated on the following indicator medium: LB agar supplemented with 5-Bromo-4-chloro-3-indolyl phosphate ($80 \mu\text{g.mL}^{-1}$), 5-Bromo-6-chloro-3-indolyl β -D-galactopyranoside ($100 \mu\text{g.mL}^{-1}$), carbenicillin ($100 \mu\text{g.mL}^{-1}$), kanamycin ($50 \mu\text{g.mL}^{-1}$), IPTG (1 mM) and phosphate buffer (50 mM , pH 7.0). Plates were incubated at 30°C during 24 to 48 h.

RT-qPCR on D-cycloserine stressed cells

Strains PAK-*gp92* was grown in LB supplemented or not with IPTG (1 mM) during 1 h prior the eventual addition of D-cycloserine (10 mM). One hour later total RNAs were extracted by using RNeasy Plus Mini® kit (Qiagen). After DNaseI treatment and purification, RNAs were reverse transcribed following manufacturer's instructions (Random Hexamers, SuperScript™ III, Invitrogen) and quantitative PCR was performed (Applied biosystem 7300) using the primers indicated in Table S8. Relative expression was assessed by the $\Delta\Delta\text{Ct}$ method and normalized against *gyrA* expression.

Samples preparation and analysis of peptides by mass spectrometry

Strain PAK-*gp92* was cultivated in the same conditions as described for the RT-qPCR experiments. One hour after the eventual addition of D-cycloserine samples were centrifuged at 8.000g for 5 min and supernatants were discarded. Pellets were resuspended in 8 M urea, 100 mM Tris HCl pH 8.5, and disulfide bonds were reduced with 5 mM Tris(2-carboxyethyl) phosphine (TCEP) for 30 minutes and alkylated with 10 mM iodoacetamide for 30 min at room temperature in the dark. Protein samples were then incubated with 500 ng rLys-C Mass Spec Grade (Promega, Madison, WI, USA) for 5 h at 30°C for the first digestion. Then samples were diluted below 2 M urea with 100 mM Tris HCl pH 8.5 and 1 ug Sequencing Grade Modified Trypsin (Promega, Madison, WI, USA) was added for the second digestion overnight at 37°C. A second incubation with the same amount of trypsin (5 h at 37°C) was performed to ensure a complete digestion. Digestion was stopped by adding formic acid and peptides were desalted and concentrated on Sep-Pak C18 SPE cartridge (Waters, Milford, MA, USA) according to manufactures instructions.

Tryptic peptides were analyzed on a Q Exactive Plus instrument (Thermo Fisher Scientific, Bremen) coupled with an EASY nLC 1000 chromatography system (Thermo Fisher Scientific). Each sample was loaded on an in-house packed 50 cm nano-HPLC column (75 µm inner diameter) with C18 resin (1.9 µm particles, 100 Å pore size, Reprosil-Pur Basic C18-HD resin, Dr. Maisch GmbH, Ammerbuch-Entringen, Germany) and equilibrated in 98 % solvent A (H₂O, 0.1 % FA) and 2 % solvent B (ACN, 0.1 % FA). Peptides were first eluted using a 6 to 22 % gradient of solvent B during 150 min and then a 22 to 50 % gradient of solvent B during 60 min all at 250 nL.min⁻¹ flow rate. The instrument method for the Q Exactive Plus was set up in the data dependent acquisition mode. After a survey scan in the Orbitrap (resolution 70 000), the 10 most intense precursor ions were selected for HCD fragmentation with a normalized collision energy set up to 28. Charge state screening was enabled, and precursors with unknown charge state or a charge state of 1 and ≥ 7 were excluded. Dynamic exclusion was enabled for 45 s.

All data were searched using Andromeda (43) with MaxQuant software (44, 45) version 1.5.3.8 against the *P. aeruginosa* strain PAK database (2989 entries) concatenated with the phage PAK_P3 proteome (142 entries), as well as usual known mass spectrometry contaminants and

reversed sequences of all entries. Andromeda searches were performed choosing trypsin as specific enzyme with a maximum number of two missed cleavages. Possible modifications included carbamidomethylation (Cys, fixed), oxidation (Met, variable) and Nter acetylation (variable). The mass tolerance in MS was set to 20 ppm for the first search then 6 ppm for the main search and 10 ppm for the MS/MS. Maximum peptide charge was set to seven. Five amino acids were required as minimum peptide length. The “match between runs” feature was applied for samples having the same experimental condition with a maximal retention time window of 0.7 min. One unique peptide to the protein group was required for the protein identification.

For the statistical analysis of one condition versus another, proteins identified in the reverse and contaminant databases and proteins only identified by site were first discarded from the list. Then, proteins exhibiting fewer than two summed intensities in at least one condition were discarded from the list to avoid misidentified proteins. After log₂ transformation of the leftover proteins, summed intensities were normalised by median centering within conditions (normalized function of the R package DAPAR (46). Remaining proteins without any summed intensities in one of both conditions have been considered as proteins present in a condition and absent in another. They have therefore been set aside and considered as differentially abundant proteins. Next, missing values were imputed using the imp.norm function of the R package norm (47). Proteins with a log₂ (fold-change) inferior to 1 have been considered as proteins which are not significantly differentially abundant. Statistical testing of the remaining proteins (having a log₂ (fold-change) superior to 1) was conducted using a limma t-test (48) with the R package limma (49). An adaptive Benjamini-Hochberg procedure was applied on the resulting p-values using the function adjust.p of R package cp4p (50) with the robust method of Pounds and Cheng (51) to estimate the proportion of true null hypotheses among the set of statistical tests. The proteins associated to an adjusted p-value inferior to a FDR level of 1% have been considered as significantly differentially abundant proteins. Finally, the proteins of interest were therefore those which emerged from this statistical analysis supplemented by those which were considered to be absent from one condition and present in another.

Bacterial two hybrid assays

Bacterial two hybrid screening against *E. coli* and *S. typhimurium* libraries as well as BACTH and dimerization assays were performed as previously described (23). Briefly, plasmid

pUT18C-*gp92* was introduced into *E. coli cya* strains BTH101, DHM1 or DHT1. The resultant strains were then transformed with the indicated plasmids (Table S8) and plated either on M63 agar supplemented with 40 $\mu\text{g.mL}^{-1}$ X-Gal or MacConkey agar supplemented with 0.2% or 1% maltose, 0.5 mM IPTG, 25 or 50 $\mu\text{g.mL}^{-1}$ kanamycin and 50 or 100 $\mu\text{g.mL}^{-1}$ carbenicillin, respectively. Plates were incubated at 30°C for 2 to 7 days until appearance of colored colonies. Screened colonies were isolated twice and further characterized by DNA sequencing of the inserts.

Adsorption assays

PAK_P3 adsorption constants were calculated as reported previously (14). Briefly, exponentially growing bacteria were infected at a MOI of 0.001 and immediately after PAK_P3 addition ($t=0$) and then every 30 s, samples of 50 μL were collected, mixed with CHCl_3 and placed on ice until phage titration on strain PAK. To calculate the adsorption constant k (mL/min), we first plotted the neperian logarithm of the free phage count against time. Then, the slope of a linear regression was divided by the bacterial initial concentration (CFU/mL) used in the experiment. Experiment was performed in triplicate.

Phage lysis kinetics and timing

Exponentially growing cultures of bacteria (until $\text{OD}_{600\text{nm}}$ reached 0.4) were infected at MOI 15 (to ensure synchronized infection (14)) before introduction in the 96-well microplate reader for 2 h. OD was recorded every 97 s after 30 s of shaking. To compare lysis timing we calculated the time interval between the maximum OD value and the reduction of this OD value by 20% to capture the early kinetics of lysis. Means of this time interval ($n=3$) for each strain were compared to the control strain.

Acknowledgments

We thank Drs Nicolas Dufour, Luisa De Sordi, Marta Lourenço and Dwayne Roach for discussions and suggestions along this work. We warmly thank Dr. Jeroen Wagemans and Dr. Anne-Sophie Delattre for providing guidance on cloning phage genes. We thank Sven van Teffelen for access to time-lapse microscope. We are grateful to Mariette Matondo and Quentin Gaii-Gianetto from the proteomic platform of Institut Pasteur.

Funding

AC and MDJ were supported by Sorbonne université, collège doctoral, Paris, France. RL and LD are members of the « PhageBiotics research community », supported by the FWO Vlaanderen.

This article is part of a project that has received funding from the European Research Council (ERC) under the European Union's ERC consolidator grant awarded to RL (Grant agreement No. [819800]).

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Figures and Tables

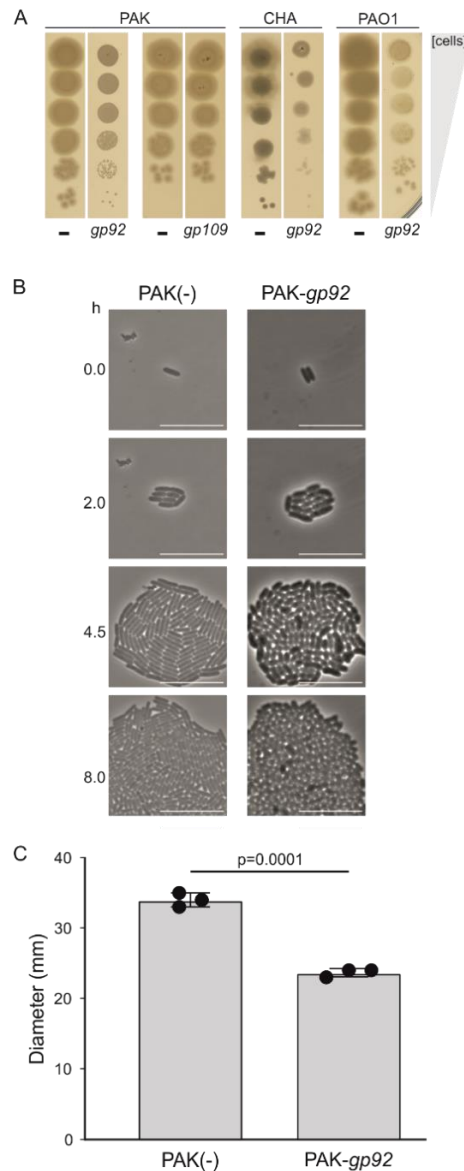


Figure 1. The expression of the phage gene *gp92* affects the morphology and the motility of *P. aeruginosa* cells. (A) Overnight culture of indicated *P. aeruginosa* strains expressing *gp92* or *gp109*, as well as respective control strains carrying an empty expression cassette (-) were serially diluted and spotted onto LB agar plates supplemented with IPTG (1 mM) and incubated overnight. (B) Time-lapse optical microscopy images of control strain PAK(-) and PAK-*gp92*, taken at indicated time after addition of IPTG 1 mM (t=0). Scale bars=10 μ m. (C) Swimming diameters measured 20 h after inoculation of control strain PAK (-) or strain PAK-*gp92* in the center of a LB soft agar (0.3%) plate containing 1 mM IPTG (n=3; error bars indicate standard deviation (sd); Welch's test).

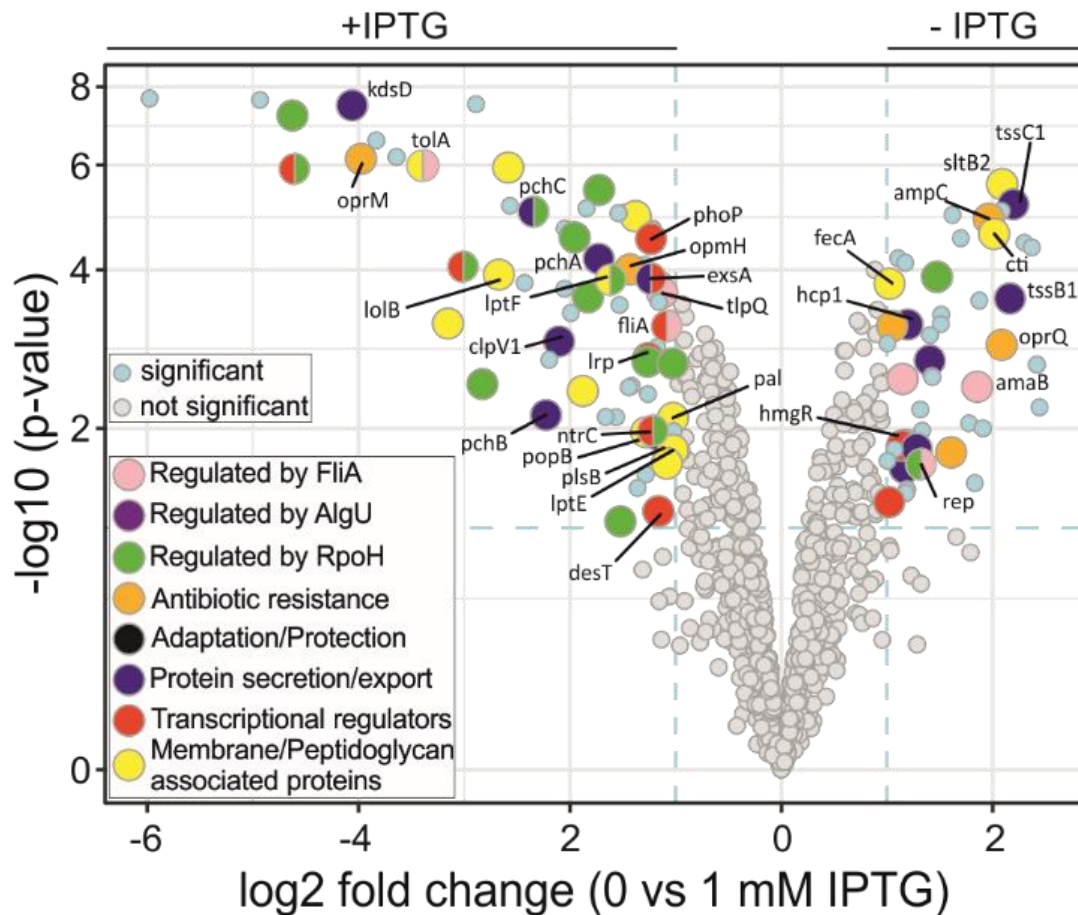


Figure 2. The expression of *gp92* massively alters the proteome of *P. aeruginosa*. Volcano plot of peptides from mass spectrometry analysis of the strain PAK-*gp92* in absence (-) or presence (+) of IPTG (1 mM). Peptides that are significantly more abundant in one condition compared to the other are represented by pale blue small dots (significance thresholds are indicated by dotted lines; see methods for statistical analysis). Large colored dots correspond to functions indicated in the inset and discussed in the text. Full dataset is available in Table S4.

