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Pseudolysogeny and sequential mutations build multiresistance to virulent bacteriophages in *Pseudomonas aeruginosa*

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Structure et dynamique des systèmes vivants

Spécialité de doctorat : Sciences de la Vie et de la Santé
Par

Mme Libera Latino

Pseudolysogeny and sequential mutations build multiresistance to virulent bacteriophages in *Pseudomonas aeruginosa*

Thèse présentée et soutenue à Orsay, le 20 septembre 2016 :

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I pictured myself as a virus...and tried to sense what it would be like.

— Jonas Salk

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ABBREVIATIONS

A: adenine

A₆₀₀: absorbance at 600 nm

bp: base pair

C: cytosine

CF: cystic fibrosis

EOP: efficiency of plating

g: grams

G: guanine

ICU: intensive care unit

kbp: kilo base pair

l: liter

LB: Luria Bertani broth

LPS: lipopolysaccharide

MOI: multiplicity of infection

PAGE: polyacrylamide gel electrophoresis

PBS: phosphate saline buffer

PCR: polymerase chain reaction

PEG: polyethylene glycol

SMG: saline magnesium gelatin buffer

SNP: single nucleotide polymorphism

T: thymine

INTRODUCTION

I BACTERIA AND BACTERIOPHAGES

I.1 PSEUDOMONAS AERUGINOSA IS AN OPPORTUNISTIC PATHOGEN

Pseudomonas aeruginosa is a gram-negative bacterium that can be naturally found in soil and water. This bacterium is able to colonize biotic and abiotic surfaces and to naturally resist different classes of antimicrobial agents. *P. aeruginosa* is a member of the normal microbial flora in humans. Representative colonization rates for specific sites in humans are up to 2% for skin, 3.3% for the nasal mucosa, 6.6% for the throat, and from 2.6 up to 24% for fecal samples (Morrison and Wenzel 1984). Generally it does not cause disease in healthy people or in people without injury. For instance, when the bacteria enter the lungs of healthy hosts, they are cleared rapidly, without the initiation of an inflammatory response, by a variety of innate host defense strategies such as mucociliary clearance or by the resident macrophages. In contrast to its harmless nature in healthy people and, more generally, in the environment, *P. aeruginosa* is an opportunistic pathogen. It dangerously affects people whose immune system is weakened by a disease, such as people with acquired immune deficiency syndrome, or burned patients, people with ulcers or intubated in intensive care units, those with postoperative infections and patients affected by cystic fibrosis (Bodey, Bolivar *et al.* 1983). Indeed, patients with impaired immunity have higher risks of contracting *P. aeruginosa* nosocomial infection (Morrison and Wenzel 1984). Colonization rates may exceed 50% during hospitalization, especially among patients with trauma, cutaneous wounds or breach in mucosal barriers due to mechanical ventilation, tracheostomy, catheters, surgery, or severe burns (Ohara and Itoh 2003; Thuong, Arvaniti *et al.* 2003; Erol, Altoparlak *et al.* 2004). Nosocomial infections, contracted from the environment or staff of a healthcare facility (by

contaminated equipment, bed linens, or air droplets), can be spread in the hospital environment, nursing home environment, rehabilitation facility, clinic or other clinical settings. Disruption of the normal microbial flora as a result of antimicrobial therapy has also been shown to increase colonization by *P. aeruginosa* (Takesue, Yokoyama *et al.* 2002).

P. aeruginosa can establish two forms of interaction with its host: acute and chronic infection (Furukawa, Kuchma *et al.* 2006). Acute infections include acute pneumonia in hospitalized and, in particular, mechanically ventilated patients, skin infections and sepsis in patients with extensive burns, corneal infections in individuals wearing contact lenses, urinary tract infections in patients with catheters, bacteremia and sepsis in immunocompromised patients, particularly neutropenic patients receiving cytotoxic therapies, and post-surgical wound infections.

P. aeruginosa is the principal pathogen in the lungs of patients with CF. In some cases, *P. aeruginosa* breaks down host defenses in tissues such as the lungs and disseminates in the bloodstream, leading to death of a patient within hours or days. Through the type III secretion system, *P. aeruginosa* is able to secrete a variety of extracellular toxins facilitating systemic dissemination via the bloodstream (Vance, Rietsch *et al.* 2005), and acute infections such as in pneumonia (Barbieri and Sun 2004; Matsumoto 2004). In contrast, persistent or chronic infection is characterized by pulmonary tissue damage and respiratory failure which even long-term antibiotic therapy does not eradicate, leading to the patient death in some cases (Burns, Ramsey *et al.* 1993; Høiby 1993).

I.I.I THE GENETIC DIVERSITY OF *P. AERUGINOSA*

The success of *P. aeruginosa* as a worldwide-spread bacterium and opportunistic pathogen is based on its broad genetic repertoire.

The complete bacterial genome of a species, the pan-genome, includes the "core genome" containing genes present in all strains, and the "dispensable genome" also called "accessory genome" containing genes present in a subset of strains (Medini, Donati *et al.* 2005). The *P. aeruginosa* pan-genome consists of a core genome of about 5000 genes (Valot, Guyeux *et al.* 2015; van Belkum, Soriaga *et al.* 2015), and a dispensable genome of more than 40 000 genes. Seventy-five percent of the latter are present only in a few clones or strains (Hilker, Munder *et al.* 2015). Several studies have described the population structure as non-clonal, panmictic with a few clones associated to diseases, environmental conditions or antibiotic resistance (Kiewitz and Tümmler 2000; Curran, Jonas *et al.* 2004). Studies of the diversity of strains present in CF patients in France showed that they are mostly non-clonal, apart from clone C and clone PA14 (Vu-Thien, Corbineau *et al.* 2007; Llanes, Pourcel *et al.* 2013). This suggested that there is continuous modification of bacterial genomes by genetic exchanges, while clones emerge in particular contexts due to evolutionary pressures. In contrast, in another study performed by Wiehlmann and colleagues, a representative strain collection of diverse habitats and geographic origin was typed to describe the global population structure of *P. aeruginosa* (Wiehlmann, Wagner *et al.* 2007). The majority of *P. aeruginosa* strains was shown to belong to a few dominant clones widespread in diseases and environmental habitats such as clone PA14 represented by the completely sequenced reference strain PA14 (Lee, Urbach *et al.* 2006), clone C (Larbig, Christmann *et al.* 2002), clone K (Klockgether, Reva *et al.* 2004), clone M (Römling, Wingender *et al.* 1994), clone TB (Tümmler, Koopmann *et al.* 1991), clone CHA (Dacheux, Attree *et al.* 1999), and clone LES (Midlands and Liverpool epidemic strains) (Scott and Pitt 2004).

I.I.II EMERGENCE OF MULTIDRUG RESISTANT *P. AERUGINOSA*

P. aeruginosa possess a high capability of developing resistance to antimicrobial agents, molecules able to kill or simply inhibit the growth of bacteria such as antibiotics and aseptics, commonly employed in hospitals. Thus, nosocomial infections due to an antimicrobial-resistant *P. aeruginosa* spread are often difficult to eradicate. Even more problematic is the development of resistance during the course of therapy, a complication which has been shown to double the length of hospitalization and overall cost of patient care (Lister, Wolter *et al.* 2009). *P. aeruginosa* can develop resistance to different classes of antibiotics such as carbapenems, aminoglycosides and fluoroquinolones (Ozer, Duran *et al.* 2012) either through the acquisition of resistance genes on mobile genetic elements such as plasmids, or through mutational processes that alter the expression and function of chromosomally encoded mechanisms (Lister, Wolter *et al.* 2009).

Carbapenem-resistance may be related to decreased bacterial outer membrane permeability due, for example, to a loss or modification of porins or to overexpression of efflux pumps, or to expression of carbapenemases (Mesaros, Nordmann *et al.* 2007; Rodríguez-Martínez, Poirel *et al.* 2009). Indeed, several studies performed on *P. aeruginosa* coming from different sources and different countries such as Europe, Asia and South America have shown that some *P. aeruginosa* isolates present a set of genes encoding antibiotic-inactivation enzymes such as carbapenemases (Nordmann and Poirel 2002; Scheffer, Gales *et al.* 2010; Vitkauskienė, Skrodenienė *et al.* 2011; Correa, Montealegre *et al.* 2012; Llanes, Pourcel *et al.* 2013). Many carbapenemase genes are carried by plasmids and are easily transferable.

Aminoglycoside resistance may be due to aminoglycoside-modifying enzymes. High level of resistance to multiple aminoglycosides can also be associated with the acquisition of a

methylase gene, able to methylate the 16S rRNA in *P. aeruginosa* (Yokoyama, Doi *et al.* 2003).

Fluoroquinolone resistance among *P. aeruginosa* isolates has been linked mostly to mutations in chromosomal genes, with alterations in the fluoroquinolone targets DNA gyrase and topoisomerase IV, or overexpression of multidrug efflux pumps (Lister, Wolter *et al.* 2009).

The simultaneous presence of aminoglycoside-modifying enzymes and chromosomal mutations in genes encoding the fluoroquinolone-target enzymes, leads to the emergence of multidrug-resistant *P. aeruginosa* (Carmeli, Troillet *et al.* 1999). Polymyxins are among the very few therapeutic options left against such strains (Levin, Barone *et al.* 1999). In the 1970s, polymyxin B agents, antibiotics produced by a strain of *Bacillus polymyxa*, were employed to treat infections caused by gram-negative bacteria, including *P. aeruginosa*, but they were soon abandoned because of reported nephrotoxicity and neurotoxicity, and replaced by other, less toxic, antibiotics (Falagas and Kasiakou 2006). Another antibiotic belonging to the polymyxin group, polymixin E or colistin, is used in clinical practice as the emergence of multidrug-resistant organisms has renewed the interest in this mildly toxic therapeutic option (Sabuda, Laupland *et al.* 2008).