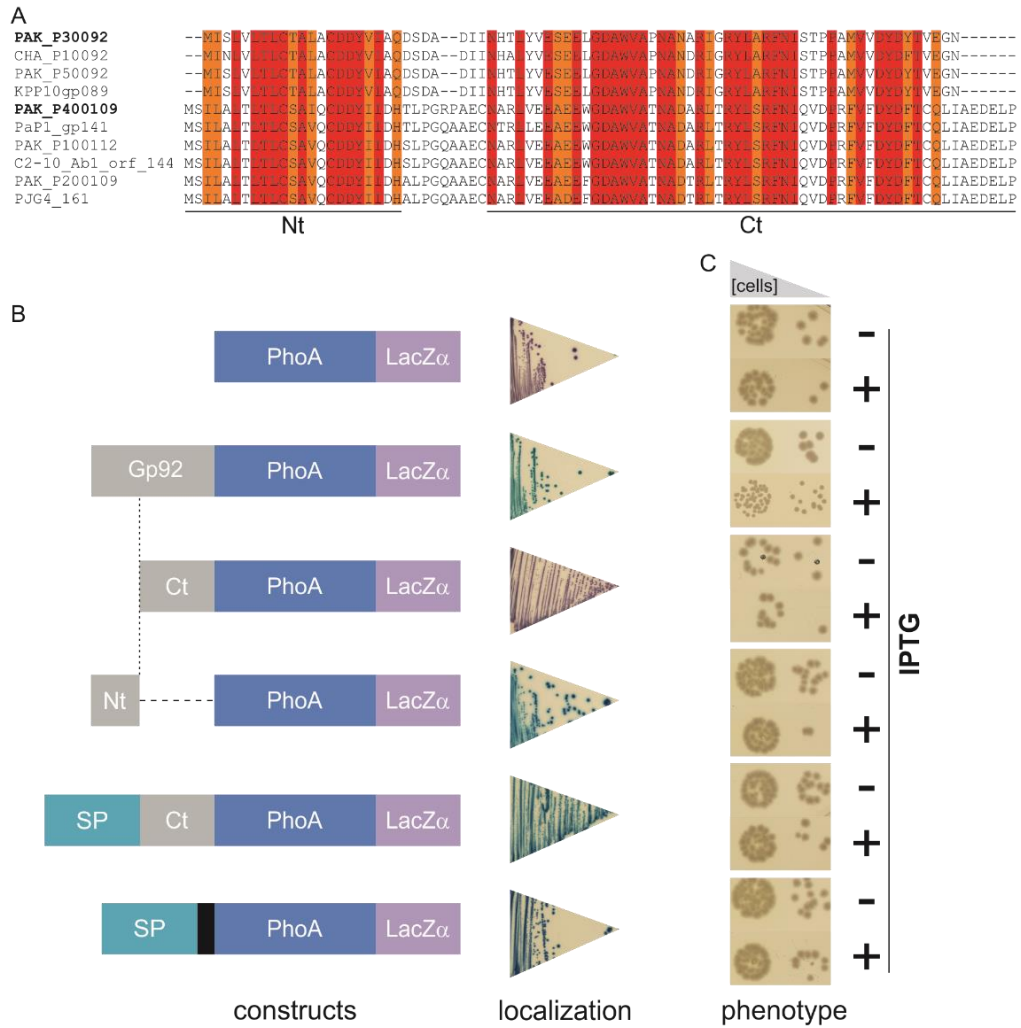


Figure 3. Gp92 is likely anchored in the cytoplasmic membrane. (A) Sequence alignments by ClustalW of Gp92 homologs encoded by phages belonging to *Nankokuvirus* (below PAK_P30092) and *Pakpunavirus* (below PAK_P400109) genera (red, identical; orange, similar). (B) Localization (assessed in *E. coli*) of indicated constructs. The Ct domain lacks the first 22 amino acids of Gp92. The Nt domain consists of the first 26 amino acids of Gp92. SP corresponds to the signal peptide of PhoA and the black area represents a linker of 14 additional amino acids added between SP and the tandem reporters. Localizations of these constructs were determined using chromogenic media (purple: cytoplasmic; blue: membrane or periplasmic) are shown in sectors of Petri dishes with individual colonies. (C) Colony morphology of *P. aeruginosa* PAK expressing the genes encoding the constructs indicated in (B) (without the PhoA-LacZ reporter), which were inserted in single copy into PAK chromosome under an IPTG-inducible promoter. Overnight cultures were spotted on plates without (-) or with (+) IPTG (1 mM).

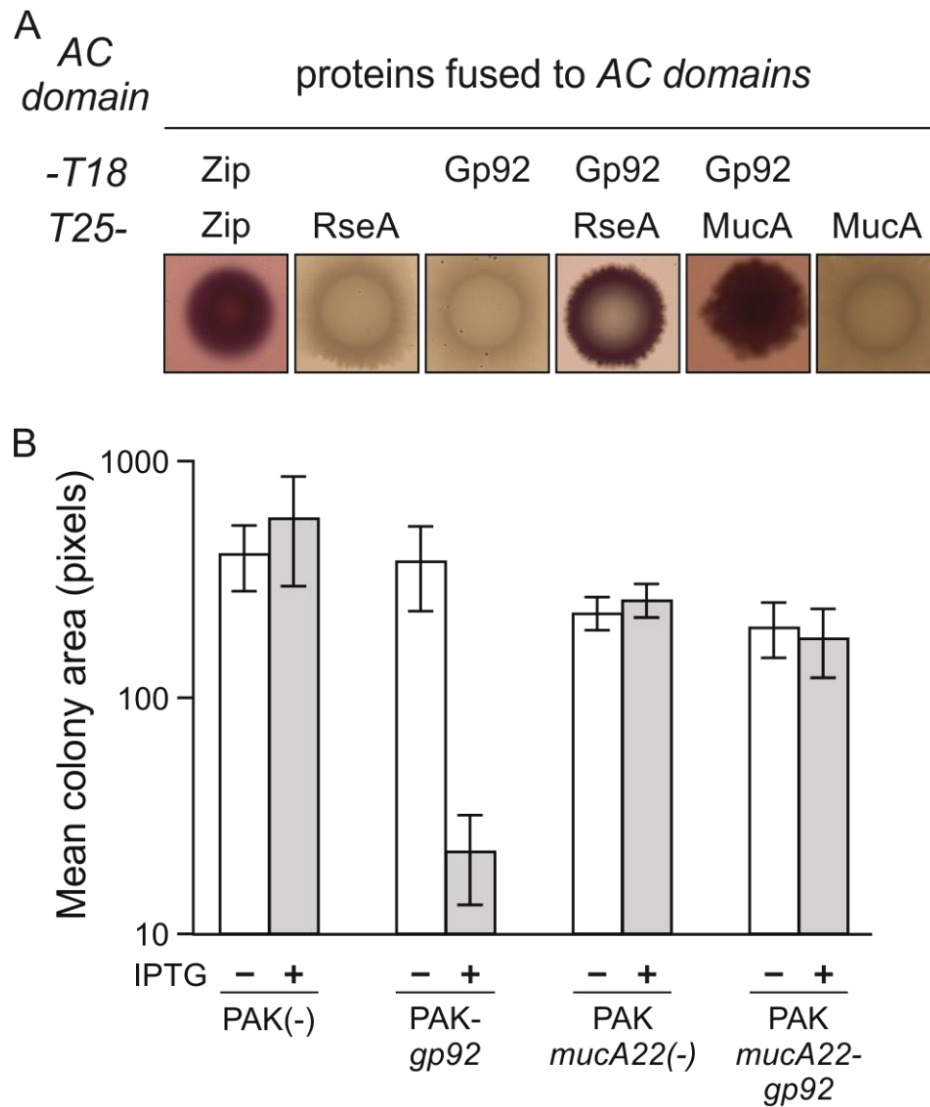


Figure 4. Gp92 interacts with the anti-sigma factor MucA involved in membrane stress response. (A) Pairwise interactions between indicated proteins. The T18 or T25 catalytic domains of the adenylate cyclase (AC) were fused to the indicated proteins and bacteria producing these fusion proteins were spotted on McConkey-maltose (1%) plates to reveal AC activity. Interactions between the fusion proteins (T18-Gp92, T25-RseA and T25-MucA) and the complementary AC domain (either T25 or T18 without proteins fused) were tested as negative controls. The dimerization of a leucine zipper domain (Zip) is shown as positive control. (B) Colony areas of indicated strains plated on LB agar with (+) or without (-) IPTG (1 mM) and incubated at 37°C for 16 h. Bars show the mean colony areas measured across three biological replicates (the total number of colonies was above 200 for each strain) with error bars (sd).

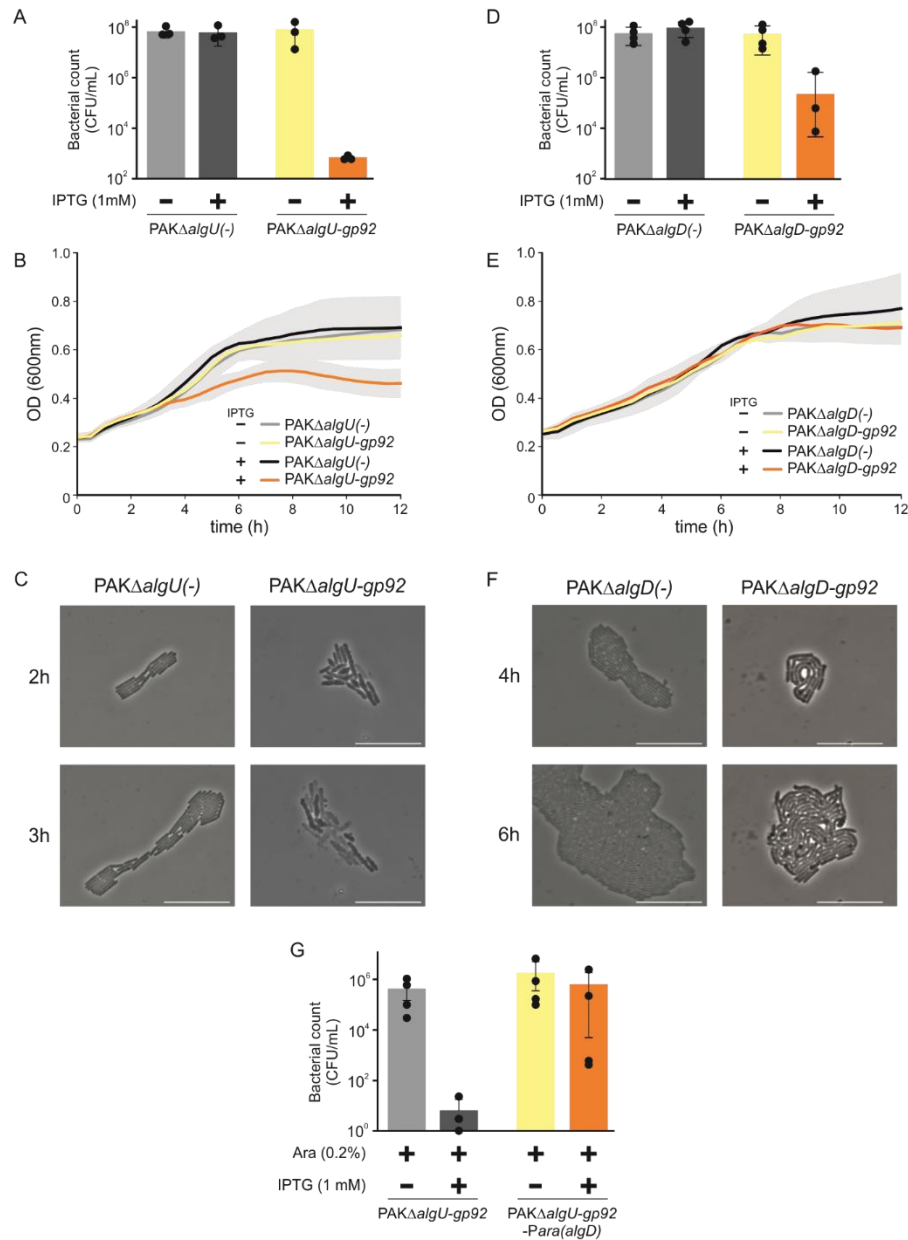


Figure 5: The expression of *gp92* affects the viability of bacteria lacking *algU* or *algD* genes. (A) Number of colonies obtained after plating exponentially growing strain PAK Δ algU(-) or PAK Δ algU-*gp92* on LB agar without (-) or with (+) 1 mM IPTG. Means, error bars (sd) and individual values from 3 to 4 independent experiments are represented. (B) Growth curves in LB of strains PAK- Δ algU or PAK Δ algU-*gp92* in absence (-) or presence (+) of IPTG (1 mM) at 37°C under agitation. Mean curves of three independent experiments are represented. Shaded areas (in grey) represent sd. (C) Time-lapse optical microscopy images of strain PAK Δ algU-*gp92* taken at indicated times upon addition of IPTG 1 mM (t=0). Scale bars=10 μ m. (D) Same as (A) but for strains PAK Δ algD(-) and PAK Δ algD-*gp92*. (E) Same as (B) but for strains PAK Δ algD(-) and PAK Δ algD-*gp92*. (F) Same as (C) but for strains PAK Δ algD(-) and PAK Δ algD-*gp92*. (G) Same as (A) but for strains PAK Δ algU-*gp92* and PAK Δ algU-*gp92*-P_{ara}(*algD*). LB agar was supplemented with 0.2% (w/v) arabinose.

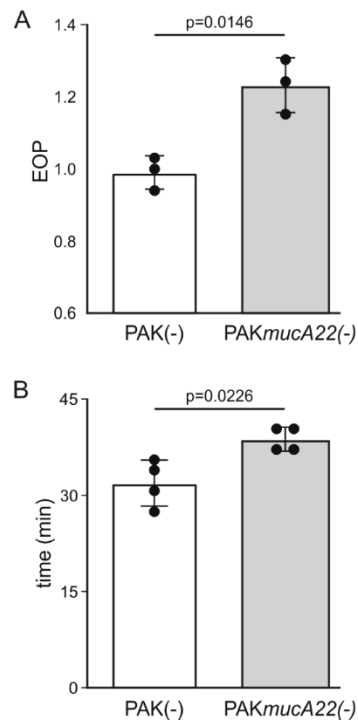


Figure 6. The MucA/AlgU membrane stress response is involved in lysis timing of cells infected by phage PAK_P3. (A) Efficiency of plaquing (EOP) of phage PAK_P3 on indicated strains (n=3, error bars=sd, unpaired t-test with Welch's correction). (B) Elapsed time between addition of phage PAK_P3 (MOI 15) and OD decrease in indicated PAK strains (n=4, error bars=sd, unpaired t-test with Welch's correction).

Target	Peptidoglycan				Protein synthesis			
	Carbapenem	Cephalosporin (2G)	Cephalosporin (3G)	Penicillin	Aminoglycoide	Macrolide	Phenicol	Tetracycline
Molecule/Strain	<u>Imipenem</u>	Cefoxitin	Ceftriaxone	Ampicillin	<u>Kanamycin</u>	Erythromycine	<u>Chloramphenicol</u>	<u>Tetracycline</u>
PAK	1	>255	>32	>255	64	>32	>255	>255
PAKgp92	0.38	>255	>32	>255	24	>32	8	6
PAKΔalgU	0.38	>255	>32	>255	48	>32	>255	>255
PAKmucA22	2	>255	>32	>255	48	>32	8	8
CHA	4.5	ND	ND	ND	ND	ND	ND	ND
CHAgp92	0.38	ND	ND	ND	ND	ND	ND	ND

Table 1. Expression of gp92 lowers the resistance of *P. aeruginosa* cells to several antibiotics. The indicated values correspond to minimum inhibitory concentrations (MIC) in $\mu\text{g.mL}$ (determined using ETEST®, n=3). Antibiotics for which gp92 lowers the MIC are underlined. ND for not determined.

Supplementary information includes

Figure S1. Phenotypic characterization of bacteria expressing the phage gene *gp92*.

Figure S2. Gp109 displays the same localisation as Gp92.

Figure S3. Expression of *gp92* modifies the physiology of membrane stressed cells.

Figure S4. Adsorption and lysis timing of phage PAK_P3

The legends for Datasets S1 to S8 (large tables)

Table S1. List of early expressed genes of phages PAK_P3 and PAK_P4

Table S2. List of homologous proteins between phages PAK_P3 and PAK_P4

Table S3. Up- and down-regulated host genes 3.5 min after infection by either phage PAK_P3 or PAK_P4

Table S4. List of the significantly more abundant proteins from the IPTG-induced strain PAK-*gp92* compared to the non-induced strain PAK-*gp92*

Table S5. List of Gp92-binding proteins identified by bacterial two-hybrid screens

Table S6. List of the significantly more abundant proteins from the IPTG-induced strain PAK-*gp92* compared to the non-induced strain PAK-*gp92* both in presence of D-cycloserin

TableS7. Resistome of strain PAK crossed with AlgU-regulated genes

Table S8. List of strains, plasmids and primers

2- The combination of bacteriophages prevents resistant outgrowth *in vitro* but does not increase pulmonary phage therapy efficacy in immunocompromised animals.

Our laboratory has previously demonstrated that monophage therapy can cure and prevent acute lung infection caused by *P. aeruginosa* in immunocompetent mice [270]. More recently, we have shown that this therapeutic success relies on antibacterial activities of both the phage and the immune system, a concept we named ‘immunophage synergy’ [292]. Indeed, monophage therapy with phage PAK_P1 failed to cure both immune signaling deficient *MyD88*^{-/-} mice and neutropenic mice, as the lack of neutrophils allows the growth of phage-resistant bacteria, not detected in immunocompetent animals.

My second PhD project aimed at investigating bacterial phage-resistance *in vitro* and *in vivo* in immunocompromised mice (*MyD88*^{-/-}), and assess the therapeutic efficacy of a cocktail composed of two phages using different bacterial receptors to prevent the growth of phage-resistant bacteria.

We found that the immunophage synergy concept is not restricted to phage PAK_P1 as monophage treatment with phage LUZ19 also failed to cure acute lung infection in *MyD88*^{-/-} mice. *In vitro*, both monophage treatments failed to prevent phage-resistant growth, but the combination of these two phages did. However, this combined treatment did not prevent phage-resistance to take place *in vivo* and was not more efficient than monophage treatments. Analysis of the phage resistance phenotypes of isolated clones from every conditions (both *in vitro* and *in vivo*) suggests that bacterial phage resistance is highly phage-dependent as PAK_P1 selects for bacteria resistant to PAK_P1 and not LUZ19. Our results also suggest a role of the experimental environment (*in vitro* or *in vivo*) on phage-resistance outcomes. A future genomic analysis of these clones should reveal to which extent phage resistance mechanisms are different between each phage treatment (PAK_P1 or LUZ19, or both) and each environment (*in vivo* or *in vitro*).

Understanding phage resistance taking place *in vivo* could help guiding the design of efficient phage combination, and overall support the development of phage therapy.

Title

The combination of two bacteriophages targeting different receptors fails to prevent the emergence of bacteriophage resistant bacteria in immunocompromised animals.

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Summary

Pulmonary infections are a major cause of death worldwide exacerbated by the increasing antibiotic resistance of bacteria. Treatment using bacteriophages, phage therapy, have been shown to improve the condition of patients but still lack mechanistic support. The success of monophage therapy in treating acute *Pseudomonas aeruginosa* lung infection in wild type mice was shown to rely on the synergistic action of phages and the immune system. In immunocompromised *MyD88*^{-/-} mice, monophage therapy failed due to the uncontrolled growth of phage-resistant bacteria. Here, to jugulate the growth of phage-resistant bacteria we evaluated two phages recognizing different bacterial receptors and their combination in both *in vitro* and *in vivo* (*MyD88*^{-/-} mice) conditions. We found that *in vitro* monophage treatments failed to prevent phage-resistance growth while the combination succeeded. In contrast, the combination was not associated with a significant higher efficacy *in vivo* compared to monophage treatments. A better understanding of phage resistance mechanisms taking place *in vivo* is required to optimize the formulation of phage cocktails and ultimately improve phage therapy efficacy in clinical settings.

Introduction

The worldwide increase of the prevalence of antibiotic resistant bacteria causing difficult to treat infections in humans led the World Health Organization to highlight a list of priority pathogens, for which new treatments are urgently needed [1]. Amongst them, the carbapenem resistant strains of *Pseudomonas aeruginosa* are worrisome as this opportunistic pathogen is ubiquitous in the environment and is a leading cause of nosocomial infections [2, 3]. Furthermore, *P. aeruginosa* is a major cause of death for immunocompromised individuals such as cystic fibrosis patients, for which antibiotics treatments are no longer sufficient to prolong lifetime [4].

Amongst solutions to defeat multidrug resistant bacteria, the use of bacteriophages (phages) to kill bacterial pathogens, phage therapy, is gaining increasing weight with the recent publication of several successful compassionate treatments in both Europe and the USA [5-10]. Despite the uninterrupted clinical experience for nearly a century, mainly from Georgia, phage therapy remains under evaluated through double blind clinical trials [11, 12]. To fill the gap between compassionate treatments and broader medical applications molecular mechanisms underlying treatments failure and success must be provided.

Decades of research performed *in vitro* identified an array of phage resistance mechanisms, from single point mutations on bacterial receptors to more sophisticated machineries (e.g. CRISPR-Cas systems) [13]. Interestingly, the relative abundance of these resistance mechanisms did not prevent the success of many proofs of concept phage therapy studies performed with animal models [14, 15]. On few occasions, phage resistant bacteria were isolated from experimentally infected animals and characterized as less virulent, which did not jeopardized the treatment efficacy [16, 17]. Recently, during the course of compassionate treatments with multiple phages on two patients with an altered immune response, phage resistant clones were isolated, showing that the growth of phage resistant clones could be of higher relevance in immunocompromised patients [8, 10].

Previously, we found that the monophage treatment of a *P. aeruginosa* pneumonia in mice was successful in wild type (WT) but failed in immunocompromised (*MyD88^{-/-}*) animals [15, 18]. The isolation of few clones from infected and treated *MyD88^{-/-}* mice revealed that the growth

of phage resistant bacteria was the main cause for the failure of this monophage treatment in immunocompromised these animals. We then introduced the concept of ‘immunophage synergy’ taking place during phage therapy in WT animals: both phages and immune cells act synergistically to obtain a successful treatment in conditions where each of the two was insufficient [18].

Here, we investigated *in vitro* and *in vivo* the consequences of adding a second phage to limit the growth of phage resistant clones to the first phage. We found that the combination of the two phages prevented the growth of phage resistant clones *in vitro*. The survival of *MyD88*^{-/-} infected mice increased with the treatment of each phage individually and also in combination, compared to untreated controls. However, the combination did not improve the clinical status of animals compared to individual phages. The phenotypic analysis of *P. aeruginosa* clones isolated at end points, revealed that the combination limited but not prevented the growth of phage resistant bacteria compared to monophage treatments.

Results

Establishing conditions for *in vitro* and *in vivo* parallel studies of phage resistance

Phage resistance is inherent to any phage-bacteria interaction, but its nature and frequency are affected by environmental conditions. Having shown that the treatment of *P. aeruginosa* (strain PAK-lumi) infected *MyD88*^{-/-} mice by phage PAK_P1 (virulent, *Myoviridae*) failed because of the growth of phage resistant clones, we wished to investigate the molecular mechanisms involved and compared them to those taking place in laboratory (*in vitro*) conditions. To perform parallel experiments (same doses of phages and bacteria) *in vitro* and *in vivo*, we refined our *in vivo* procedure (see methods) and used a bacterial inoculum of 2×10^6 colony forming units (CFU) and a phage dose of 2×10^7 plaque forming units (PFU) administered 2 h post infection (p.i.). During preliminary experiments, WT animals controlled the infection and survived, while 100% of *MyD88*^{-/-} infected mice experienced an acute and fatal pneumonia within 24 h p.i., leading us to set an end point at 22 h p.i. for subsequent experiments. Compared to our previous conditions, the survival of *MyD88*^{-/-} mice decreased from 27% at day 3 to 0% at 24 h, thus allowing a clearer assessment of phage therapy effect on mice survival.

In these conditions, we observed that the intensity of light emitted from the pulmonary area of infected *MyD88*^{-/-} mice increased continuously up to 22 h, at which point the median pulmonary bacterial load reached 5.85×10^7 [3.80×10^7 - 3.39×10^8] CFU/lungs (Fig. 1A,1B and Fig. S1). In WT animals the median pulmonary bacterial load recovered at 22 h p.i. was much lower with 6.17×10^2 [1.75×10^2 - 3.31×10^3] CFU/lungs, testifying that in these animals' immune cells control bacterial growth while in *MyD88*^{-/-} mice cannot (Fig. 1A,1B and Fig. S1). When infected *MyD88*^{-/-} mice were treated by a single dose of phage PAK_P1 the emitted light increased less rapidly compared to untreated animals (Fig. 1A). The median pulmonary bacterial load at 22 h p.i. reached 3.40×10^5 [1.33×10^4 – 4.70×10^5] CFU/lungs and the median phage load was 3.33×10^9 [1.25×10^9 – 1.00×10^{10}] plaque forming unit (PFU) (Fig 1B and Fig. S1), which represents a 167-fold increase compared to the administered dose of 2×10^7 PFU demonstrating that phage PAK_P1 amplified at the expenses of strain PAK-lumi.

These data are consistent with our previous observations that phage PAK_P1 treatment jugulates the infection during the first hours. In addition, out of 257 clones collected from the lungs of PAK_P1 treated mice 89.11% are resistant to phage PAK_P1, confirming that in these immunodeficient animals the growth of phage resistant clones is largely taking place within 20 h (Table S1). As a comparison, only 0.83% of 360 clones from the lungs of untreated animals at 22 h p.i. are resistant to phage PAK_P1, which is similar to the proportion found in the PAK-lumi inoculum (0.55% out of 182 clones) (Table S1).

For the parallel *in vitro* experiments we recorded the OD_{600 nm} over time. While the growth of strain PAK-lumi without phage reached the stationary phase within 10 h, the presence of phage PAK_P1 rapidly stopped the growth from 5 h until 18 h at which time point the OD_{600 nm} started to increase exponentially (Fig. 2). As for *in vivo* experiments, samples were collected at 22 h, CFUs determined and susceptibility to phage PAK_P1 tested. From samples not exposed to phage PAK_P1, none of the 188 clones was resistant to PAK_P1, which is consistent with the low frequency of such clones in the inoculum (0.55%) (Table S1). By contrast, the frequency of PAK_P1 resistant clones from samples exposed to this phage reached 90.96 % (177 clones) (Tables S1). Therefore, in both *in vitro* and *in vivo* conditions, the addition of phage PAK_P1 led to the growth of phage resistant clones. When comparing over time the intensity of emitted light from animals and the OD values from *in vitro* plates, we observed that the kinetics of the bacterial decay was faster *in vitro*, which is expected because of the lack of activated immune

cells and the difference between the *in vitro* homogenous liquid environment compared to the heterogeneous semi-solid environment in the animal lungs.

Phage LUZ19v infects PAK_P1 phage-resistant clones

One solution to lower the growth of phage resistant clones consist in the addition of a second phage that recognizes a different bacterial receptor. Indeed, mutations affecting phage receptors were shown to be the main mechanism of cross-resistance in *P.aeruginosa* exposed to 27 different phages [19]. Previous work on a highly similar phage to PAK_P1, as well as our unpublished observations, suggested that PAK_P1 uses the LPS as receptor [20]. Phage LUZ19, which we previously assessed for its efficacy to infect strain PAK-lumi both *in vitro* and in the murine pneumonia model [21], is known to use type IV pilus as a receptor [22]. However, our data indicated that both *in vitro* and in WT mice, phage LUZ19 was less efficient than phage PAK_P1 [21]. As this could be a confounding factor in this study, we performed serial *in vitro* passages in liquid culture and isolated a LUZ19 variant (LUZ19v) that displayed an efficiency of plaquing (EOP) on strain PAK-lumi of 1 instead of 0.2 for the original phage LUZ19 [21]. Next, we tested the ability of phage LUZ19v towards PAK_P1-resistant clones collected above from both *in vitro* and *in vivo* samples. We found that 100% of PAK_P1-resistant clones were susceptible to LUZ19v, which showed a lack of cross-resistance (Table S1).

Phage LUZ19v controls more rapidly pneumonia than phage PAK_P1

Using the same *in vitro* condition as for phage PAK_P1, we observed that LUZ19v rapidly lysed and prevented the growth of strain PAK-lumi from 4 h until 12 h at which time point the OD_{600nm} started to increase exponentially to almost reached the OD of the control without phage (Fig. 2). The clones collected at 22 h were tested for their susceptibility to phage LUZ19v and we found that 98.90% of them (182 clones tested) were resistant (Table S1). Therefore, *in vitro* phage LUZ19v treatment resulted in the same outcome than *in vitro* phage PAK_P1 treatment, but with faster kinetics. Interestingly, 5.49% of these clones were resistant to PAK_P1 and overall, 4.95% were resistant to both phages. These results contrast with those obtain with

PAK_P1 where no cross-resistance was observed. Next, administered to infected *MyD88*^{-/-} mice, we observed that phage LUZ19v controlled the infection more quickly than phage PAK_P1 (Fig. 3A). Indeed, the light emitted barely increased within 22 h p.i. at which point the median pulmonary bacterial load in lungs reached 1.13×10^2 [1.04×10^2 - 2.12×10^2] CFU/lungs (Fig. 3B). Unfortunately, we were unable to recover enough clones from these animals to assess their phenotypic characterization. We also observed a lower phage replication compared to phage PAK_P1 as the median amount of phage LUZ19v recovered at 22 h p.i. (1.17×10^8 [9.57×10^7 - 2.17×10^8]) was only 5.85-fold higher than the amount administered (Fig. 3B). This may be due to the rapid action of LUZ19v that quickly limits the number of host available for its replication but could also be caused by a higher rate of elimination by the animals as LUZ19 is a *Podoviridae*, smaller than *Myoviridae* PAK_P1. Overall, we found that the reduction of PAK-lumi growth in both *in vitro* and *in vivo* conditions was faster with LUZ19v compared to PAK_P1. These observations are in agreement with the link between *in vitro* and *in vivo* phage efficacies that we previously established from experiments performed with WT mice [21].