The emergence and spread of multidrug-resistant *P. aeruginosa* strains has significantly increased during the past years and this can be attributed to the wide use of new generation antibiotics and to the action, at the genetic level, of horizontal transfer of plasmids carrying different combinations of antibiotic-resistance determinants (Poole 2005; Nordmann, Poirel *et al.* 2011). It constitutes a serious threat for future therapy (Barbier and Wolff 2010).

I.I.III BIOFILM FORMED BY *P. AERUGINOSA* IN PATIENTS

The failure of antibiotic treatment of some chronic *P. aeruginosa* infections has been hypothesized to depend from two main events: the formation of a biofilm known, in general, to protect bacteria from environmental stressful conditions (Costerton, Stewart *et al.* 1999), and the selection, through prolonged use of antibiotics, of antibiotic-resistant variants.

Biofilms constitute a protective mode of growth that allows survival in a hostile environment. The biofilms contain channels in which nutrients can circulate, and cells in different regions of a biofilm exhibit different patterns of gene expression (Davies, Chakrabarty *et al.* 1993). These sessile biofilm communities can give rise to non-sessile individuals, planktonic bacteria that can rapidly multiply and disperse (Figure 1) (Mizan, Jahid *et al.* 2015).

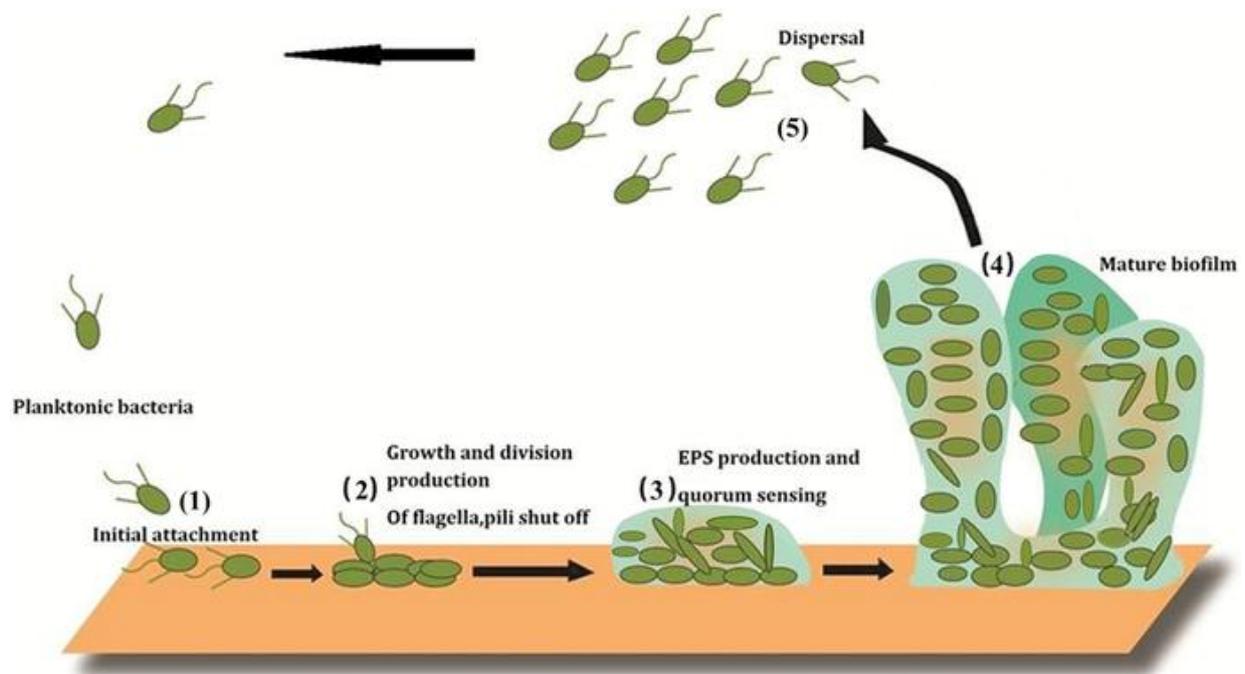


FIGURE 1. HYPOTHETICAL DEVELOPMENT OF A BIOFILM. The mature biofilm is produced through different steps: **1)** the planktonic bacterial cells attach on a surface; **2)** the production of structures devoted to the attachment is arrested and the bacterial cells start to proliferate; **3)** the bacterial cells produce an exopolysaccharide matrix and “communicate” with other bacterial cells through quorum sensing mechanism; **4)** the mature biofilm is formed and **5)** some cells disperse and colonize other free surfaces. Figure extracted and modified from (Mizan, Jahid *et al.* 2015).

Planktonic bacteria expose themselves to deleterious agents in their environment, such as phages or potent antimicrobial agents in a clinical setting. Thus, it is not surprising that chronic bacterial infections could involve bacterial biofilms, which are not easily eradicated by conventional antibiotic therapy.

Analysis of quorum sensing signals released by the bacteria in the sputum of CF patients chronically infected with *P. aeruginosa* suggested that this bacterium exist in the form of biofilms (Singh, Schaefer *et al.* 2000). The factors responsible for the induction of biofilm formation in lungs of CF patients are still not completely clear. In patients affected by this disease, the transmembrane regulator chloride channel located at the apical membranes of epithelial cells is defective, thus the fluid present in the airways of CF people is characterized by high salt concentration; the salt is believed to inhibit the activity of antimicrobial peptides and proteins of the innate immunity present in the airways, therefore the bacterium become free to colonize the epithelium in the form of a biofilm (Welsh and Smith 1993).

Antibiotic treatment during chronic *P. aeruginosa* infections of CF patients sometimes allows the disappearance of some symptoms usually associated with acute infections. This could be explained by the fact that planktonic cells released by the biofilm matrix and provoking the typical symptoms of acute infection, are susceptible to the antibiotic being used and can be killed. However, it is possible that the use of antibiotic could also select for planktonic cells resistant to antibiotics that can, in turn, colonize other parts of the epithelium giving rise to an antibiotic-resistant biofilm, determining the failure of the ongoing infection treatment (Costerton, Stewart *et al.* 1999; Singh, Schaefer *et al.* 2000; Drenkard and Ausubel 2002).

Current knowledge about bacterial biofilms suggests that effective eradication depends upon the use of therapeutic agents able to penetrate the exopolysaccharide matrix, and promote the detachment of bacterial cells, thus impairing the emergence of antibiotic-resistant variants.

I.II BACTERIOPHAGES

Bacteriophages (or phages) are the most abundant and most diversified microorganisms on Earth. They can be seen as obligate bacterial predators since they need bacteria for their multiplication, and can be found in all reservoirs populated by bacterial hosts including soil, aquatic environments (Srinivasiah, Bhavsar *et al.* 2008), human gut (Mills, Shanahan *et al.* 2013) etc.

Interactions between phages and their bacterial hosts are complex and play significant roles in shaping the structure of environmental microbial communities. Phage survival depends on its ability to infect the bacterial host. Adaptation to selective pressures such as host resistance, determines its abundance and diversification. Co-evolution of the phage tail fibers and bacterial receptors determines bacterial host ranges, mechanisms of phage entry, and other infection parameters. Phage host ranges have been shown to be highly variable in terms of specificity, ranging from phages with extremely narrow ranges of hosts within a single species to those that can infect bacteria across genera (Weitz, Poisot *et al.* 2013).

In natural habitats, phages and bacteria are in a constant arms race that proceeds in continuous cycles of co-evolution. As soon as the bacteria develop mechanisms to prevent phage infection, for example, bacterial receptor modification and degradation of invading phage DNA (Labrie, Samson *et al.* 2010), phages can evolve mechanisms to target such resistant bacteria (Samson, Magadán *et al.* 2013). This arms race continues and become one of the major forces to both widen the genetic diversity and maintain the equilibrium within microbial communities.

I.II.I DISCOVERY OF PHAGES AND FIRST PHAGE THERAPY ASSAYS

The very first evidences of an antibacterial activity that could be attributed to bacteriophages were obtained by Ernest Hankin. In 1896 this British bacteriologist reported the presence of an antibacterial agent in the water of two Indian rivers. The agent was able to pass through a porcelain filter, was inactivated by heat, and could impede the spread of a cholera epidemic (Hankin 1896). Twenty years later (in 1915), Frederick Twort, a medically trained bacteriologist from England, suggested that the antibacterial activity previously mentioned, could be due, among other possibilities, to the presence of a virus. Two years later (in 1917), Félix d'Hérelle, a French-Canadian bacteriologist working at the Pasteur Institute in Paris, reported the discovery of bacteriophages (see (Duckworth 1976) for an analysis of the controversy of who should be credited for the discovery of bacteriophages) and subsequently developed their therapeutic use (phage therapy). In 1915 d'Hérelle had been assigned to conduct an investigation on the dysentery outbreak among the French troops stationed at Maisons-Laffitte (on the outskirts of Paris). He filtered some patient fecal samples and incubated them with a *Shigella* strain isolated from the same patients, then he inoculated the mixture in rabbits in order to develop a vaccine against dysentery, and spread it also on agar plates to look at the bacterial growth; he observed that the bacterial growth on the plate was not uniform due to the presence of small, clear areas that he first called *taches* and only later *plaques* (Summers 1991). This discovery opened the way to a new therapeutic approach of bacterial infections. D'Hérelle was the first to use phages to successfully treat, in 1919, at the Necker-Enfants-Malades Hospital in Paris, five children affected by dysentery (Dublanquet and Fruciano 2008). However, the results of the treatment were published only later and were preceded by the first publication on the subject made by Richard Bruynoghe and Joseph Maisin in 1921, who used phages to treat a staphylococcal skin disease. After that, different treatment employing phages were performed and all were shown to be quite efficient in

eradicating the infection, such as those performed by d'Hérelle in India, on thousands of people affected by cholera or bubonic plague (Sulakvelidze, Alavidze *et al.* 2001).