The combination of PAK_P1 with LUZ19v is more efficient than either phage *in vitro* but not *in vivo*

Next, we assessed the efficacy of the combination of phage PAK_P1 with phage LUZ19v (hereafter named cocktail) by mixing an equal amount of each of them to obtain a dose of 2×10^7 PFU identical to the dose of either phage used during experiments reported above. When incubated in liquid broth with strain PAK-lumi, the cocktail stopped the growth from 4 h until 22 h (Fig. 2). This is in sharp contrast with either phage alone for which the growth of bacteria resumed after some delays and nearly reached the density of the control without phage (Fig. 2). The amount of bacteria collected at 22 h was significantly lower from the cocktail condition compared to any other conditions (Fig. S1). The phenotypic analysis of the few clones (n=45) recovered following exposition to the cocktail revealed that 97.78% of them are resistant to PAK_P1 but only 64.44% are resistant to LUZ19v. Interestingly, 100% of clones resistant to LUZ19v are also resistant to PAK_P1. These results demonstrate that cross-resistance is largely taking place but does not, yet within the 22 h time window, favor the growth of these clones,

which supports the conclusion that at this time point the cocktail is more efficient than either phage.

When the cocktail was administered to infected *MyD88*^{-/-} mice, we observed at 22 h p.i. that the amount of emitted light reached a level intermediate between PAK_P1- and LUZ19v-treated mice (Fig. 1A and 3A). This could reflect the 2-fold lower abundance of each phage in the cocktail. The median pulmonary bacterial load at 22 h p.i. confirmed the low intensity of bioluminescent data (1.33×10^3 [$3.50 \times 10^2 - 6.77 \times 10^4$] CFU/lungs) (Fig. 3B). Likewise, the median pulmonary phage load (7.50×10^8 [$1.59 \times 10^8 - 1.46 \times 10^9$] PFU/lungs) also reached an intermediate level compared to those observed with PAK_P1- and LUZ19v-treated mice (Fig. 3B). The clones from these mice were further tested for their phenotypic resistance to either phages. From the 90 clones recovered, we found that 70.00% of them are resistant to PAK_P1, while 98.89% are resistant to LUZ19v and consequently 70.00% of these 90 clones are resistant to both phages. Overall, within the time window of the above experiments the results obtained *in vivo* did not allow to confirm the benefit of using two phages instead of one compared to *in vitro* conditions. However, we noticed that clones exposed to the cocktail from the *in vitro* condition were mainly resistant to phage PAK_P1, while those from the *in vivo* condition were mainly resistant to LUZ19v.

The phage cocktail controls pneumonia but does not cure immunodeficient mice

Next, we questioned whether the treatments of infected *MyD88*^{-/-} mice by single phages or the cocktail could have a prolonged impact on preventing bacterial growth. To this end we performed additional experiments using the same settings as above but extended our observations up to 44 h p.i. For most of the phage-treated animals, either by PAK_P1 or LUZ19v or the cocktail, the emitted light remained very close to the infected and untreated WT animals (as mentioned earlier infected and untreated *MyD88*^{-/-} mice do not survive over 24 h) (Fig. 4A,B,C). However, we observed for at least for one animal in each group a continuous increase of emitted light between 24 and 44 h p.i. suggesting that the growth of phage resistant clones was not fully controlled (Fig. 4A,B,C). This observation was confirmed by the median pulmonary bacterial load observed at 44 h p.i. with 1.25×10^3 [$9.17 \times 10^1 - 3.58 \times 10^3$] CFU/lungs for PAK_P1-treated animals, 1.37×10^3 [$1.34 \times 10^2 - 2.32 \times 10^7$] CFU/lungs for

LUZ19v-treated animals and 1.84×10^2 [5.00×10^1 - 1.22×10^4] CFU/lungs for cocktail-treated animals (Fig. S1). These low values are more than 1-log below the bacterial inoculum (1×10^5 CFU) that is lethal for 50% of *MyD88*^{-/-} mice 48 h p.i. [18]. Nevertheless, between 24 and 44 h the clinical status of most of the animals worsened and reached end points without being associated to an increase of the bacterial load except for few animals in each group. It is therefore most likely that damages to the pulmonary tissues caused by *P. aeruginosa* during the first hours could not be efficiently repaired in *MyD88*^{-/-} mice. Indeed, Skerrett *et al* found that at 24 h p.i. the lungs of *MyD88*^{-/-} mice infected by strain PAK displayed widespread peribronchial and bronchiolar neutrophilic infiltrates, necrosis of bronchial epithelium, extensive neutrophilic alveolitis, and alveolar hemorrhage [23]. We also previously reported that strain PAK-lumi administered to WT mice damaged epithelial cells as soon as 6 h p.i., including in mice treated with phage PAK_P1 2 h p.i. [15]. Finally, *MyD88* deficiency has been linked with impaired epithelial repair following tracheal injury [24]. Therefore, despite the efficacy of all phage treatments to control bacterial growth *in vivo*, the virulence of strain PAK coupled to the *MyD88* deficiency led to fatal outcomes.

From the lungs of the above phage-treated animals we recovered bacterial clones that were tested for their susceptibility to each of the two phages. From PAK_P1-treated animals we found that 63.03% of the 119 clones tested are resistant to this phage while they all remain susceptible to LUZ19v. From LUZ19v-treated animals 81.82% of the 121 clones tested are resistant to this phage while 0.83% of are resistant to PAK_P1, and none of them are resistant to both phages. Finally, from cocktail-treated animals 83.19% of the 119 clones tested are resistant to LUZ19v, 58.82% are resistant to PAK_P1 and 51.26% are resistant to both phages. All of these values are similar to those obtained at 22 h p.i from infected and treated animals, suggesting that phage bacteria interactions were not evolving between 22 and 44 h.

Genomic analysis of population and individual clones revealed resistance mechanisms taking place *in vitro* and *in vivo*.

The genome sequencing of some individuals as well as populations of phage-resistant clones is planned in the near future. DNA will be extracted in May and sequencing will be performed in June and analyzed by the bioinformatics hub of Institut Pasteur. These data will inform on the

resistance mechanisms taking place both *in vivo* and *in vitro* and may guide the next steps to control the growth of phage resistant clones during phage therapy in immunodeficient animals.

Discussion

The success of monophage therapy to treat *P. aeruginosa* pneumonia in WT mice was found to rely on the synergistic action of the phage and the immune system. While the phage treatment rapidly decreases the bacterial load at the expense of the growth of phage resistant clones, the immune system targets both phage susceptible and phage resistant bacteria. These data led us to propose the concept of immunophage synergy. This concept explains the failure of phage therapy observed in *MyD88*^{-/-} infected mice and treated with phage PAK_P1, a *Myovirus* using LPS as a receptor. Here we showed that this failure is not restricted to this particular phage as similar results were obtained with phage LUZ19v, a *Podovirus* using the type IV pili as a receptor, which broadens the immunophage synergy concept. *In vitro* tests performed with the same initial bacteria and phage densities than during *in vivo* experiments showed similar results: monophage treatments led to an initial reduction of the bacterial density followed, after a few hours, by the growth of phage resistant clones.

To evaluate the strategy of designing a phage cocktail combining phages using different receptors we selected phage LUZ19v, which infected all PAK_P1 resistant clones, isolated following *in vivo* and *in vitro* treatments. The lack of cross-resistance following monophage treatments indicates that phage resistance emerges in a phage-specific manner. Moreover, when tested *in vitro* the cocktail successfully lysed bacteria and prevented the growth of phage resistant clones during at least 22 h. However, analysis of the few viable clones isolated at this time point revealed that the majority (64%) were resistant to both phages, showing that, in these conditions, the apparent success of the cocktail may not last very long.

All the phage treatments administered to infected *MyD88*^{-/-} mice had a significant impact on the lung bacterial density compared to PBS-treated animals (Fig. S1) (ANOVA followed by Tukey's multiple comparison test, p-values < 0.005). However, there was no significant differences between the lung bacterial density between the phage treatments. Thus, despite the synergic effect of phages PAK_P1 and LUZ19v observed *in vitro*, their combination was not

more efficient than either monophage treatment *in vivo*. Moreover, the bacterial load in the lungs of phage-treated animals did not significantly increase between 22 and 44h p.i., which suggests a growth defect of the phage-resistant populations in the lungs. Finally, phage resistant clones recovered following exposition to the cocktail are, *in vitro*, mainly resistant to PAK_P1 while *in vivo* they are mainly resistant to LUZ19v. Altogether, these observations are in agreement with a role of the environment (*in vitro* vs. *in vivo*) in the selection of phage resistance.

Several studies have reported the influence of environmental factors on bacterial phage resistance mechanisms. For example, *P. aeruginosa* strain PA14 was shown to favor cell surface modifications to acquire resistance to phage DMS3vir in nutrient-rich medium, whereas it relied on CRISPR–Cas to acquire phage resistance in nutrient-limited conditions [25] or in presence of other bacteria [26]. Additionally, Meaden *et al* demonstrated that *Pseudomonas syringae* mutants that acquired phage resistance via single mutation paid a substantial cost for this resistance when grown on their tomato plant hosts but not in nutrient-rich media [27]. In addition to physiological conditions and fitness costs, physical constraints can also influence phage bacteria interactions. We previously showed that taking into account the spatial heterogeneity of phage and bacterial populations in the lungs was pivotal for the accurate modelling of phage lysis kinetics during pulmonary phage therapy [18]. The faster kinetics of phage mediated lysis *in vitro* compared to *in vivo* conditions further support this model.

Against the initial hypothesis and *in vitro* data, the combination of the two phages did not provide a significant advantage over single treatment *in vivo* and was not sufficient to cure infected *MyD88*^{-/-} mice. This may be due to the broad consequence of the lack of MyD88 protein that has been linked to higher susceptibility to *P. aeruginosa* infections (in both mice and humans [23, 28]). Indeed, the central role of MyD88 in the inflammation process [29] leaves only alveolar macrophages as antibacterial immune cells in the lungs of *MyD88*^{-/-} animals. Interestingly, these cells are still able to phagocytose bacteria and provide good protection against *Staphylococcus aureus*, but not *P. aeruginosa* [23]. We previously demonstrated that neutrophils are the key immune cells involved in the immunophage synergy [18], but these cells are significantly less abundant in the lungs of *MyD88*^{-/-} animals infected with *P. aeruginosa* [23]. Neutropenia is also associated with the inefficacy of classical antibiotics (*e.g.* Aminoglycosides) [30], and systematic reviews have concluded that antibiotic

prophylaxis (treatment before an infection is detected) should be used in neutropenic patients to reduce the incidence of bacterial infections [31, 32]. Overall, *MyD88*^{-/-} mice are a suitable model to study the growth of phage resistance *in vivo* (as these resistant bacteria are usually rapidly cleared by the immune system) but these animals might be too susceptible to *P. aeruginosa* pulmonary infections to study the efficacy of phage treatments.

Methods

Ethics statement

Wild-type C57Bl6/J (B6) (n=7), and myeloid differentiation primary response gene 88 (*MyD88*^{-/-}) (n=31) [33] male and female mice were used between the ages of 10 to 12 weeks. Corresponding groups of mice were age and gender matched and housed under pathogen-free conditions with ad libitum access to food and water. Animal experiments were conducted in accordance with European directives on animal protection and welfare. Protocols were approved by the veterinary staff of Institut Pasteur animal facility (Ref.#20.173) and the National Ethics Committee (APAFIS#26874-2020081309052574 v1).

Microorganisms

The bioluminescent *P. aeruginosa* strain PAK-lumi constitutively expresses the *luxCDABE* operon from *Photobacterium luminescens* [34] and was cultured in lysogeny broth (LB) at 37°C. Phages were amplified and purified as described in [21]. Briefly, early-log growing PAK-lumi strain was infected by phages at a multiplicity of infection (MOI) of 0.001. Cell lysates were filtered and then concentrated by ultrafiltration (Vivaflow 200™, Sartorius) in PBS before to be purified by cesium chloride density gradients and finally dialyzed in Tris buffer (10 mM Tris, 150 mM NaCl, pH 7.5). Endotoxin was removed by three passages through an EndoTrap HD™ column (Hyglos, Germany). Endotoxin levels (31,8EU/mL for PAK_P1 and 13 EU/mL for LUZ19v) were measured using the Endonext™ kit (Biomérieux, France). Purified phage stocks were stored at 4°C until use.

Bacteria and phages quantifications

Samples were serially diluted in PBS and 100µL of each dilution were spread on LB agar plates to count bacteria. Conversely, 4µL of each dilution were spotted on LB agar plates covered by a layer of strain PAK-lumi to count phages. The PAK-lumi layer was obtained by damping 1mL of a mid-log growing culture in order to cover the entire surface of the agar. Then the plate was inclined to remove the excess of liquid and put under a safety cabinet for at least 20 min before use. To quantify the phage content from homogenized lung samples, an aliquot was taken and centrifuged at 5.000g for 10 min before serial dilution.

Adaptation of phage LUZ19 to strain PAK-lumi

Serial passages of the original phage LUZ19 on strain PAK-lumi were performed daily during 10 days. Briefly, early-log growing PAK-lumi strain in LB was infected at a MOI of 0.001 and incubated for 5 h. Cells were centrifuged (5 min. at 5.000g) and 10-fold dilutions of the supernatants were spotted on both PAO1 and PAK-lumi lawns. Each supernatant was used the next day to infect a new culture of PAK-lumi at a MOI of 0.001. At the end of this process several individual plaques were randomly picked and their EOP was measured on both PAO1 and PAK-lumi strains. As all plaques had the same EOP on both strains, one was chosen, labelled LUZ19v and further amplified using strain PAK-lumi. Note that the EOP of LUZ19v on strain PAO1 is identical to the original phage LUZ19.

Quantification of the light emitted from animals

Before measurements with the IVIS™ platform (PerkinElmer) mice were anesthetized by isoflurane inhalation. The luminescent signal was recorded by means of a charge-coupled device camera managed by the LivingImage software (version 4.7; Xenogen). Photons were counted within a defined area corresponding to the whole lung region. The average radiance of regions of interest was recorded and expressed as photons/second/cm²/steradian.

Murine pneumonia model

Compared to our original procedure [18], we increased the inoculum of strain PAK-lumi from 1×10^5 to 2×10^6 colony forming units (CFU) and proceeded with an intra-tracheal (i.t.) administration instead of intranasal (i.n.), but kept the same phage:bacterium ratio (10:1) and administration (i.n.) applied 2 h post-infection (p.i.). These modifications were intended to increase the reproducibility of the lung infection procedure as i.t. has been shown to provide more reproducible delivery in the lungs [35] and allowed us to perform parallel experiments (same doses of phages and bacteria) *in vitro* while reading OD_{600nm} over time with a plate reader (limit of detection around 10^6 CFU). Mice were infected via non-chirurgical i.t. instillation using a BioLITE™ (BioSeb) intubation illumination system [35]. First, mice were anesthetized by intraperitoneal injection of a ketamine (100mg/kg) xylazine (10mg/kg) mix. Then, mice

were placed on a custom built mice intubation stand and were infected using the BioLITE™ system using 20 G, 1.5cm intra-venous (i.v.) catheters (BD Insyte). The bacterial dose was prepared from a mid-log growing PAK-lumi strain in LB that was washed twice in PBS and adjusted to obtain 2×10^6 CFU in 20 μ L for each mouse. The phage or PBS treatments (20 μ L) were administrated i.n. 2 h p.i., following a light anesthesia via isoflurane inhalation. The phage dose was 2×10^7 PFU. At specified end points (22 or 44 h p.i.) lungs were aseptically collected and homogenized (gentleMACS™ Octo Dissociator, Milteny Biotec) in PBS.

***In vitro* phage infection dynamics**

Bacteria were prepared following the exact same procedure as for an animal experiment (see above). Overall, 20 μ L of PAK-lumi (2×10^6 CFU) were added in a flat bottom 96 wells plate (Falcon) filled with 140 μ L of LB. The plate was then incubated for 2 h at 37°C with shaking. Next, 20 μ L of PBS (control), or 20 μ L of phage PAK_P1 (2×10^7 PFU), or 20 μ L of phage LUZ19 (2×10^7 PFU), or 20 μ L of a PAK_P1 + LUZ19 phage cocktail (1×10^7 PFU of each phages) were added before the plate was sealed with a transparent cover and placed in an automatic plate reader (GlomaX, Promega) at 37°C for 20 h with OD at 600nm recorded every 30 min after 30 s of shaking. Next, a sample of every well was 10-fold diluted and serial dilutions spotted on LB agar plates to count CFU, and on LB agar plates covered with lawns of mid-log growing PAK-lumi strain in order to count PFU.

Phage resistance assay

Phage resistance of isolated bacterial clones was assessed through double spot assay. Mid-log growing culture of a clone was spotted (10 μ L) on LB agar plate, allowed to dry for 10 min under a safety cabinet. Then 4 μ L of phage solution (10^7 PFU/mL of phage PAK_P1 or LUZ19v) was spotted over the previous bacterial spot and dry once again. A control plate with only bacterial spots was also prepared. Plates were then incubated overnight at 37°C. A clone was considered as phage resistant if no difference was observed between the two plates (with and without phage spots).

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Figures and Tables

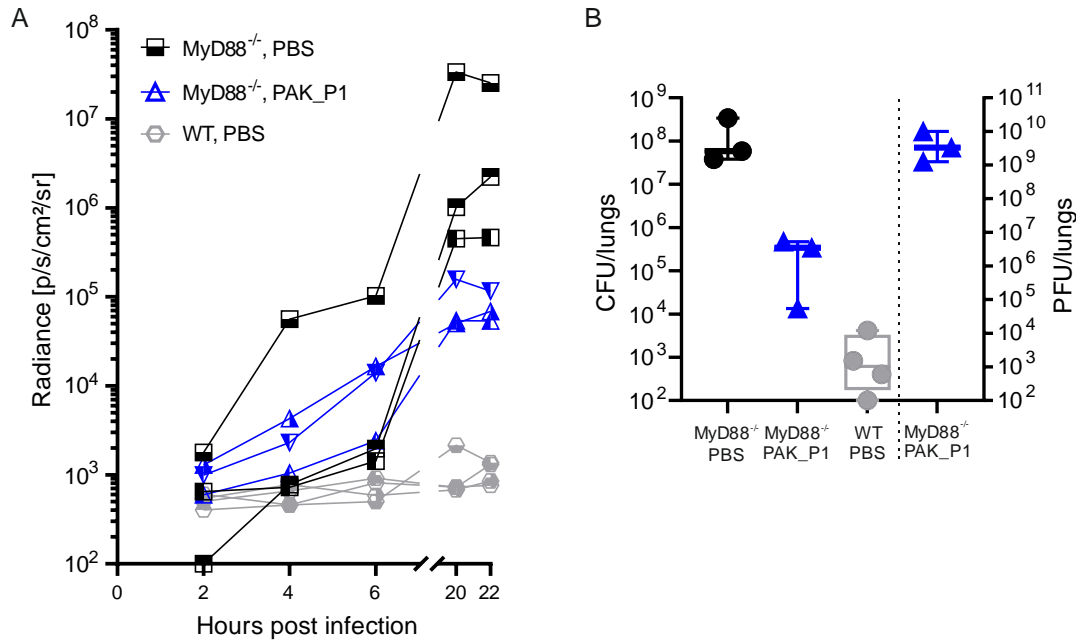


Figure 1. Phage PAK_P1 reduces the burden of *P. aeruginosa* pneumonia in *MyD88*^{-/-} mice. Mice (n= 3 to 4 per group) were infected by strain PAK-lumi (2×10^6 CFU) and treated 2 h later by either PBS or PAK_P1 phages (2×10^7 PFU). **A.** Plotted is the light emitted from the pulmonary area as mean radiance (p/s/cm²/sr) at the indicated time points for both WT and *MyD88*^{-/-} mice. Colors are indicative of the treatment and each symbol represents a unique animal. **B.** Bacterial and phage counts (in homogenates of lungs at 22 h p.i. from mice reported in panel A. The individual values are plotted and box plots represent the median with the 25th to 75th percentiles and whiskers extend from the lowest to the highest values.

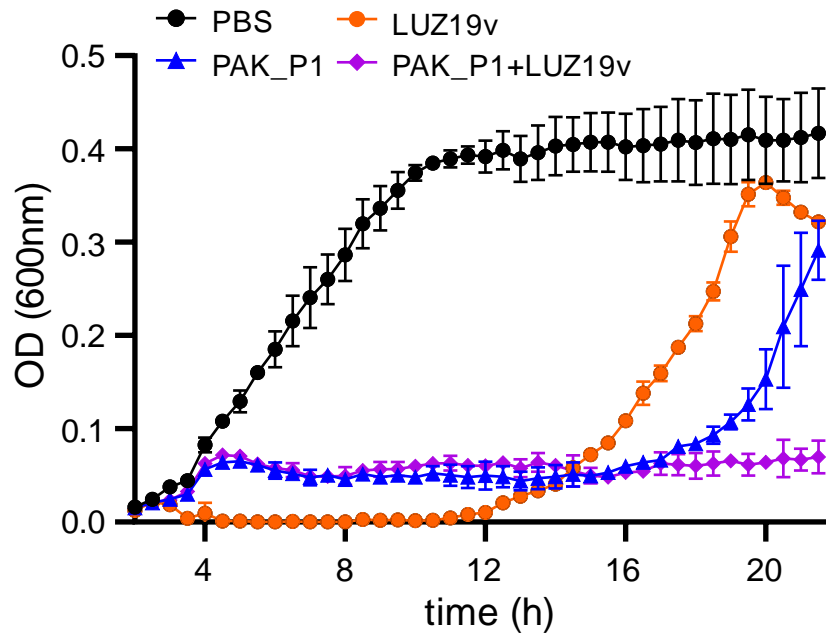


Figure 2. The growth of phage-resistant clones *in vitro* is prevented by the combination of two phages. An inoculum of strain PAK-lumi prepared as for animal experiment was used to fill 96well plates and incubated for 2 h at 37°C with shaking before the addition of either, PBS, or phage PAK_P1, or phage LUZ19v, or the combination of the two phages (all with a total phage dose of 2×10^7 PFU). OD values at 600nm recorded every 30 min during 22 h p.i. (T0=the addition of bacteria) were plotted as means with SD of triplicates wells from one representative experiment.

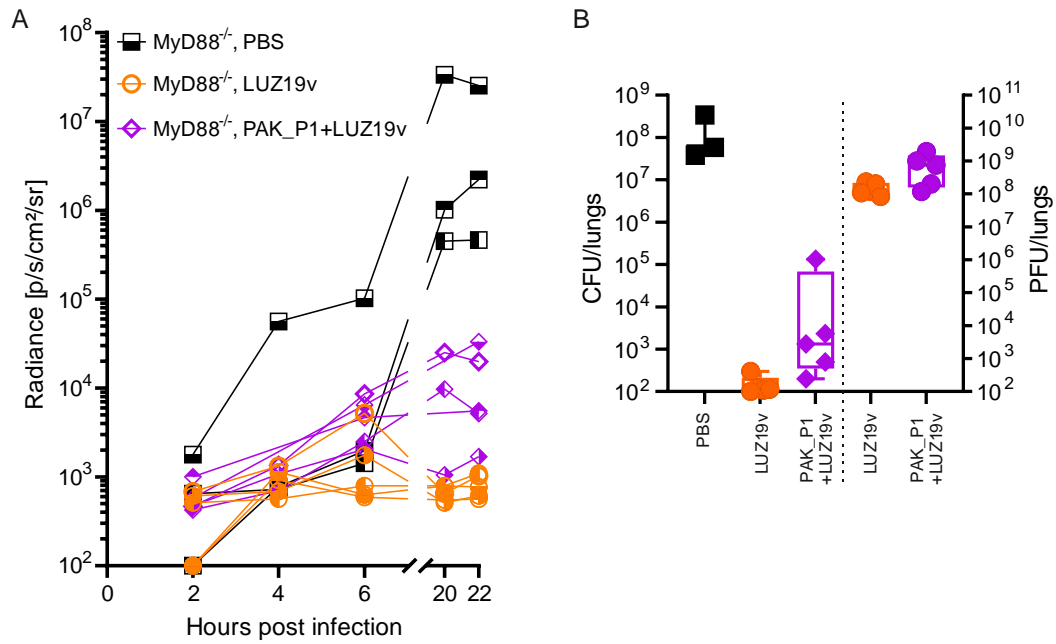


Figure 3. Phage LUZ19v controls quickly *P. aeruginosa* pneumonia in *MyD88*^{-/-} mice. *MyD88*^{-/-} mice (n= 3 to 5 per group) were infected by strain PAK-lumi (2×10^6 CFU) and treated 2 h later by either PBS or phages LUZ19v or the combination of PAK_P1 and LUZ19v (2×10^7 PFU total). **A.** Plotted is the light emitted from the pulmonary area of each mouse as mean radiance (p/s/cm²/sr) at the indicated time points. Colors are indicative of the treatment and each symbol represents a unique animal. **B.** Bacterial and phage counts (in homogenates of lungs at 22 h p.i. from mice reported in panel A). The individual values are plotted and box plots represent the median with the 25th to 75th percentiles and whiskers extend from the lowest to the highest values.

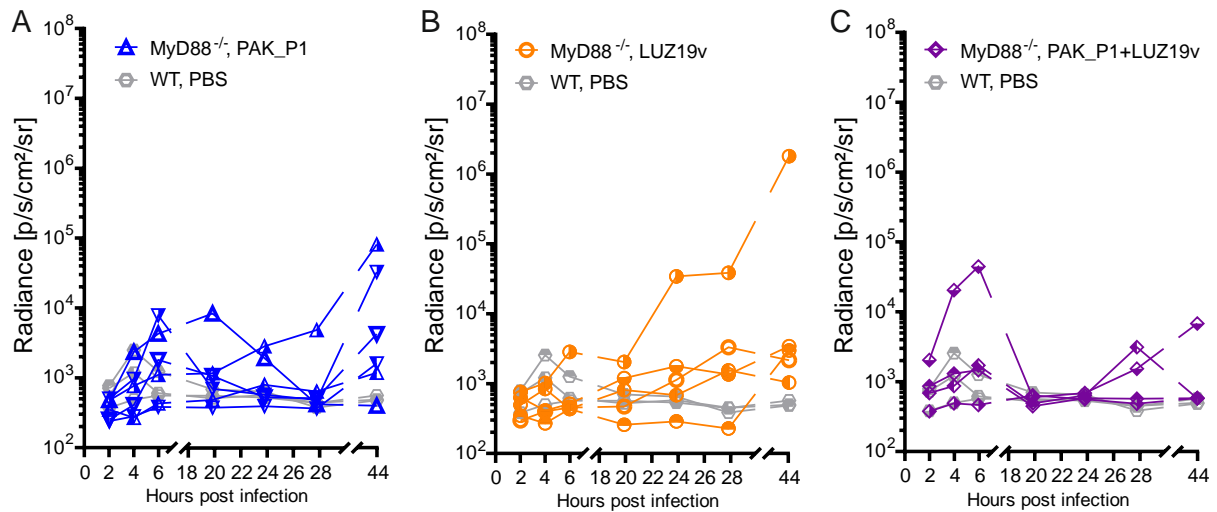


Figure 4. The cocktail of two phages is not more efficient than monophage treatment for pneumonia in *MyD88*^{-/-} mice. Mice (n= 3 to 6 per group) were infected by strain PAK-lumi (2×10^6 CFU) and treated (2×10^7 PFU) 2 h later by either PBS, phages PAK_P1 (A) or LUZ19v (B) or a cocktail of these two phages (C) (all with a total phage dose of 2×10^7 PFU). Plotted is the light emitted from the pulmonary area as mean radiance (p/s/cm²/sr) at the indicated time points for both WT and *MyD88*^{-/-} mice. Each symbol represents the same individual mouse followed over time.

Supplementary Data

Table S1. Resistance profiles of clones recovered from different experimental conditions.

Condition	Clones tested	PAK_P1-resistant (%)	LUZ19v-resistant (%)	Cross-resistant (%)
Inoculum	182	0.55	1.61	0.00
PBS	360	0.83	1.60	0.00
PAK_P1 (22 h)	257	89.11	0.00	0.00
LUZ19v (22 h)	ND	ND	ND	ND
cocktail (22 h)	90	70.00	98.89	70.00
PAK_P1 (44 h)	119	63.03	0.00	0.00
LUZ19v (44 h)	121	0.83	81.82	0.00
cocktail (44h)	119	58.82	83.19	51.26
<i>in vitro</i> PBS	188	0.00	0.53	0.00
<i>in vitro</i> PAK_P1	177	90.96	0.00	0.00
<i>in vitro</i> LUZ19v	182	5.49	98.90	4.95
<i>in vitro</i> cocktail	45	97.78	64.44	64.44

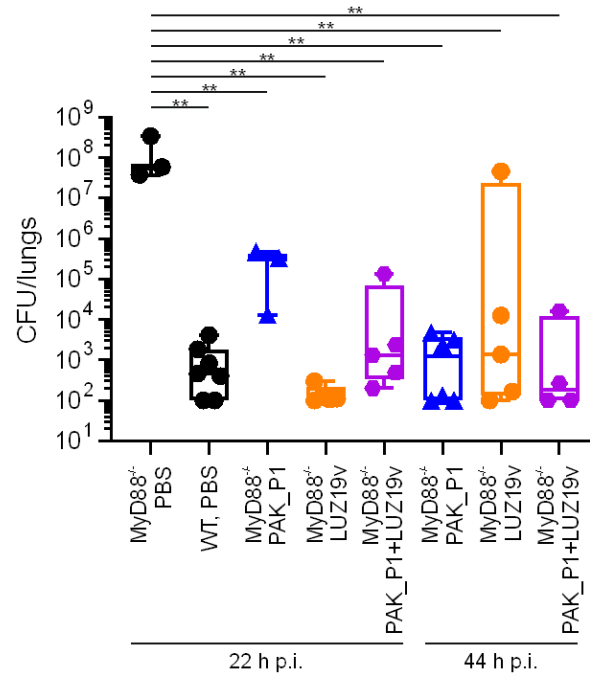


Figure S1. Loads of strain PAK in lung of mice at end points. The amount of CFU from the lungs of WT or *MyD88*^{-/-} mice that received PBS, or phage PAK_P1, or phage LUZ19v, or the cocktail of these two phages is plotted from animals reaching end point at either 22 or 44 h p.i. Colors correspond to different treatments. **, p<0.005, ANOVA followed by Tukey's multiple comparison test. The individual values are plotted and box plots represent the median with the 25th to 75th percentiles and whiskers extend from the lowest to the highest values.