Despite its initial success, with the advent of the first antibiotics and, consequently, the discovery of several new molecules from 1940 to 1990, phage therapy was abandoned in Western Europe and the Americas, and re-considered only recently (Dublanche and Fruciano 2008; Abedon, Kuhl *et al.* 2011). However, it became a major anti-bacterial therapy in the former USSR and is still in use in Russia and different countries in Eastern Europe (Abedon, Kuhl *et al.* 2011).

Phage therapy is also being considered to treat animal and plant infections. For instance, different strains of the bleeding canker bacterium, *Pseudomonas syringae* pv. *actinidiae*, devastating kiwi plantations in New Zealand, threatening to cause significant economic damage to the country. The group of Peter Fineran at the University of Otago published an investigation of more than 200 phages that show activity against this pathogen, laying the foundation for the design of a phage cocktail to deal with this problem (Frampton, Taylor *et al.* 2014). Further potential for the application of phages lies in aquaculture (Richards 2014), such as shrimp farming, where the current approach to fighting bacterial disease is an unsustainable release of antibiotics into the water. Moreover, phages can be used to treat farm animals carrying human pathogens as, for example, bacterial species of the genus *Campylobacter*, frequently responsible for human enteric disease with occasionally very serious outcomes (Adak, Meakins *et al.* 2005), therefore a lot of efforts are devoted to the isolation of bacteriophages for these bacteria (Sørensen, Gencay *et al.* 2015).

I.II.II PHAGE LIFE STRATEGIES

Phages are viruses composed of a genome made of a single- or double-strand DNA, or RNA molecule, packaged into a capsid and, in some cases possessing a tail and tail fibers. In this

manuscript I will discuss the largest group of bacteriophages, the *Caudoviridae*, which are constituted of a head containing the phage double-stranded DNA genome and a tail that, according to its characteristics allow the distinction of three phage families: the *Myoviridae* with long and contractile tail, the *Siphoviridae* with long non-contractile, flexible tail, and *Podoviridae* with short non-contractile tail (Figure 2) (Ackermann 2007).

These phages are generally separated into “lytic” (alias “virulent”) and “lysogenic” (alias “temperate”), on the basis of their capacity to integrate their genome into the bacterial chromosome (Hobbs and Abedon 2016). Whatever the characteristics of the considered phages, a number of capacities are required for a successful life cycle: adsorption to the bacterial surface, injection of the nucleic acid into the cell, replication and expression of proteins encoded by the phage genome, assembly of the virion structure, cell lysis, release of phage particles and further opportunities for transmission of infection to another host.

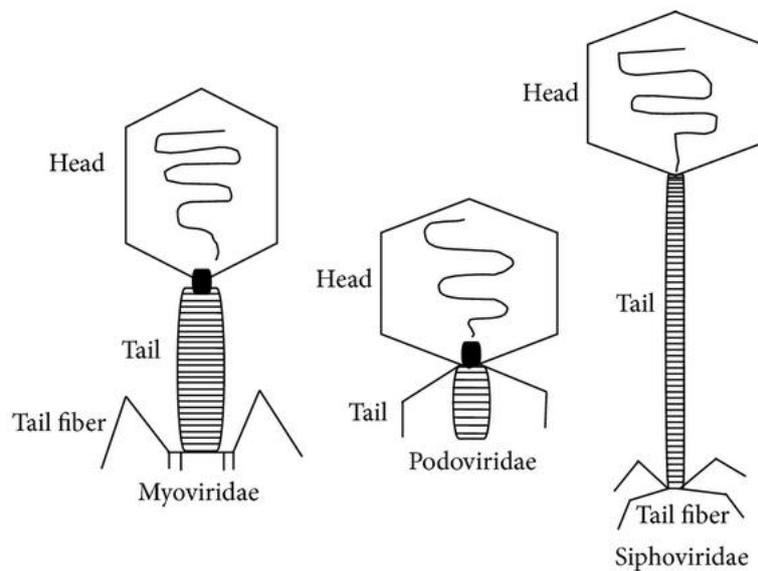


FIGURE 2. THREE TAILED PHAGE FAMILIES. Phages belonging to the *Myoviridae* family possess a contractile tail with terminal tail fibers; those belonging to the *Podoviridae* family have a short tail with tail spikes and those belonging to the *Siphoviridae* family possess a long flexible non-contractile tail. Figure extracted and modified from (Elbreki, Ross *et al.* 2014).

Adsorption is the first step of physical interaction between a phage and its host: a reversible adsorption on the cell surface is followed by a second irreversible and stable interaction between phage tail structures, such as tail fibers and the phage receptor. Then, the cell wall is permeabilized by the action of enzymes present in the viral capsid or on the phage tail, and the phage nucleic acid is internalized in the bacterial cell. At this stage, the phage can enter into different cycles according to its nature (lytic, temperate or pseudolysogenic). However, all the phages go through similar phases during their replication and virion maturation. After phage adsorption, the so-called latent period starts and ends with the cell lysis. During the latent period the phage DNA is replicated and phage proteins are expressed, resulting in the assembling of functional phage particles (eclipse period) which are, then, released during the rise period, basically consisting in the bacterial cell lysis. The number of phage particles released by each infected cell defines the phage burst size, and can be different not only considering phages belonging to the same genus, but also when the same phage is cultured on different bacteria. Phages can use different mechanisms to lyse the bacterial cells. For some of the tailed phages, cell lysis is accomplished through a two steps process mediated by two phage-encoded proteins: the holin, an enzyme that damage the bacterial membrane allowing the endolysin to hydrolyze the peptidoglycan and release of the virions (Ackermann 1998).

Lytic phages cannot integrate their genome into the bacterial chromosome and therefore can only perform a productive cycle after injection of their genome (Figure 3). In contrast, lysogenic phages have the possibility to enter a lysogenic cycle, during which the phage genome is integrated into the bacterial chromosome to become a prophage, and persist in a latent or dormant state that does not promote cell death or the production of phage particles (Figure 3). Some prophages persist as low copy number plasmids and do not integrate into the bacterial chromosome (for example, the coliphages P1 and N15) (Edlin, Lin *et al.* 1977; Ravin, Ravin *et al.* 2000). Prophages are replicated together with the bacterial host

chromosome, and this lysogenic state is maintained by the repression of the phage lytic genes. A switch to lytic production is initiated when stressful conditions (Little 1984) induce the excision of the phage genome, which is followed by the expression of lytic genes that promote DNA replication, phage particle assembly, DNA packaging and bacterial lysis.