Perspectives

1- A bacteriophage protein favors bacterial host takeover by impairing stress response.

A) Hypotheses and Complementary Work

About the protein conformation: our initial hypothesis of a functional conservation between Gp92 and Gp109, based on sequence homologies (50% amino acids identity) was not confirmed by our data since Gp109 had no impact on the morphology of WT cells and on the viability of an *AalgU* strain. This calls for a closer examination of these two proteins. First, the length of Gp109 is 10 AA longer than Gp92. Most of these extra AA are located at the carboxylic extremity of the protein and could affect its folding or stability. Moreover, sequence alignment of the two proteins using Clustal Omega [299] (**Figure 10**) allows the definition of two domains for which sequence identity is higher: The N-terminal domain (amino-acids 1 to 21) has a 69% sequence identity whereas the C-terminal domain (amino-acids 35 to 74) has a 65% sequence identity.

```

PAK_P3_gp92      --MISLVLTLCALACDDYVIAQSDA--DIINHNTLYVESEELGDAWVAPNANARIGRYL 56
PAK_P4_gp109    MSILALTLTLCSAIQDDYIIDHTLPGRPAECNARLVEEAEEWGDWVATNADARLTRYL 60
                  :::*.*****: : *****:  :  .      *  *  *:* ** ***** **:*: ***

PAK_P3_gp92      ARFNISTPPAMVVDYDYTVEGN----- 78
PAK_P4_gp109    SRFNIQVDPFRFVFDYDFTCQLIAEDEL 88
                  :****.. * :*.***:* :

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Figure 10 : Gp92 and Gp109 protein sequences alignment using Clustal Omega. Amino acids sharing physicochemical properties are shown in the same color. A star * indicates identical amino acids. Two dots (:) indicates a strong conservation of the physicochemical properties. A dot (.) indicates weak conservation of physicochemical properties.

As our experiments indicate that both proteins share a similar localization and that the N-terminal domain is likely involved in the localization of the protein to the membrane, the lack of function of Gp109 is probably the consequence of amino acids divergence located on the C-terminal domain. In particular, the AA substitutions (written as Gp92AApositionGp109AA) P46T, P64D and A66R, involve amino acids with different physicochemical properties, including two proline residues, which often play a critical role in protein conformation [300]. These substitutions are good candidates to explain the lack of observed effects of Gp109. Additionally, the mutation L39W, located in the middle of a stretch of identical AA could also be involved as well since tryptophan is a much larger amino acid

than leucine. To test these hypotheses, site directed mutations on the *gp92* gene could be made and proteins expressed in the WT strain PAK. Assessment of the morphology of colonies could then revealed which AA are necessary for this phenotype. This targeted approach could also be complemented by a more systematic approach (*e.g.* alanine scanning), where one by one, every single amino acid is replaced by an alanine residue, to identify the residues needed for Gp92 activity.

As phages PAK_P3 and PAK_P4 showed different kinetics of host take over, it is also possible that despite their sequence homology, Gp92 and Gp109 serves different functions for each phage. If we concur that gene reduction is linked to optimized viral infection, we can hypothesize that Gp92 diverged from Gp109 and gain a function for controlling the host membrane stress response. Alternatively, Gp109 might require other phage proteins to support the same function. Note that both *gp92* in PAK_P3 and *gp109* in PAK_P4 are part of operons. Unfortunately, attempt to clone these operons was unsuccessful.

About the impact of Gp92 on cell morphology: our data have shown that the synthesis of Gp92 changes cell morphology of WT strain PAK from rod-shape to spherical bacteria. As this effect was abolished in a *mucA22* background (characterized by constitutive expression of the AlgU membrane stress response) and led to cell bursting in a Δ *algU* background, a link between Gp92 and this stress response was established. However, the precise mechanism remains to be deciphered. Indeed, despite the involvement of AlgU in the regulation of the biosynthesis of the peptidoglycan and some components of the bacterial cytoskeleton (*e.g.* *mreB*), both crucial for cell shape determination and maintenance [301], Δ *algU* cells still display a rod-shape. Therefore, the disruption of the AlgU stress response by Gp92 cannot solely explain the impact on cell morphology. This points out that Gp92 has a broader impact than disrupting the AlgU stress response. Our results suggest that Gp92 is both causing a membrane stress and disrupting the stress response responsible for mitigating this effect. One hypothesis could be that the overexpression of an inner-membrane protein (*e.g.* Gp92) might disrupt the cell morphology, but the absence of a similar effect when expressing Gp109 does not support this hypothesis. A direct assessment of Gp92 localization in the cell (by fluorescence microscopy) could help defining its localization and help drawing hypothesis regarding its mechanism of action. Unfortunately, fusions of Gp92 with GFP led to the abolition of the colony phenotype on plates, probably due to a loss of function of these fused proteins.

About the role of Gp92 in phage infection: the phenotypes observed in different bacterial backgrounds (WT, *mucA22* and *ΔalgU*) pushed us to study the phage infection process in these cells. These studies revealed that EOP and lysis timing were affected, with *mucA22* cells showing an increased EOP and a slightly delayed lysis. We hypothesize that during phage infection Gp92 disrupts the AlgU stress response in order to keep the control on the timing of cell lysis. It could be interesting to explore this phenomenon in more details, perhaps through one-step growth experiments or video microscopy (to time precisely the first cell lysis). We also checked if the activation of the AlgU stress response might be relevant for other *P. aeruginosa* phages. We found that variations of the EOP between the different genetic backgrounds were similar for phage PAK_P3 and PAK_P4, whereas they were different for both phages LUZ19 and PhiKZ. Indeed, the EOP of LUZ19 was reduced on both *mucA22* and *ΔalgU* strains compared to WT, while the EOP of PhiKZ was reduced on *mucA22* but increased on *ΔalgU* strains. Due to time constraints these experiments were not pursued but these initial results showed that the AlgU stress response impacts the replication process of these phages in different ways. Finally, the role of Gp92 during phage infection could also be more easily assessed if we had access to a PAK_P3 Δ gp92 phage. Unfortunately, genetic tools, using CRISPR/Cas enzymes, have not yet been implemented for *P. aeruginosa*.

About the link between phage replication and bacterial stress: we have found that activation of AlgU stress response can impact phage lysis. Interestingly, other studies have described impact of cellular stresses (provoked by antibiotics) on phage replications. First, Comeau *et al* [302] described the Phage Antibiotic Synergy (PAS) phenomenon in which the use of sub-lethal concentrations of some antibiotics can significantly increase the production of a specific virulent phage in Gram-negative bacteria. They observed this PAS with diverse host/phage pairs, including T4-like phages, with β -lactams and quinolone antibiotics. As these antibiotics inhibit cell division and trigger the SOS system, the authors investigated the PAS effect in these contexts. They found that PAS effect was SOS-independent and was primarily a consequence of cellular filamentation and suggested that this elongated cells provided a favorable environment for increasing phage production. As Gp92 also affects cellular morphology, we hypothesize that the effect of stressor on the bacterial membrane could impact phage lysis.

Later, Kim *et al* [303] demonstrated a similar PAS in Gram-positive bacteria and showed that cell filamentation did not significantly increase phage adsorption, but might actually affect the lysis timing. Indeed, they observed a delayed lysis and an increased burst size in presence of specific antibiotics. They proposed that the increase of cell surface during filamentation was not matched by an increased holin production, thus delaying the lysis. This interesting hypothesis does not however explain their observation of PAS in absence of filamentation, nor the increased phage production when bacteria were stressed with reactive oxygen species (H₂O₂). In our Gp92 study, we observed an effect on the cell morphology (from rod-shape to spherical), however we did not evaluate the effect on the cell volume, which could affect lysis timing by “dilution” of the lysis effectors. Moreover, the authors observed an increased phage production following stress with reactive oxygen species, which supports further the hypothesis of a link between bacterial stress responses and phage infectious process.

Overall, the underlying molecular mechanisms of the PAS phenomenon are still not clear, but its use for increased phage production has been patented (patent number: US8178087B2) as well as the production of phage in presence of H₂O₂ (patent number: KR101909831B1).

About the broad impact on cellular proteome: the major shift of the proteome following Gp92 induction is puzzling regarding its underlying mechanism: how a single phage protein could have such a broad impact? It is unlikely that Gp92 is interacting directly with the hundreds of affected proteins. The direct interactions between Gp92 and a sigma/anti-sigma regulatory system points towards the origin of a broad impact via interactions with several regulatory proteins. We indeed found that numerous regulators were affected by Gp92 expression, including sigma factor like FliA (responsible of flagellum regulation) and RpoH (responsible of the heat shock stress response). Since sigma factors show high structural conservation [304] it is possible that Gp92 recognizes a conserved structural motif in such regulators, although double hybrid assays detected only direct interaction with MucA and AlgU. However, the original screening was performed using banks made in *E. coli* and *S. typhi*. A screen performed with a *P. aeruginosa* bank might yield a higher number of hits in regulators but could also identify potential new targets of Gp92. Interactions with regulators is a very potent way to profoundly affect bacterial physiology as most of them do not simply regulate a specific function, but present cross-talks with other regulators. For example, regarding the networks of

extra-cytoplasmic sigma factors in *P. aeruginosa* (like AlgU or RpoH), Schulz *et al* [305] found a highly modular network architecture (a module per sigma factor), and a limited but highly function-specific, crosstalk of the various sigma factor networks (*e.g.* between AlgU and RpoH), allowing the orchestration of complex cellular processes. Although experiments to explore the mechanism of action of Gp92 are limited, we could nevertheless expand our understanding of its impact on bacterial cell physiology. For example, one could explore the effect of Gp92 on the virulence of *P. aeruginosa* using an animal model (mice acute lung infection for example). Indeed, *gp92* expression affects the levels of several virulence factors (including different secretions systems, flagellum motility, pyocyanin production...). If a *gp92* expressing strain is less virulent it would represent an additional argument, with the effect of Gp92 on antibiotic resistance, to pursue applications from Gp92. Here, Gp92 could be considered as an antivirulence drug, a strategy now being developed for worrying pathogens including *P. aeruginosa* [306].

B) From a Protein of Unknown Function to a Potent New Drug

Our data show that Gp92 increases the susceptibility of *P. aeruginosa* to imipenem a last resort antibiotic, in both the PAK strain and the CHA strain, a cystic fibrosis isolate. This result demonstrates that understanding new phage hijacking mechanisms could provide novel strategies to fight against antibiotic resistance. It also provides a new way to increase antibiotics efficacy. Indeed, looking at the history of the development of β -lactams (the oldest and most used class of antibiotics) we can see an impressive evolution from using a natural fungal molecule (*e.g.* Penicillin [135]) to modified molecules with enhanced activity against Gram negative bacteria and resistance to β -lactamases (*e.g.* Cefoxitin [307]), ending with today's β -lactam/ β -lactamases inhibitor combinations (*e.g.* Piperacillin/Tazobactam [308]). Could this story continue with the addition of a phage derived peptide (or a small molecule mimicking its effects) disrupting bacterial defense systems and increasing antibiotics efficacy? Only time will tell...

More broadly, this could be achieved through a method initially described by Liu *et al* [309] and later reframed by Wagemans and Lavigne [310] (Figure 10). From 26 sequenced *Staphylococcus aureus* phages, Liu *et al* [309] identified 31 novel polypeptide families inhibiting growth upon expression in *S. aureus*. One of them targets DnaI and this interaction

was used to screen for small inhibitors to finally identify 11 compounds that inhibit both bacterial growth and DNA synthesis with a MIC \leq 16 $\mu\text{g/ml}$. After this pioneer work, Wagemans and Lavigne [310] described a general method to go from an uncharacterized phage gene to an antimicrobial compound (**Figure 11**).

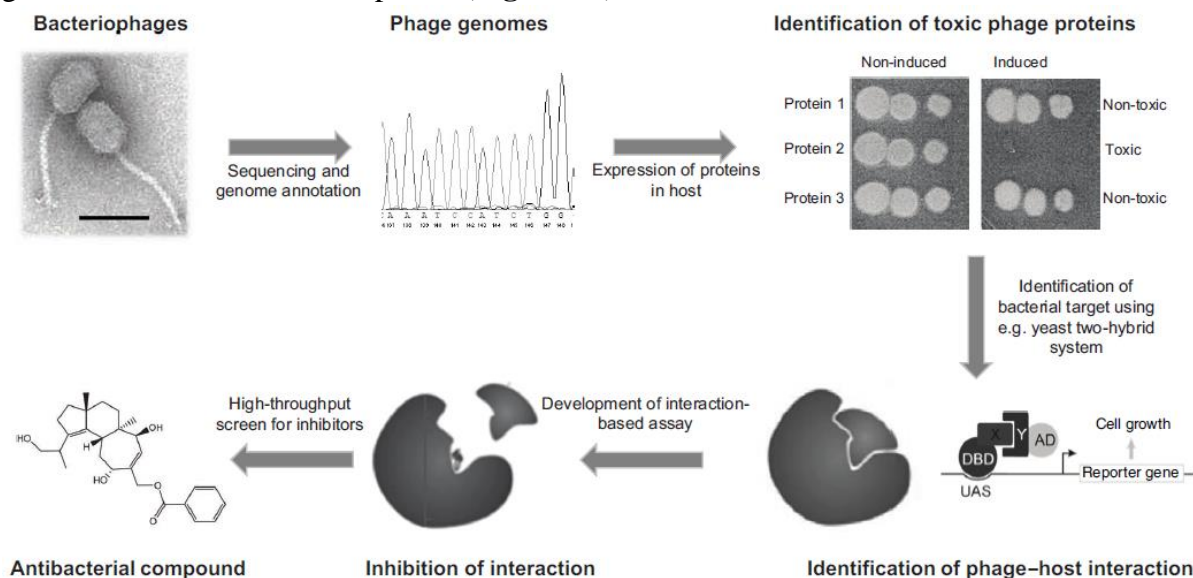


Figure 11 : Bacteriophage–host interaction-based development of new antibacterial compounds (from [310]): After sequencing of phage genomes, uncharacterized genes are cloned under an inducible promoter to assess their toxicity. Then, the identification of the bacterial target can be achieved via different techniques, including double-hybrid assays. Once the phage/host interaction has been characterized, a screening for inhibitors of this interaction can be carried. Among these inhibitors, molecules mimicking the phage protein antibacterial effects might be found.