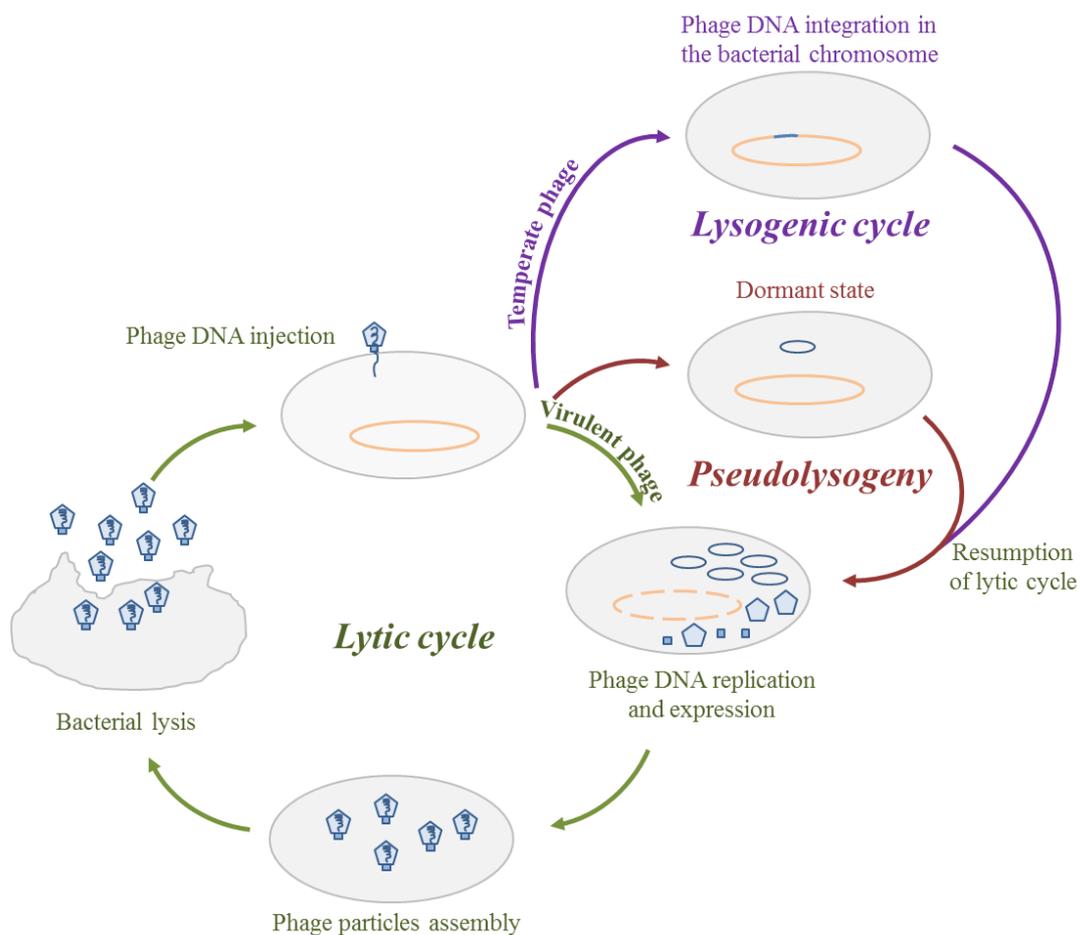


FIGURE 3. PHAGE LIFE STRATEGIES. Lytic cycle: the phage adsorbs on the bacterial surface and injects its DNA into the cell. The phage DNA is immediately replicated and the phage proteins are expressed in order to produce functional phage particles, then released at the end of the lytic cycle terminating with cell lysis. **Lysogenic cycle:** after adsorption and DNA injection in the host cell, a temperate phage can integrate its genome inside the bacterial chromosome becoming a prophage and entering a dormant state persisting until an external stress, inducing bacterial DNA damage, resume the phage lytic cycle. **Pseudolysogeny:** after adsorption and DNA injection in the host cell, the phage DNA remains in an episomal form, not integrating in the bacterial chromosome as a prophage nor entering in a productive lytic cycle. It has been frequently observed in starved cell and when the environmental conditions become again favorable for the host replication, the phage can enter a lytic cycle or lysogenic cycle according to the phage nature.

There exists a third, even if still poorly understood, phage life strategy called pseudolysogeny, represented by an unstable situation in which the virulent phage fails to finish the reproductive cycle and lyse the host or, in case of temperate phage, to become established as a prophage (Baess 1971). This phenomenon has been frequently observed under nutrient-deprived conditions, when bacterial cells cannot support DNA replication or protein synthesis. The phage genome is maintained, until the nutritional status is restored, at which point it enters either a lysogenic or a lytic life cycle (Fuhrman 1999) (Figure 3).

I.II.III THE IMPORTANCE OF PHAGE RESEARCH

As previously mentioned, phage research has seen many peaks over the past century, particularly in relation with their use as therapeutic agents. The vast majority of clinical trials involving the use of phages have been conducted in Eastern Europe, especially in Poland, Georgia, and Russia, where phage therapy has been employed for decades without interruptions (Sulakvelidze, Alavidze *et al.* 2001); in the rest of the World, after the initial successes, phage research dedicated to clinical use was abandoned due to the discovery of antibiotics. It has regained some attention as a therapeutic approach against the problematic rise in antibiotic-resistant pathogens mostly in the past decade.

Basic research on different aspects of bacteriophages life cycles and diversity has been abundant. The development of molecular biology and biotechnology techniques has contributed to, but also benefited from, the development of this research, as phages encode enzymes and promoters with peculiar characteristics that make them good tools for genetic manipulation (Haq, Chaudhry *et al.* 2012).

Most of the research was performed on phages targeting gram-negative bacteria, in particular on *Escherichia coli*, as one particular strain of this bacterium became the main model organism for microbiology. Consequently, coliphages such as T phages (Demerec and Fano

1945) and lambda (Lederberg and Lederberg 1953) served as model phages in the development of molecular tools and the fundamental understanding of phage-host interactions.

T-even phages (T2, T4 and T6) have been major model systems in the development of modern genetics and molecular biology since the 1940s. Coliphages T2 and T4 were fundamental for the recognition of nucleic acids as genetic material and for the definition of gene by mutational, recombinational, and functional analyses (Miller, Kutter *et al.* 2003). These phages were instrumental to demonstrate that the genetic code is constituted of triplets. The phages allowed the discovery of mRNA, of the importance of recombination in DNA replication, of light-dependent and light-independent DNA repair mechanisms, of restriction and modification of DNA, of self-splicing introns in prokaryotes, of translational bypassing etc (Brenner, Jacob *et al.* 1961; Crick, Barnett *et al.* 1961).

One of the characteristics that make the phage T4 a good tool for molecular studies is the total inhibition of the host gene expression mediated by the phage during the infection. Studies performed to elucidate the virion assembly mechanisms, phage DNA replication and recombination led to important insights into macromolecular interactions between phage and host proteins (Alberts 1987; Alberts and Miake-Lye 1992) that constitute nowadays a standard reference for similar studies performed on phages belonging to different genera.

T-even phages possess many proteins with redundant functions and this could explain their ability to exploit a broad range of potential hosts and to resist different sorts of antiviral mechanisms imposed by the host (Kutter, d'Acci *et al.* 1994; Abedon, Herschler *et al.* 2001). Phage T4 research led to the identification of several enzymes with widespread applications in genetic engineering, such as DNA and RNA ligase, polynucleotide kinase, and DNA polymerase.

T5 and T7 phages have been model phages for structural analyses and mechanisms of DNA injection into the bacteria, respectively. The overall structure of T5 has been revealed by cryo-electron microscopy and image reconstruction. The early events of T5 capsid assembly have also been, even if partly, deciphered; the initial prohead I is assembled from a precursor protein possessing an N-terminal scaffolding domain which is cleaved by the T5-encoded head protease, yielding the mature prohead II (Huet, Conway *et al.* 2010). Packaging of DNA into prohead II is accompanied by expansion of the capsid, which involves large structural rearrangements of the coat protein subunits and allows accommodation of the full-length genome (Preux, Durand *et al.* 2013). The mature capsid is then decorated with some proteins, which bind as monomers to the center of the hexamers (Effantin, Boulanger *et al.* 2006). T5 properties are generally applicable to the large *Siphoviridae* family of dsDNA tailed phages.

As previously mentioned, T7 has mostly been studied to clarify the molecular mechanisms acting during phage interaction with the bacterial host. Details about the molecular interaction between the phage and its host during infection are provided in the following paragraph.

I.II.IV MOLECULAR INTERACTION BETWEEN VIRULENT PHAGES AND BACTERIA DURING INFECTION

Phage host range is defined by looking at which bacterial genera, species and strains a given phage is able to lyse (Kutter 2009). Bacteriophages generally target their hosts at the strain-specific level. For this reason they have often been used as genotyping tools to classify bacterial strains at the subspecies level.

In addition to the initial interaction between the tail and its receptor, bacteriophages rely on the action of bacterial proteins at different stages of their life cycle. For example, during a normal lytic infection cycle by phage T7, the viral genome is injected in two steps. In an initial phase after phage adsorption on the surface, only part of the viral genome enters the

bacterial cell (García and Molineux 1995). Transcription is started by the RNA polymerase of the host, recognizing strong promoters located at the very beginning of the phage DNA early region. After transcription and translation of the phage RNA polymerase, the remaining part of the phage genome is internalized (Figure 4) (García and Molineux 1999). The viral cycle proceeds using the phage-encoded RNA polymerase.

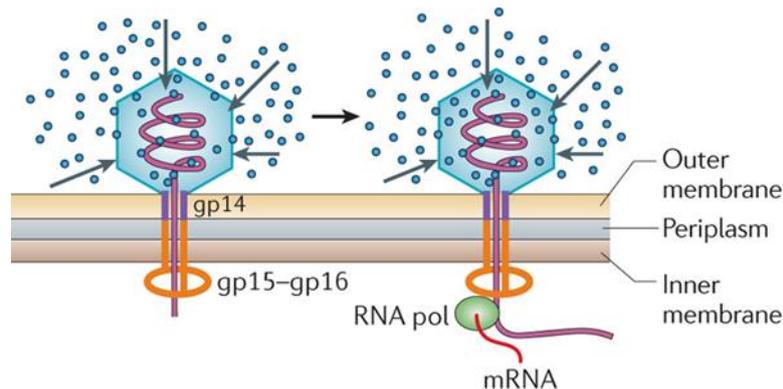


FIGURE 4. PHAGE T7 DNA INJECTION IN THE HOST CELL. Three phage proteins, Gp14, Gp15 and Gp16, are ejected from the phage capsid and form a channel and a motor that allow the injection of ~ 1 kbp of phage DNA inside the host cell. The remaining phage DNA is pulled inside the cell after the action of the bacterial RNA polymerase (RNA pol). Figure extracted and modified from (Molineux and Panja 2013).

The mRNAs of phage early genes produced by the host RNA polymerase code for several phage proteins involved in the shut-off of host defenses. Among them are the Gp0.3 coding for an anti-restriction protein (Studier 1975) or Gp0.7, a protein kinase that phosphorylates the β' subunit of host RNA polymerase and affects its termination properties (Severinova and Severinov 2006). Once the T7 RNA polymerase is synthesized, middle and late regions are transcribed including the Gp2 gene encoding a potent inhibitor of host RNA polymerase (Hesselbach and Nakada 1977).

More generally, phages use the bacterial machinery for their replication and gene synthesis and in some cases they might not find the perfect environment to perform a full productive cycle, thus initiating a pseudolysogenic stage. The pseudolysogenic life cycle will be described in more details in the paragraph “II.I PSEUDOLYSOGENY”.

I.III PHAGES EMPLOYED IN PHAGE THERAPY

I.III.I PHAGES OF *P. AERUGINOSA*

More than 97% of isolated phages active against *P. aeruginosa* strains belong to the *Caudoviridae*. The other phages that infect *P. aeruginosa* have single-stranded DNA genomes or RNA genomes and they will not be described in this manuscript.

Given the wide distribution of *P. aeruginosa* clones, it is possible to imagine that their phages could be commonly found and distributed in few defined genera able to cover, when combined, a large host spectrum. To date, the characterized *P. aeruginosa* phages have been distributed in at least 7 genera of virulent phages (T7-like, Φ KMV-like, LUZ24-like, LIT1-like, PB1-like, Φ KZ-like, JG004-like) and into a similar number of temperate genera (Ceysens and Lavigne 2010). Within each genus, phages can show a different host spectrum according to the specificity of their tail receptor-recognizing proteins (Chaturongakul and Ounjai 2014). Although several studies performed using *P. aeruginosa* strains have shown that combining different phages allows to cover a large host range spectrum, almost 15% of clinical strains were shown to be resistant to all phages tested (Essoh, Blouin *et al.* 2013).

THE *MYOVIRIDAE*

The ϕ KZ-like viruses are *myoviruses* also defined as giant phages due to the big size of their genome: the representative of this genus, the ϕ KZ phage, possesses a genome of ~280 kbp in length (Mesyanzhinov, Robben *et al.* 2002). Like phage ϕ KZ, phage EL possesses a genome of ~211 kbp in length and a similar morphology (Hertveldt, Lavigne *et al.* 2005). Phages of this genus have a broad host range and high burst size.

Phages belonging to the PB1-like genus present high similarities at genomic (>96%) and morphological level (Ceysens, Miroshnikov *et al.* 2009). They have a wide host-range and have been shown to target LPS as receptor.

The PAK-P1-like (Debarbieux, Leduc *et al.* 2010) and KPP10-like (Uchiyama, Rashel *et al.* 2012) phages are worldwide spread genera. Previously, these two groups of phages constituted the Felix O1-like genus since they share structural characteristics and genome organization similar to that of the *Salmonella* phage Felix O1 (Whichard, Weigt *et al.* 2010). Performing a phylogenetic analysis of the sequenced genomes of phages belonging to the Felix O1 group, Henry and colleagues proposed to distinguish two main genera: the *Pakpunaviridae*, and the *Kpp10viridae* (Henry, Bobay *et al.* 2015).

In contrast to the virulent behavior of the phages belonging to the four genera just described, phages belonging to the CTX genus are temperate *myoviruses* carrying the cholera-toxin (CTx) that provides the lysogen bacteria with new virulent characteristics (Hayashi, Baba *et al.* 1990).

THE SIPHOVIRIDAE

The *siphoviruses* of *P. aeruginosa* are mostly temperate phages. Phages D3112, B3, DMS3, PM105 and PA1Ø (genome sequences reported by (Wang, Chu *et al.* 2004), (Braid, Silhavy *et al.* 2004), (Budzik, Rosche *et al.* 2004), (Pourcel, Midoux *et al.* 2016) and (Kim, Rahman *et al.* 2012), respectively) are all Mu-like phages which replicate by transposition and are capable of transferring fragments of bacterial DNA from one strain to another (Harshey 2014). This ability to carry fragments of bacterial DNA from a donor cell (the phage-infected cell) to a recipient (the cell infected by a phage released by a primarily infected cell) is called transduction. With the exception of phage PA1Ø (lacking phage repressor), they are all temperate.

The D3-like phages including phage D3 (Kuzio and Kropinski 1983) and PAJU2 (Uchiyama, Rashel *et al.* 2009), are temperate phages with similar virion structure, able to change the bacterial serotype upon lysogenization.

The YuA-like phages carry anti-restriction genes enabling the phage DNA to be protected against the bacterial restriction endonucleases (Ceysens, Mesyanzhinov *et al.* 2008).

Apart from phage PA1Ø, two lytic phages belonging to the *Siphoviridae* family have been isolated: phage Kakheti25 isolated in Georgia (Karumidze, Thomas *et al.* 2012) and phage KPP23 isolated in Japan (Yamaguchi, Miyata *et al.* 2014).

THE *PODOVIRIDAE*

Most *podoviruses* of *P. aeruginosa* are lytic phages. ϕ -KMV-like phages possess unique characteristics: their packaged DNA possesses single-strand interruptions whose biological function is still unknown (Kulakov, Ksenzenko *et al.* 2009). ϕ -KMV-like phages encode an alginate-degrading enzyme located at the C-terminal of the tail spike protein providing the phage with the ability of penetrating deeply in biofilms (Glonti, Chanishvili *et al.* 2010) and making them suitable for therapeutic cocktail preparations. Phages belonging to this genus target the type IV pilus as a receptor (Chibeu, Ceysens *et al.* 2009). The genome of all the ϕ -KMV-like phages encodes an RNA polymerase responsible for the transcription of genes located in the middle and late region of their genome (Lavigne, Burkal'tseva *et al.* 2003).

Phages of the LUZ24 genus do not encode an RNA polymerase, thus their multiplication relies entirely on the bacterial transcription machinery (Ceysens and Lavigne 2010). Similarly to ϕ -KMV-like phages, their packaged genome possesses single-stranded DNA breaks (Essoh, Latino *et al.* 2015).

The LIT1-like phages are morphologically similar to the coliphage N4 and constitute another genus of the *Podoviridae* family; they are known to carry a large virion-associated RNA polymerase that is injected with the phage DNA inside the host cell (Wittmann, Klumpp *et al.* 2015).

A number of phages not yet assigned to a specific genus also belong to the *Podoviridae*. F116, a temperate generalized transducing phage, is particularly interesting as it possesses an alginate-degrading activity that it uses to reach its receptor, the type IV pilus (Byrne and Kropinski 2005). Despite the presence of an integrase encoding gene in its genome, this phage is believed to be carried by the host strain in a plasmid form, the so-called carrier state (Miller, Pemberton *et al.* 1977) discussed in “II.I PSEUDOLYSOGENY”.

I.III.II SELECTION OF PHAGES FOR THERAPEUTIC USE

The first steps in implementing a phage therapy protocol is phage isolation and selection according to the characteristics of the pathogen to be targeted (Gill and Hyman 2010; Goodridge 2010). The phage choice will be oriented by the therapeutic objectives. Pyophage or Intestiphage are multi-species phage cocktails that cover a wide host spectrum. They are commercialized in some countries to allow the simultaneous elimination of different bacterial targets (genera, species and strains) (Kutter, De Vos *et al.* 2010). A second approach targets a specific bacterial species either with a cocktail of well-characterized phages (Merabishvili, Pirnay *et al.* 2009; Gill and Hyman 2010), or with single phages. In the latter situation, also called “sur mesure” (Pirnay, De Vos *et al.* 2011; Ravat, Jault *et al.* 2015), single phages could be successful in eliminating particular strains. However, several studies have demonstrated that the use of a phage cocktail is advantageous not only to provide wider host range target, but also to prevent or delay the emergence of mutations in the bacterial population that can

result in the selection of phage-resistant clones and consequent therapeutic failure (Tanji, Shimada *et al.* 2004; Gu, Liu *et al.* 2012).

Three main characteristics define the suitability of a phage for therapeutic use: a strictly virulent behavior, the absence of encoded toxins, and the inability to perform generalized transduction. However, the virulent behavior of therapeutic phages does not ensure total safety of the treatment; indeed virulent phages could recombine with the host prophages forming chimeras with temperate properties. Chimera phages, such as the *P. aeruginosa* phage Ab31, isolated in Abidjan by our group (ANNEX I) (Essoh, Latino *et al.* 2015) do exist in nature although the frequency of such events has not been quantified. Phage Ab31 seems to derive from a recombination event between a phage similar to temperate *P. aeruginosa* phage PAJU2 and a phage similar to the lytic phage AF of *P. putida* (Latino, Essoh *et al.* 2014). In the ANNEX II, we show that phage Ab31 could select phage-resistant variants with genome rearrangements and phenotypic characteristic such as mucoidy that, in a phage therapy context, could give an unfavorable prognosis (Latino, Essoh *et al.* 2014).

Some phages, such as the Shiga toxin-converting phages of *E. coli* (Tozzoli, Grande *et al.* 2014), are known to encode toxins or other proteins that could somehow be acquired by the bacteria, producing a more virulent variant with a competitive advantage over other bacteria. Concerning this point, it is essential to sequence and annotate the phages that are going to be employed and try to assign functions to each of the genes. Once each phage has been sufficiently annotated and the absence of putative toxins or integrases has been confirmed, their behavior *in vitro* should be studied.