During the study of Gp92, we already performed most of the described steps of this strategy (genome screening, cloning, toxicity assessment, and identification of the bacterial target), but the most challenging, *i.e.* devising a robust screen to search for inhibitors of the Gp92/MucA interaction, has not yet been achieved.

The idea of not using the whole phage but only some of its parts (e.g. phage proteins) is actually not new, and a lot of research has been particularly dedicated to the enzymes catalyzing the cell lysis: the phage endolysins.

Indeed, the use of endolysin as a new therapeutic strategy seems promising as numerous successes both *in vitro* and *in vivo* have been reported [311-314], and a recent review on the global preclinical antibacterial pipeline [315] found that out of the 407 preclinical projects

identified, 33 projects involved phages or phage-derived peptides, including 11 natural phage cocktails projects, 11 engineered phage cocktails projects, and 7 engineered endolysin projects (the remaining 4 projects utilize phages as cargo for drug delivery). Note that the interest in endolysins has not only been fueled by successful laboratory results, but also by an “easier fit” into the medicinal products regulatory framework (still a big hurdle for classical phage therapy) [316]. As the first lysins targeting *Staphylococcus aureus* (CF-301, SAL200, P128, and Staphefekt™) are currently being evaluated in clinical trials, phage-derived enzymes might end up being widely used while phages may remain restricted to punctual compassionate use.

2- The combination of bacteriophages prevents resistant outgrowth *in vitro* but does not increase pulmonary phage therapy efficacy in immunocompromised animals.

A) Hypotheses and Complementary Work

About the differences between *in vitro* and *in vivo* results: the *in vitro* environment (a well of 96-well plate filled with LB) is a liquid homogenous environment, characterized by a rather small volume (180 μ L) compared to the lungs of mice (total lung capacity of C57BL/6J = 1400 μ L [317]), which is a semi-solid heterogeneous environment. These differences of physical properties between *in vitro* and *in vivo* environment could be responsible of the differences of kinetics observed (quicker *in vitro* than *in vivo*) as they impact the frequency of phage/bacteria interactions. Our previous work showed that *in silico* modelling of phage therapy needed to incorporate the heterogeneity of the lung environment to correctly depict phage lysis kinetics in our experimental settings compared to well-mixed environments (*in vitro*) [292]. To explore the contributions of these physical parameters, we can design *in vitro* experiments in which either the volume (larger wells), or the viscosity (addition of mucins) of the medium is increased.

Additionally, we observed that the phage cocktail treatment was superior to monophage treatment *in vitro*, but not *in vivo*. This indicates that bacteria *in vitro* and *in vivo* can have different biological response to the same event (phage predation) according to their environment. As already discussed in the article, different groups have reported that environmental conditions (*e.g.* nutrient concentrations, or presence of competitor bacteria) can influence phage-resistance in *P. aeruginosa*, by tipping the balance towards cell surface modifications or CRISPR-Cas based resistance [318, 319]. Since our strain of interest (PAK) does not possess a functional CRISPR-Cas system, we are left with the hypothesis of the involvement of a different bacterial physiology between *in vitro* and *in vivo* environments. Indeed, the *in vitro* experiments were conducted in rich medium, whereas the mice lungs are certainly not as rich in term of nutriment. To explore the impact of nutriment concentrations on phage resistance, we could perform *in vitro* experiments with increasingly diluted medium and measure the rate at which phage resistant clones grow. We could also perform an *ex-vivo*

experiment using a “lung medium” made with filtered-sterilized supernatants of lungs homogenates from *MyD88*^{-/-} mice in order to approximate the lung environment.

Furthermore, the fitness cost associated with phage resistance has been shown to be linked to the environment in which the resistant bacteria are growing [320]. Thus, we can hypothesize that some phage-resistant bacteria found *in vitro* would not be selected *in vivo* as they could be more susceptible to the immune defenses left in *MyD88*^{-/-} mice (*e.g.* alveolar macrophages, defensins, surfactant, lactoferrin...)[321]. This hypothesis is supported by reports of phage-resistant variants with increased susceptibility to immune effectors (*e.g.* human complement) [322, 323]. It could be interesting to compare the growth rates of the resistant clones we isolated after the same treatment (*e.g.* PAK_P1) but in different environment (*in vitro* vs *in vivo*) to evaluate the existence of an environment dependent fitness cost.

Overall, it is important to understand if the mechanisms and outcomes of phage resistance *in vitro* and *in vivo* differ too much, as it would mean that *in vitro* experiments cannot inform on *in vivo* efficacy.

About the limitations of our infection model with *MyD88*^{-/-} mice: Our previous work demonstrated the pivotal contribution of the immune system (especially neutrophils) in the success of phage therapy in our experimental conditions. This result had serious implications: if phage therapy needs a competent immune system to be efficient, it might not be a good treatment against opportunistic pathogens (such as *P. aeruginosa*) targeting people with compromised immunity. To address this possible limitation of phage therapy, we decided to research a phage combination able to limit bacterial resistance and cure immunocompromised mice. In other world we wanted to demonstrate that immunophage synergy was not essential to phage therapy success, and that a specially designed phage combination might actually be curative even in the absence of a potent immune response. In this regards the *MyD88*^{-/-} mice provided an interesting experimental model as these animals have been characterized for their very limited innate immune response and inability to control *P. aeruginosa* infections [293, 294, 324]. Interestingly, both the lack of control of *P. aeruginosa* infections and the lack of efficacy of phage therapy in *MyD88*^{-/-} mice have been linked to their severe neutropenia [292, 293].

In the end, we were unsuccessful in creating a phage cocktail able to cure *MyD88*^{-/-} mice, as phage treatment only delay the death of the animals. However, this failure needs to be contextualized. Indeed, as neutropenia has also been linked to a limited efficacy of antibiotics in both neutropenic rats [325] and neutropenic human patients [326], the observed limitation of phage therapy efficacy might not be as critical as initially thought. For example, Lumish *et al* found that when treating neutropenic rats with Carbenicillin, Gentamicin or both, animals death was only shifted by a day, and overall the survival of treated-mice at 5 days post infection (i.p. of 1.8×10^8 CFU of *P. aeruginosa*), only increased from 5 to 15% (the antibiotic combination being the most efficient treatment) [325] (**Figure 12**).

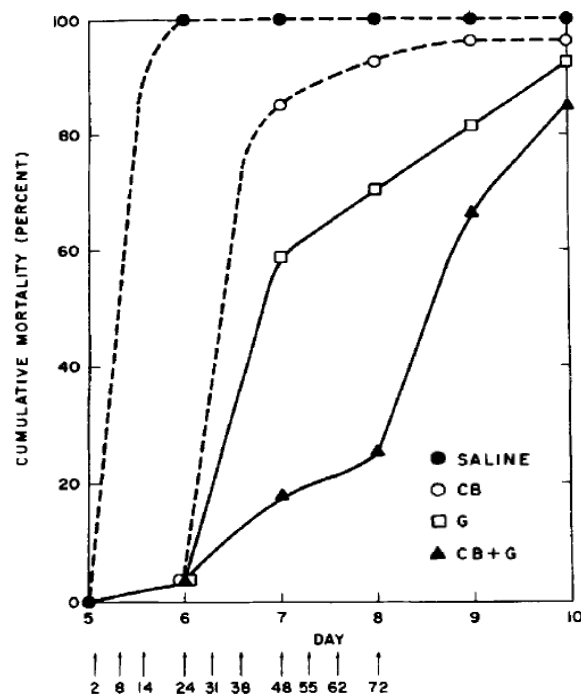


Figure 12: Limited efficacy of antibiotic treatments in neutropenic rats (adapted from [325]): Cumulative mortality rate (percentage) of neutropenic rats challenged with 1.8×10^8 CFU of *P. aeruginosa* on day 5 and treated for 72 hr at the intervals (hr) indicated by the arrows on the abscissa. The dose of carbenicillin (CB) was 400 mg/kg (27 rats) and that of gentamicin (G) was 10 mg/kg (27 rats); 400 mg of carbenicillin/kg and 10 mg of gentamicin/kg were given for combined therapy (CB + G) (27 rats). As control 10 rats received saline im.

An interesting observation coming from systematic reviews on antibiotics use in neutropenic patients is the efficacy of prophylaxis (*e.g.* the administration of antibiotics before an infection is detected) to reduce bacterial infection incidence [327, 328]. We have already demonstrated the efficacy of phage prophylaxis in our acute lung infection in WT mice [270], so it is tempting to predict that such treatment in *MyD88*^{-/-} mice could be successful.

It is important to note that *MyD88*^{-/-} mice are not just neutropenic, but lack a proper activation of the inflammation process, which limits the recruitment and activations of numerous immune cells [294]. This does not only impact antibacterial activity, but could also

disrupt lung tissue repair mechanisms, as cells first recruited during the inflammatory process are also responsible for tissue repairs [329, 330]. Notably, *MyD88* was found to be essential for tracheal epithelial remodeling following acute epithelial injury [331]. This is very relevant for our experiments, as the clinical signs of phage treated-animals did not improved despite the significant reduction of the bacterial loads in their lungs. We hypothesize that this could be due to the accumulation of tissue damages that might be exacerbated in *MyD88*^{-/-} mice.

Overall, the “extreme phenotypes” of *MyD88*^{-/-} mice might be suitable to study phage resistance *in vivo* (as it is not observed in WT mice), but might not be the best model to investigate phage therapy efficacy. To address these limitations of the *MyD88*^{-/-} mice model, future experiments will be carried in WT mice with various degree of neutropenia using different doses of Anti-granulocyte receptor-1 (Gr1) monoclonal antibodies. This strategy will help defining the levels of neutrophils actually needed for phage therapy success. These results could guide phage therapy clinical applications by defining an immune status suitable for phage therapy.

Finally, our results demonstrate that formulating a phage cocktail able to limit phage-resistance both *in vitro* and *in vivo* is a difficult task. In the following sections we will discuss other ways to formulate potent therapeutic cocktails. Hopefully, this information will help us design a phage cocktail with superior efficacy notably in *MyD88*^{-/-} mice.

B) Phage Receptor Cocktail Design

Our strategy to design a potent phage cocktail was based on phage receptor, meaning that we combined two phages (PAK_P1 and LUZ19v) using two different receptors on the bacterial surface (LPS and Type IV pili). As cell surface modification is utilized by bacteria to gain phage resistance, using phages with different receptors allows to limit the cross-resistance. In our study we have isolated bacteria resistant to both phages *in vitro* and *in vivo* pointing out the limit of our two phages cocktail. If this cross-resistance is catalyzed by two independent mutations (one for each phage receptor), we can hypothesize that adding new phages using other receptors (*e.g.* porins) would decrease the probability of resistance and increase the efficacy of the treatment. However, in order to achieve such a goal, the identification of the phage receptors is mandatory. The classical method for phage-receptor determination relies on

the isolation of phage-resistant bacteria (following phage and bacteria incubation), whole-genome sequencing of these clones, and deduction of the phage receptor from the identified mutations. Once a candidate receptor is found, its gene is cloned into a plasmid and its expression in phage-resistant clones should restore their susceptibility to the phage (**Figure 13b**).

However, this approach is time consuming and become unmanageable as the number of phages to be tested increases, especially if we want to create a phage library using a wide array of receptors. A more high-throughput method utilizes library of random bacterial variant (generated for example via transposon mutagenesis [332]) with genomic tags, allowing a more targeted sequencing (cheaper and easier to analyzed). Incubation of the phage with this library will lead to the selection of phage-resistant variants, and once again genomic analysis of the mutations will reveal the potent phage receptors (**Figure 13c**).

In the future, once sufficient knowledge regarding phage receptors is acquired, we may design genetic mutations to change the phage host range by the modification of base-plate or tail proteins, or even generate semi-random sequences to target different bacterial structures (**Figure 13e**). Moreover, once a high number of phage receptors has been characterized, we might even be able to predict a phage's receptor from its genome (**Figure 13f**), which would incredibly speed up the process of phage characterization, thus helping a fast design and production of efficient phage cocktails.

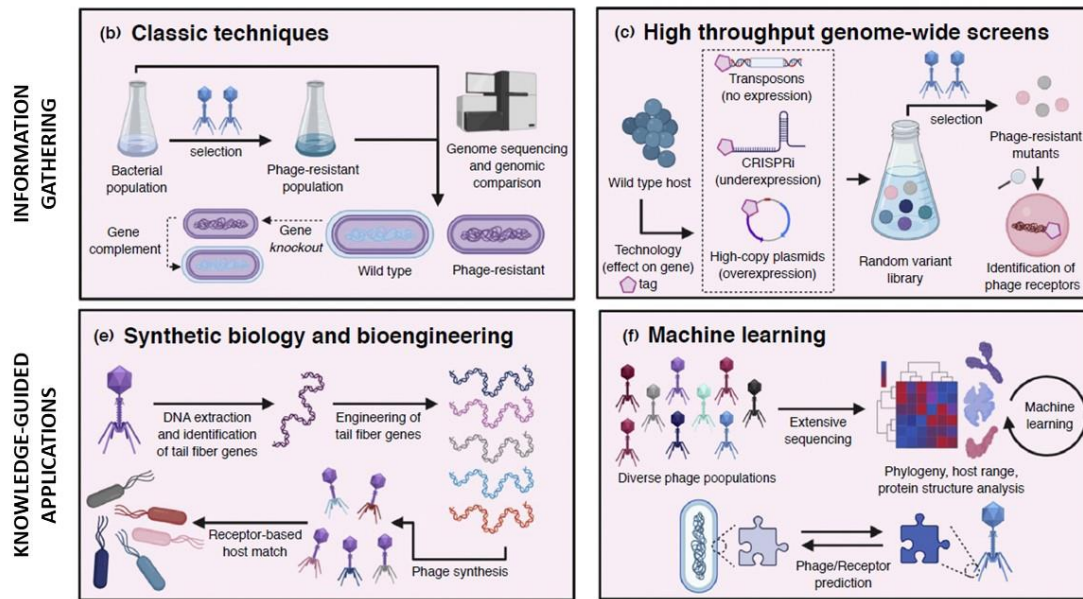


Figure 13: Methods for phage receptor determination and their applications (adapted from [289]). (b) Classic techniques rely on genome comparison between wild type and phage-resistant strains to identify genes (receptors) involved in phage-resistance. The potent receptor can be confirmed through knockout and/or complementation experiments. (c) High-throughput genome-wide screens utilize molecular technologies to generate random bacterial variant libraries. Barcodes on the used probes facilitate and accelerate the downstream identification of phage resistance genes (including receptors). (e) Guided synthetic biology could allow us to modify or de novo produce phages with tail-fibers specifically designed towards a receptor or host. (f) As knowledge on phage receptors and phage tail fibers increases, data regarding phylogeny, host range, or protein structure could feed machine learning algorithms and in the end allow accurate prediction of a phage receptor from its genome.

Despite an increased efficacy, even a carefully design phage cocktail might only delay phage resistance. On the other hand, if the selection of phage-resistant bacteria is unavoidable, we might be able to take advantage of this process. Indeed, as previously mentioned, bacteria can become phage resistant by preventing phage adsorption but this loss of the phage receptor might disrupt the bacterial physiology, thus creating an evolutionary trade-off associated to phage resistance. This phenomenon creates an opportunity to use phages with known receptors to ‘steer’ bacterial evolution towards clinically exploitable phenotypes (*e.g.* avirulence, antibiotic susceptibility...).