Other secondary characteristics should also be considered when selecting phages for therapeutic purposes. Those with a broad host range would be good candidates for phage therapy as it would increase the chances to target different bacterial strains. Phages belonging

to *P. aeruginosa* PB1 and ϕ -KMV genera possess a wide host spectrum, and are commonly employed for the constitution of phage cocktails. PB1-like phages have been recently used to treat infections caused by *P. aeruginosa* in burn patients (Merabishvili, Pirnay *et al.* 2009) and in laboratory simulated chronic pneumonia conditions (Garbe, Wesche *et al.* 2010). However, it has been reported that isolated clinical *P. aeruginosa* strains are frequently resistant to such phages (Pleteneva, Shaburova *et al.* 2008; Ceysens, Miroshnikov *et al.* 2009). Phages belonging to the *P. aeruginosa* ϕ KZ genus are also characterized by a broad lytic spectrum and have been commonly employed for the preparation of cocktails for therapeutic use (Krylov, Shaburova *et al.* 2013). Unfortunately they have been shown to be able to persist inside the bacterial host in a pseudolysogenic state and to perform generalized transduction (Krylov, Miroshnikov *et al.* 2010; Pleteneva, Burkal'tseva *et al.* 2011; Krylov, Kropinski *et al.* 2012). The persistence of phage DNA in a pseudolysogen host may favor its recombination with prophages carried by the infected host, producing a phage with undesirable characteristics. It may also induce a change in the bacterial phenotype: *P. aeruginosa* PAO1 pseudolysogenized by ϕ KZ phages, converts to a mucoid phenotype, a characteristic very undesirable during the course of *P. aeruginosa* infection (Krylov, Miroshnikov *et al.* 2010). To safely use ϕ KZ-like phages, it might be interesting to select mutants losing the ability to pseudolysogenize the host. Although ϕ KZ-like phages and their variants seem to be good candidates for the constitution of phage cocktails due to their broad host spectrum, they should be avoided.

The adsorption properties of phages are also important when it comes to use them to constitute a cocktail because of their strict correlation with selection of phage-resistant mutants. Synergy between phages takes into account the adsorption characteristics of each single phage constituting the cocktail (Schmerer, Molineux *et al.* 2014). For instance, one phage can synergistically affect the infection of a second phage impacting three main phases

of its infection cycle: adsorption rate, burst size or latent period. The synergy impacting the phage adsorption on the bacterial surface has been frequently observed, especially for phages carrying depolymerizing activity that can degrade the bacterial capsule (Azeredo and Sutherland 2008) allowing other phages to better adsorb and infect the host (Bull, Vimr *et al.* 2010). At the same time, phages can also negatively interfere with each other; for example, during coinfection, one phage can strongly affect the burst size of a second phage. So, it is necessary when combining different phages, to carefully choose them according to their adsorption characteristics, such as the nature of their target receptor (LPS, type IV pilus, etc.), in order to lower the possibility of cross-resistant variant selection or phage interference.

Studies on *P. aeruginosa* suggest the potential utility of phages to reduce or eliminate biofilms produced by different strains, including PAO1 (Pires, Sillankorva *et al.* 2011), and to prevent biofilm formation on medical devices, such as catheters (Fu, Forster *et al.* 2010). In particular, phages belonging to PB1-like, LIT1-like and ϕ -KMV-like genera have been shown to be efficient in the *in vitro* dissociation of *P. aeruginosa* biofilm (Alves, Perez-Esteban *et al.* 2015).

I.III.III PHAGE PROPHYLAXIS AND THERAPY TO TREAT *P. AERUGINOSA* INFECTIONS

The advantages of using phages as a therapy to treat infections are numerous; indeed, phages are highly specific for the infecting bacterium, thus being harmless for the host; phages can be used to kill antibiotic resistant bacteria since resistance to phages and to antibiotics seems not to be mutually dependent; moreover it has been shown that the combination of both antimicrobials, phages and antibiotics, can have a synergistic effect on the eradication of infection (Torres-Barceló and Hochberg 2016); it is not necessary to administer high doses of phages, since phages multiply in the bacterial cells releasing new phage particles; this process continues until all the susceptible cells are completely destroyed.

Attempts to treat *P. aeruginosa* infections with phages have been shown to efficiently help to reduce the frequency of local nosocomial infections in clinical settings. Ahiwale and colleagues isolated the *P. aeruginosa* lytic phage BVPaP-3, able to efficiently disperse biofilm formed *in vitro* by a multidrug-resistant *P. aeruginosa* strain isolated in the hospital. Used at a multiplicity of infection (MOI) of 0.001, it behaved as a disinfectant to prevent biofilm formation on medical devices (Ahiwale, Tamboli *et al.* 2011). In a similar study, the frequency of hospital infections caused by *P. aeruginosa* was shown to drop from 40.8% to 8.93% when virulent phages adapted to local *P. aeruginosa* were employed (Aslanov, Iafaev *et al.* 2003).

Phages have been shown to be efficient not only as disinfectants, thus impeding the spread of *P. aeruginosa* nosocomial infections, but also as a therapeutic treatment. Different animal models have been developed to investigate the efficiency and safety of phage therapy. For instance, Wang and colleagues, showed that a virulent phage chosen among 29 phages isolated from hospital sewage, was able to reduce the mortality of mice infected intraperitoneally with an imipenem-resistant *P. aeruginosa* strain (Wang, Hu *et al.* 2006). KPP10-like and PAK-P1-like phages have been employed to conduct experiments *in vivo*, on mice model, to assess their efficacy in the treatment of induced murine gut-derived sepsis, or *P. aeruginosa* pulmonary infections, respectively (Watanabe, Matsumoto *et al.* 2007; Debarbieux, Leduc *et al.* 2010; Uchiyama, Rashel *et al.* 2012). Mice infected with *P. aeruginosa* strain PAK were shown to be cured when the lytic phage PAK-P1 was administrated a few hours after infection (Debarbieux, Leduc *et al.* 2010). In the course of the same study, different results were obtained when the phage was tested *in vitro* on CF *P. aeruginosa* strains derived from patients with primary colonization or chronic infection. Phage PAK-P1 effectively lysed 50% of the primary colonization strains, but it only moderately lysed 10% of the chronic ones. This could be related to the fact that phage PAK-

P1 was isolated using a planktonic culture, and was thus not adapted to kill bacteria deriving from biofilm-like structure typical of chronic infections (Debarbieux, Leduc *et al.* 2010). In this case it is worth to mention that, although phages could give positive results in the treatment of *P. aeruginosa* infections, the choice of the phage should be rationalized according to the stage of infection. In chronically infected patients, it would be recommended to use phages carrying a depolymerizing activity able to lyse bacteria embedded in a biofilm (Hanlon, Denyer *et al.* 2001).

Although phages seem to represent a promising tool to fight some bacterial pathogens, as previously outlined, the use of chemotherapy (antibiotics) should not be abandoned. Phages used in combination with antibiotics, have been shown to have a synergistic effect on the killing of the bacteria and in the lowering of the resistant variants emergence (Torres-Barceló and Hochberg 2016). Alone or in association with antibiotics, phages seem to constitute a promising treatment for pathogens, but there are only few large-scale clinical studies performed to evaluate their safety and efficacy, according to modern rules. Although during almost a century, large amount of treatments were performed in Easter Countries, they were not conducted following the legislation dictating the rules to perform clinical trials. Moreover, most of the reports about the results obtained from these treatments were written in Russian and only few have been translated in English (N. Chanishvili 2012).

In 2009 in the United Kingdom, a clinical trial to treat an antibiotic-resistant *P. aeruginosa* causing chronic otitis was conducted and reported to be effective and safe (Wright, Hawkins *et al.* 2009). Another clinical study, Phagoburn, evaluating the efficacy of the treatment of antibiotic-resistant infections of burn wounds, is ongoing in France, Belgium and Switzerland (Kingwell 2015).

II PHAGE-BACTERIA INTERACTIONS

The interactions most frequently observed between bacteria and their predators are beneficial (synergistic) or adverse (antagonistic) ones. The beneficial interactions, including mutualism, can be observed in nature mainly between temperate phages and their hosts. For example, phage conversion, described as a change in the bacterial phenotype when lysogenized by a temperate phage, may result in an increased fitness of the host. Examples of this advantageous condition have been reported for various phages infecting *E. coli* (Edlin, Lin *et al.* 1977). An increased fitness of the host should also result in higher replication of the phage genome, and this might be considered a mutualistic interaction. Moreover, the establishment of a lysogenic interaction always confers immunity to the lysogenic cell against superinfection with the same or related phage types (Ackermann and DuBow 1987). In contrast, virulent phages mainly establish an antagonistic interaction, better known as predator–prey interaction, with their host.

Overall, a description of phages as parasites of bacteria seems to be an oversimplification regarding the sophisticated and diverse interactions that they can have with their host. Interactions between bacteria and phages are complex and comprise a continuum from mutualistic to parasitic, even among the same set of actors at different stages of their life cycles (Dennehy 2014). In some cases, phages provide bacteria with critically important genes, including antibiotic resistance, metabolic and virulence genes (Chibani-Chennoufi, Bruttin *et al.* 2004; Comeau and Krisch 2005), and protect their host from infection by other viruses (Bondy-Denomy and Davidson 2014). Phages are also lethal parasites of bacteria, they modulate host populations and drive their diversification by selecting for resistant mutants in co-evolutionary arms race (Brockhurst, Buckling *et al.* 2005; Weitz, Hartman *et al.* 2005; Rodriguez-Valera, Martin-Cuadrado *et al.* 2009; Dennehy 2012).

II.I PSEUDOLYSOGENY

Much work has been devoted to characterizing classical phage-bacteria interactions, such as the lysis-lysogeny paradigm of temperate phages and the strictly lytic life cycle. However, it has long been known that other phage-bacteria interactions exist, most prominently pseudolysogeny, which has been described as an intermediate state between the lytic and lysogenic lifestyles (Ripp and Miller 1998; Abedon 2009). For historical and technical reasons, this strategy remains poorly understood. Given the significant ecological impacts of pseudolysogenic phages discussed below (Wommack and Colwell 2000; Paul 2008; Clokie, Millard *et al.* 2011; Łoś and Węgrzyn 2012; Maura and Debarbieux 2012), a better understanding of pseudolysogeny is sought.