C) Exploiting Phage Resistance to Steer Bacterial Evolution

In contrast to antibiotic resistance that is mostly acquired via plasmids carrying resistance genes or by the overexpression of resistance determinants, phage resistance mainly occurs via the loss or dysregulation of cell surface components. This resistance mechanism, although very efficient, is sometimes costly for the bacteria. Indeed, bacterial surface components can play critical roles in infection (*e.g.* motility, attachment, secretion...) as they often contribute to virulence, or to antibiotics resistance (*e.g.* efflux pumps). For example, a list of 306 virulence factors has been reported for *P. aeruginosa*, from which 45% are predicted *in silico* (using PSORTb) to be localized to the outer-membrane [333].

Numerous studies have reported phage resistant clones with reduced growth rates, lower virulence or increased susceptibility to antimicrobials. The first report of this phenomenon was published in the 1980's by Smith and Huggins in an article entitled "Successful Treatment of Experimental *Escherichia coli* Infections in Mice Using Phage: its General Superiority over Antibiotics" [334]. Despite the emergence of phage-resistant bacteria, the efficacy of phage treatments was not jeopardized because these phage-resistant clones had lost the K1 capsule and were then avirulent in mice. Almost 40 years later, Gordillo Altamirano *et al* [323] reported similar findings when they used *A. baumannii* phages recognizing the capsule as receptor. Phage-resistant clones that lost the capsule were unable to form biofilms, avirulent in a bacteremia murine model, and re-sensitized to multiple antimicrobials they were previously resistant to, including the complement system, other phages, and antibiotics. Besides capsules, the LPS is another frequent phage receptor determinant that bacteria can modify to resist to phage predation. Filippov *et al* [286] identified a direct correlation between truncation of LPS molecules and reduction of virulence using eight phages infecting *Yersinia pestis*.

Moreover, the acquired vulnerability to antibiotics following phage treatment, is not constrained to Gram negative bacteria as a report by Chatterjee *et al* [335] described how phage-resistant mutants lacking enterococcal polysaccharide antigen became susceptible to cell wall targeting antibiotics (*e.g.* vancomycin). Interestingly, these phage-resistant mutants were also deficient in a murine model of intestinal colonization.

Finally, Chan *et al* [336] reported a direct link between phage and antibiotic resistance when studying the *P. aeruginosa* phage OMKO1 that uses a membrane component of an efflux pump (the porin OprM) as a receptor. They found that phage resistance results in loss-of-function of this pump, restoring antibiotic susceptibility of otherwise Tetracycline-resistant clinical strains. This report is of particular interest as the bacterium is put in an “evolutionary dead-end” as it cannot become resistant to both the phage and the antibiotic, as shown on **Figure 14**. Indeed, in presence of antibiotic, a strain with an efficient efflux system can grow (in blue), but will be lysed in presence of the phage (in green), whereas the absence of the efflux system provides phage-resistance (in yellow) but does not allow growth in presence of antibiotic (in red).

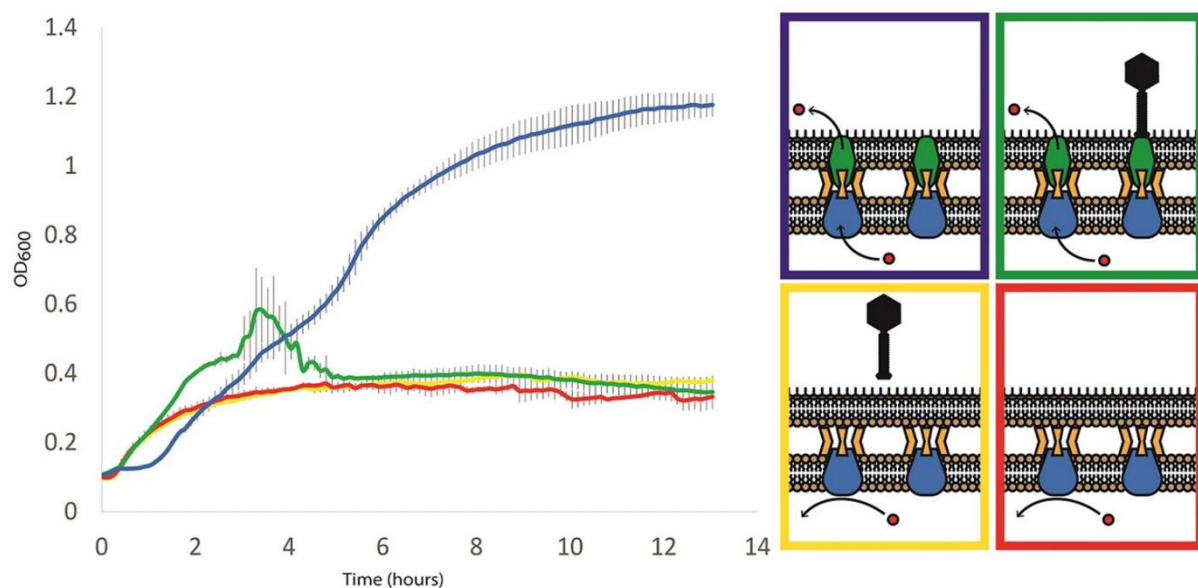


Figure 14 : Bacteria are unable to grow in presence of both phage and antibiotics (from [336]). Average cell densities (OD_{600}) of PAO1- $\Delta mexR$ and PAO1- $\Delta oprM$ over time in the presence of Tetracycline (10 mg/L) and phage OMKO1 (green and yellow lines). PAO1 $\Delta mexR$ (blue, green) overexpresses mex-OprM and readily grows in TET to high densities alone due to active efflux of TET (blue) but is susceptible to phage infection (green). On the other hand, PAO1 $\Delta oprM$ grows poorly in the presence of TET (red) but is resistant to phage OMKO1 (yellow).

Moreover, these encouraging *in vitro* results were followed by a clinical success as a single dose of phage OMKO1 combined with ceftazidime successfully treated a chronic *P. aeruginosa* infection of an aortic Dacron graft [337]. This positive outcome may be very specific to this phage/bacteria system, but it clearly demonstrates that phage/antibiotic combinations can mitigate both phage and antibiotic resistance issues, and thus could represent

a potent therapeutic option to successfully treat infections in our immunocompromised mice model.

D) Phages/Antibiotics Combinations: Everything Can Happen

Currently, clinical applications of phages are almost always paired with antibiotic treatments as clinicians use everything at their disposal to treat patients in life-threatening conditions. Thus, it is of the utmost importance to better understand the interactions between phages and antibiotics. Moreover, as mentioned in the previous section, phage/antibiotic combination may be a very potent solution against both phage and antibiotic resistance [336, 337], and could be efficient even in the absence of a functional immune system. Unfortunately, a limited number of studies have investigated phage/antibiotic combinations, and their conclusions are as diverse as the phage/bacteria/antibiotic systems evaluated. Hence, no general rule can yet be drawn, but an overview of described effects will be presented.

Synergic activity of phages and antibiotics exploiting their differential efficacies on sessile bacteria and biofilm was described by multiple groups. Indeed, Kirby *et al* [338] reported that effective gentamycin treatments on *S. aureus* planktonic cultures promotes aggregation and biofilm formation, leading to increase antibiotic resistance. On the other hand, the phage alone had limited effect on the planktonic cultures, but were efficient on biofilms. When antibiotics and phages were combined an efficacy on both planktonic bacteria and biofilms was reported. Similar results were observed in other studies on *E. coli* biofilms using a *Myoviridae* associated with cefotaxime [339], or on *S. aureus* biofilms, but this time using a *Siphoviridae* combined with rifampicin [340], demonstrating that these synergistic effects are not limited to a specific phage type or antibiotic class.

However, the combination of both phage and antibiotics does not always has positive outcomes. Indeed, Moulton *et al* [341] studied the combination of a phage and gentamycin in long term evolutionary experiments and they found that *P. aeruginosa* developed a resistance against both gentamycin and the phage, nine days after the beginning of the treatment. The combination performed worse than the antibiotic alone from the ninth day, demonstrating that phages can impede the action of antibiotics. Likewise, phage replication was consistently lower in the combination treatment compare to phage alone, pointing out a negative effect of the

antibiotic on phage replication. The most striking result of this study is that exposure to phage had a significant positive effect on bacterial growth in the presence of antibiotic, meaning that phage selection could lead to lower antibiotic susceptibility. Conversely, Allen *et al* [342] discovered through a wide screening of phage and antibiotic combinations (94 *E. coli* strains, 10 antibiotics and 7 phages) that the resistance against gentamycin in *E. coli* isolates was correlated with the resistance against phage T6 (a *Myoviridae*). This association was then confirmed by the deletion of *tsx* (encoding a protein channel used as a receptor by phage T6), which conferred resistance to both T6 and gentamicin. However, the authors did not find an association between overall phage resistance and antibiotic resistance profiles across their isolates, indicating that despite rare associations among individual pairs of phage and antibiotic resistance phenotypes, susceptibility to antibiotics and phages are not generally correlated.

Overall, our current knowledge of phage/antibiotic interactions is still hazy as both positive and negative interactions have been described regarding antibiotic resistance and overall treatment efficacy. In absence of general rules for potent combinations, more research is needed to identify solid phages/antibiotics combinations and in particular which phage functions are involved.

To conclude, the logical follow up of my work would be to exploit the upcoming genomic sequences from phage resistant clones I isolated in order to design the next generation of cocktails with improved therapeutic efficacy in our immunocompromised mice model. In addition, to find the best combination of phages, we could utilize the knowledge of their receptors, and select phage cocktails driving phage-resistance towards lower bacterial virulence or increased antibiotic susceptibility. Finally, this newly designed phage cocktail could be tested in combination with different antibiotics to further increase its therapeutic efficacy. Hopefully, such a treatment will be able to successfully treat acute lung infection in immunocompromised animals.

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Abstract (English)

Pseudomonas aeruginosa is an increasingly antibiotic resistant pathogenic bacterium. Here, we studied bacteriophages infecting this bacterium to develop antibacterial strategies. We focused on two objectives: (1) the characterization of an early-expressed phage protein that increases the susceptibility of *P. aeruginosa* to antibiotics and (2) the development of a bacteriophage cocktail to optimize phage therapy efficacy in immunocompromised *MyD88^{-/-}* mice.

The first objective aimed at understanding the molecular mechanisms underlying viral strategies to hijack bacterial functions. We characterized *gp92*, an early-expressed gene of phage PAK_P3 during its infection of *P. aeruginosa*. The ectopic expression of *gp92* alters bacterial morphology, motility and provokes a major shift of the cell proteome, without affecting growth. Via its interaction with the anti-sigma factor MucA, Gp92 impairs the membrane stress response mediated by the sigma factor AlgU. We hypothesize that Gp92 disrupts this stress response to control phage lysis timing. Additionally, expression of *gp92* increased the susceptibility of *P. aeruginosa* to antibiotics, including Imipenem, thus revealing a new target to increase bacterial susceptibility to Carbapenems.

The second objective focused on phage therapy efficacy and bacterial resistance to monophage treatments in immunocompromised *MyD88^{-/-}* mice. In these mice monophage therapy fails due to the uncontrolled growth of phage-resistant bacteria. To limit this growth, we evaluated two phages recognizing different bacterial receptors and their combination, both *in vitro* and *in vivo*. *In vitro*, monophage treatments failed to limit phage-resistance growth while the combination succeeded. In contrast, the combination was not associated with higher efficacy in treating *MyD88^{-/-}* mice, compared to monophage treatments. Analysis of the resistance pattern of bacteria isolated in each experimental conditions suggests that phage resistance is phage- and environment-dependent. We found that bacteria will not resist to each phage the same way and that *in vitro* experiments are not predictive of phage therapy success *in vivo*.

Résumé (Français)

Pseudomonas aeruginosa est une bactérie pathogène de plus en plus résistante aux antibiotiques. Nous avons étudié les bactériophages infectant cette bactérie afin de développer des stratégies antibactériennes. Nous nous sommes concentrés sur deux objectifs : (1) la caractérisation d'une protéine de phage exprimée précocement qui augmente la sensibilité de *P. aeruginosa* aux antibiotiques et (2) le développement d'un cocktail de bactériophages pour optimiser l'efficacité de la phagothérapie chez les souris immunodéprimées *MyD88^{-/-}*.

Le premier objectif visait à comprendre les mécanismes moléculaires sous-jacent aux stratégies virales de détournement des fonctions bactériennes. Nous avons caractérisé *gp92*, un gène exprimé par le phage PAK_P3 au début de son infection de *P. aeruginosa*. L'expression ectopique de *gp92* modifie la morphologie et la motilité bactérienne. Elle provoque aussi une modification majeure du protéome cellulaire, mais n'affecte pas la croissance bactérienne. Par son interaction avec le facteur anti-sigma MucA, Gp92 altère la réponse au stress membranaire médiée par le facteur sigma AlgU. Nous émettons l'hypothèse que Gp92 perturbe cette réponse au stress pour contrôler le moment de déclenchement de la lyse par le phage. De plus, l'expression de *gp92* augmente la sensibilité de *P. aeruginosa* aux antibiotiques, y compris l'Imipénem, révélant ainsi une nouvelle cible pour accroître la sensibilité bactérienne aux Carbapénèmes.

Le deuxième objectif est centré sur l'efficacité de la phagothérapie et la résistance bactérienne aux phages chez des souris *MyD88^{-/-}* immunodéprimées. Chez ces souris, la thérapie via un seul phage échoue à cause de la croissance de bactéries résistantes aux phages. Pour limiter cette croissance, nous avons évalué *in vitro* et *in vivo*, deux phages reconnaissant différents récepteurs bactériens et leur combinaison. *In vitro*, les traitements par un seul phage n'ont pas réussi à limiter la croissance de clones résistants aux phages, tandis que la combinaison a réussi. En revanche, cette combinaison n'avait pas une plus grande efficacité que les traitements avec un seul phage chez les souris *MyD88^{-/-}*. L'analyse du profil de résistance des bactéries isolées dans toutes les conditions expérimentales suggère que la résistance aux phages dépend du phage utilisé et de l'environnement. Nous avons observé que les bactéries ne résistent pas à tous les phages de la même manière et que les expériences *in vitro* ne sont pas prédictives du succès de la phagothérapie *in vivo*.