Pseudolysogeny is believed to play an important role in the long-term survival of phages as it might prevent poor replication or even degradation of the phage chromosome in a host that is too starved to support further steps in lytic or lysogenic development. In addition, it provides a transient intracellular refuge for the phage chromosome in environments characterized by low host densities and short capsid half-lives (Ripp and Miller 1998). Despite its ecological importance (Łoś and Węgrzyn 2012), few formal molecular evidences currently exist for the regulation of such a state and its possible impact on the physiology of the cell (Cenens, Mebrhatu *et al.* 2013).

Until now, there is no literature about the molecular mechanism (phage-bacterial proteins interaction and regulation) that could constitute the basis for the establishment of pseudolysogeny with lytic phages. In contrast, for temperate phages, even if only in few cases, the regulation of carrier state, defined by Ackermann and DuBow (Ackermann and DuBow 1987) as lysogeny by a plasmid phage, has been reported. Cenens and colleagues identified in *Salmonella* Typhimurium phage P22 a locus, named *pid*, expressed only in what

they called “phage carrier cells” and able to specifically derepress the *dgo*-operon of the host involved in the galactonate metabolism (Cenens, Makumi *et al.* 2013). The reason why Pid would specifically target galactonate metabolism and whether or not this interaction is beneficial for the phage and/or the host so far remains unclear.

II.I.I HISTORY ABOUT THE DEFINITION OF PSEUDOLYSOGENY

The definition of pseudolysogeny has always been controversial and nowadays is still ambiguous. The first definition of pseudolysogeny, could be associated with the observations made by Delbrück in 1946 on “pseudolysogenesis”, during which the contaminating phage reproduced at the expense of phage-susceptible bacteria produced by mutation during growth of a phage-resistant culture (Delbrück 1946). These observations were in accordance with the hypothesis made by D’Herelle (1930) saying that in a “symbiose bactérie-bactériophage” there were bacteria covering a certain spectrum of susceptibility to phage action, while the phage population covered a considerable range of virulence.

However, the first historical discussion about the concept of pseudolysogeny, mentioned as “carrier strain” was presented by Lwoff (Lwoff 1953). Lwoff pointed out the distinction between “lysogenic strains”, in which all the bacteria are lysogenic and perpetuate hereditarily the power to produce phage that cannot be eliminated by cultivating the strain in anti-phage serum, and “carrier strains”, also called “pseudolysogenic strains”, constituted of a mixture of bacteriophages and bacteria in a more or less stable equilibrium. The majority of the bacteria are phage-resistant, some are sensitive variants that could be infected by extrinsic phages and allow phage multiplication. In contrast to “lysogenic strains”, the “carrier strains” could lose their phage-producing power if treated with phage-antiserum or simply purified through colony re-isolation (Lwoff 1953).

Some years later Stent (Stent 1963) described the pseudolysogens as bacteria capable of adsorbing the phage they were carrying but being resistant to infection. Stent hypothesized that all the bacterial cells in the pseudolysogen population carried extracellular free phages on their surface and that phage multiplication was ensured by occasional phage-sensitive variants that appear during bacterial growth (Stent 1963).

A more detailed description of pseudolysogeny was provided by Baess in 1971 (Baess 1971). Baess stated that in pseudolysogens, the phage DNA, possessed by a fraction of cells, is not integrated in the bacterial chromosome and cannot be induced applying the techniques commonly used for the prophage induction in “lysogenic strains”. Moreover, as previously observed by Lwoff, the pseudolysogens could be cured by culturing them in anti-serum against the bacteriophage or simply by purification through colony re-isolation (Baess 1971).

Another, more complete, hypothesis about the mechanism underlying pseudolysogeny, was given a few years later by Ackermann and DuBow (Ackermann and DuBow 1987) and recently, even if under another name (carrier state*) by Abedon (Abedon 2009). Pseudolysogeny is a result of phage maintenance within a culture via lytic infection of only a portion of the bacteria present (Abedon 2009). The phage-resistant and phage-sensitive bacteria can differ for mutations in the bacterial chromosome or they can possess the same genome but be phenotypically different due to phage-dependent or phage-independent action. Abedon hypothesized that the infected bacterial cells could release soluble factors that can temporarily modify the surface of the sensitive bacteria increasing the number of cells that cannot support phage growth; this factor is then diluted as more bacteria became temporarily surface-modified (phage-resistant) and the bacteria can recover their susceptibility to phage allowing its multiplication. Alternatively, the modification of bacterial surface can be phage-independent: the bacterium itself can modify the structure of phage receptors such as pili or

LPS by a reversible mechanism becoming temporarily resistant to phage attack (Abedon 2009).

II.I.II IMPACT OF PSEUDOLYSOGENY ON BACTERIAL POPULATIONS

Several studies suggested that pseudolysogeny is connected to host physiology. For example, Ripp and Miller showed that the frequency of pseudolysogeny by some *P. aeruginosa* phages was inversely proportional to nutrient supplementation: they hypothesized that in starving conditions bacterial cells do not have enough energy to allow the phage to initiate infection cycle (Ripp and Miller 1998). In another example, the *E. coli* phage T4 was known to pseudolysogenize starved, slowly growing cells (Los, Wegrzyn *et al.* 2003; Golec, Karczewska-Golec *et al.* 2013). However, it would be incorrect to assume that pseudolysogens form only in starved cells. Moebus reported that mutants of the phage H24 showed pseudolysogenic behavior at high nutrient concentrations and high phage densities (Moebus 1997). Similar results were obtained in other studies performed with phages targeting different hosts such as *E. coli*, *Propionibacterium acnes*, *Campylobacter jejuni* and *Salmonella* Typhimurium, where nutrient supplementation was not limited (Lood and Collin 2011; Cenens, Mebrhatu *et al.* 2013; Kulikov, Golomidova *et al.* 2014; Brathwaite, Siringan *et al.* 2015). Moreover, pseudolysogeny could be favored in certain host strain rather than others. For instance, the ability of ϕ KZ-phages to form pseudolysogens seems to depend on the bacterial strain being infected. Klylov and colleagues showed that the phage SER, a ϕ KZ-like phage, is able to lyse C8-14 strain, a bacterial strain isolated from a CF patient, with higher efficiency than phage ϕ KZ, suggesting that SER could have lost its ability to pseudolysogenize this particular host (Krylov, Miroshnikov *et al.* 2010). These latter results are at odds with a scenario where pseudolysogeny is merely a consequence of nutrient

deprivation leading to a lack of molecular machinery to transcribe and translate phage genetic material.

Collectively all the observations about pseudolysogeny suggest that it can occur in presence of sufficient phage replication machinery and that phages must be actively suppressing the transcription and translation of their own genes. This perspective presents a conundrum. In actively growing cells in high quality environments, pseudolysogenic phages will remain quiescent in host cells and reproduce later. In such situations, phages engaging in reproductive restraint would be at a competitive disadvantage to lytic phages that maximize their own reproductive effort over time. This conundrum may be resolved by the introduction of periodic environmental catastrophes that eliminate all free-living phages, but permit the survival of phages within hosts. In such situations, pseudolysogeny may be a bet-hedging strategy against phage population extinction in the event of a catastrophe (Evans and Dennehy 2005). Bet-hedging theory addresses how individuals should optimize fitness in varying and unpredictable environments by sacrificing mean fitness to decrease variation in fitness (Olofsson, Ripa *et al.* 2009). In the case of pseudolysogeny, some phages in a population enter a protected state, at a cost to their own reproductive effort, in order to recolonize a habitat following an extinction event (Stewart and Levin 1984; Avlund, Dodd *et al.* 2009; Maslov and Sneppen 2015).

A successful pseudolysogenic bet-hedging strategy requires that: 1) some members of the phage population temper their multiplication even during ideal conditions (i.e., induce pseudolysogeny); 2) the probability of pseudolysogeny is a function of the probability of an environmental catastrophe; 3) pseudolysogenic phages must prevent other phages from infecting and lysing these host cells (immunity); 4) reproductive restraint must be of sufficient duration for the host population to recover and for some host cells to be cured from infection.

A similar strategy is commonly observed with temperate phages for which lysogeny operates regardless of ambient conditions (Ptashne 2004; Kobiler, Rokney *et al.* 2005; Oppenheim, Kobiler *et al.* 2005; Amir, Kobiler *et al.* 2007; Joh and Weitz 2011). In the case of phage λ , the set point of the switch between lytic and lysogenic cycle varies with phage and host densities, such that frequency of lysogeny is directly proportional to host and phage density, but inversely proportional to environmental quality. One could imagine that virulent phages follow this strategy as well and that pseudolysogeny is an intermediate stage in a continuum between temperate and fully lytic phages. It is possible to assume that, although virulent phages do not possess the full suite of lysogenic tools (e.g. integrases, excisionases) as do temperate phages, they possess other tools, such as those that induce a transiently quiescent state and provide immunity to superinfection. This subject will be discussed in the following paragraph.

II.II SUPERINFECTION EXCLUSION: A MECHANISM OF BACTERIAL IMMUNITY AGAINST PHAGES

Traditionally, the immunity system of higher animals could be defined as adaptive (specific, acquired or anticipatory) or innate (aspecific and evolutionarily more primitive) (Turvey and Broide 2010). Concerning bacteria, the term immunity is used to describe the capacity to build resistance to a virus after a first encounter. Superinfection exclusion in lysogens can be considered as an adaptive immunity. Another adaptive system which does not rely on the continuous presence of the phage genome is the CRISPR-Cas system and it will be further described in another paragraph.

Superinfection exclusion (Sie) systems are characterized by proteins able to block the entry of phages DNA into the bacterial cells preventing the infection by specific phages. Commonly,

these proteins are encoded by genes located on the prophages sequences integrated in the host chromosome but they can also be carried by virulent phages.

Some of the most well-known and characterized Sie systems are those (Imm and Sp) carried by phage T4 (Lu and Henning 1994). The Imm protein acts at the level of the inner membrane, changing the conformation of the entry site of phage DNA, thus preventing its injection in the bacterial cell (Lu, Stierhof *et al.* 1993). The Sp protein has been shown to be able to block the action of the lysozyme, an enzyme carried on the tail of the phage T4. Lysozyme degrades bacterial peptidoglycan allowing the formation of a hole in the inner membrane and the consequent entrance of phage DNA (FIGURE 5) (Moak and Molineux 2000).

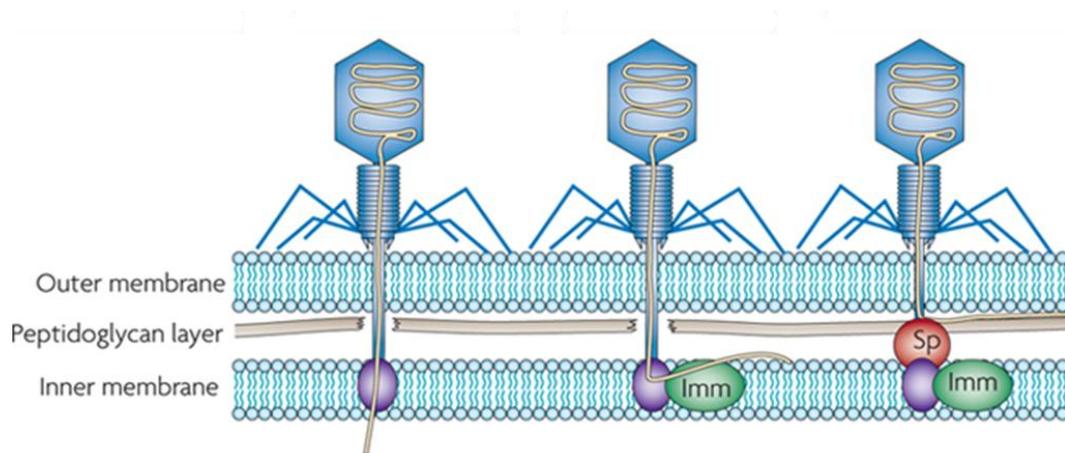


FIGURE 5. SUPERINFECTION EXCLUSION SYSTEM OF COLIPHAGE T4. Normal phage T4 infection of the host cell (left); blocking of T4 DNA injection by the Imm system localized at the bacterial inner membrane (center); blocking of the phage peptidoglycan-depolymerizing activity mediated by the Sp protein (right). Figure extracted and modified from (Labrie, Samson *et al.* 2010).

Figure 6 illustrates some of the mechanisms employed by phages to avoid superinfection by the same phage or other related ones. Temperate *Salmonella* phage P22 and *E. coli* phage P1 have been shown to block phage DNA translocation into host cytoplasm by SieA and Sim systems, respectively, similar to the Imm system of phage T4 (Kliem and Dreiseikelmann 1989; Hofer, Ruge *et al.* 1995). The lytic coliphage T5 encodes a protein called Llp able to

inactivate, through conformational change, the T5 receptor FhuA, making the receptor temporarily unavailable, and consequently the infected cells become resistant to superinfection by the same phage or other phages (Braun, Killmann *et al.* 1994; Decker, Krauel *et al.* 1994). As this mechanism is driven by a lytic phage, Decker and colleagues named it lytic conversion. Similarly, it is known that temperate phages are able to modify the bacterial surface by a mechanism named lysogenic conversion. For instance, the temperate *P. aeruginosa* phages D3 and D3112 have been shown to modify the host LPS and type IV pili, respectively, when lysogenizing the host (Kuzio and Kropinski 1983; Chung, Jang *et al.* 2014).

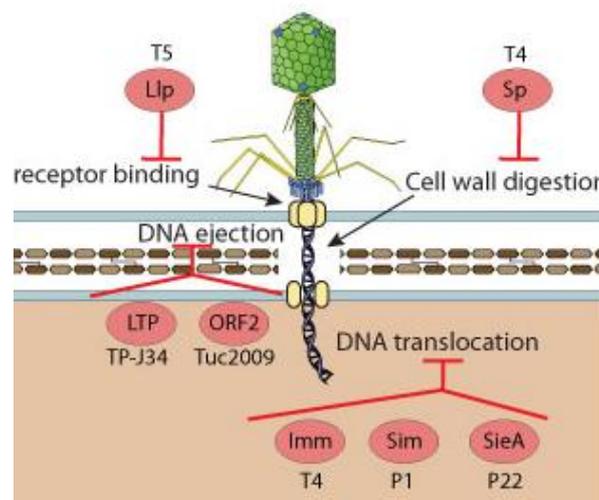


FIGURE 6. EXAMPLES OF SUPERINFECTION EXCLUSION MEDIATED BY DIFFERENT PHAGES. Phage P1 and phage P22 use a Sie system similar to the Imm system of phage T4, blocking DNA entrance modifying the site of phage DNA injection; phage T5 acts by changing the conformation of the phage receptor, impeding the first steps of infection (adsorption); phage T4 also possesses another Sie system, the Sp system, able to block the peptidoglycan degradation, mechanism that the phage uses to open a way through the bacterial wall and to access the inner membrane for injecting DNA. Figure extracted and modified from (http://viralzone.expasy.org/all_by_species/3971.html).

One can imagine that all these mechanisms could act during pseudolysogeny in order to protect pseudolysogens from superinfection by the same phage or others that might infect and kill the cell.

Thus, it is of importance to clarify the molecular mechanisms underlying pseudolysogeny, annotate phage genomes to assign functions to phage ORFans and try to understand their possible involvement in the interaction with the bacterial machinery during the replication cycle.

II.III BACTERIAL RESISTANCE AGAINST PHAGES

Resistance is defined as the bacterial capacity to survive phage infection. It can be accomplished by different mechanisms acting separately, sequentially or in association during the phage life cycle.

Most often, resistant bacteria will be spontaneous mutants that cannot sustain viral infection, and this, in the majority of cases, involves the receptor. Immunity provided by phages is rather specific. Bacteria can protect themselves from invasive DNA using restriction-modification systems or using the CRISPR-Cas system. The latter necessitate that the viral DNA has been encountered previously.

II.III.I MUTATIONS OF PHAGE RECEPTORS

A phage is generally able to infect a narrow host range within a microbial species. Such specificity in interaction of phages with bacterial cells is determined by specificity of adsorption, which in turn is dependent on the nature and structural peculiarities of receptors on the bacterial cell surface (Rakhuba, Kolomiets *et al.* 2010).

Mutations that prevent phage attachment to the cell surface can consist in a lack of receptor expression, alteration or masking of receptors. The bacterial LPS and the extracellular appendages, such as pili or flagella, are good candidates for being recognized as receptors in gram-negative bacteria such as *P. aeruginosa* and, if modified, can constitute a barrier against phage infection (Wilkinson 1996).

THE BACTERIAL LIPOPOLYSACCHARIDE

LPS is a complex polymer made up of monosaccharides and fatty acids. Structurally, it is made of three parts: lipid A, core oligosaccharide and the O-antigen (Wilkinson 1996).

There are two types of LPS: the Smooth (S) type is characterized by typical LPS structure comprising lipid A, core and O-antigen chains, and the Rough (R) type that lacks O-antigen but contains lipid A and the core. Some bacteriophages adsorb to either LPS types. Phages specific for S-type LPS display an extremely narrow host range specificity determined by large variability of O-antigen structure in bacteria of different taxonomic groups. For example, the lytic coliphage G7C possesses a very narrow host range as it is able to recognize and infect only *E. coli* 4s strain by adsorbing on the O-acetylated O-antigen; in absence of this modification the bacterial cells become completely resistant (Knirel, Prokhorov *et al.* 2015). Phages, such as *Salmonella* phage SSU5 (Kim, Kim *et al.* 2014) and *Pseudomonas* phages ϕ CTX and ϕ PLS27 (Jarrell and Kropinski 1981; Nakayama, Kanaya *et al.* 1999), recognizing a conserved structure of the R-type LPS, show a broader host range since the structure of the LPS core is rather conservative in various species and genera of gram-negative bacteria.

Most *P. aeruginosa* strains simultaneously produce two distinct O-antigen chains. One of these is a heteropolymer composed of repeated units of two to five distinct sugars and is termed O-specific antigen (OSA or B-chain). The other form of O-antigen contains a homopolymer of D-rhamnose. Since the latter structure is produced by the majority of *P. aeruginosa* strains, it has been named the common polysaccharide antigen (CPA or A-chain) (Figure 7) (King, Kocíncová *et al.* 2009).

Multiple motifs on the LPS molecule of *P. aeruginosa* have been identified as receptors for various phages. The *P. syringae* phage A7 was found to bind and hydrolyze the D-rhamnose of A-chains in a *P. aeruginosa* mutant strain devoid of B-chains (Rivera, Chivers *et al.* 1992). In contrast, *P. aeruginosa* phage D3 has been shown to specifically adsorb on the B-chains, and lysogenization with this phage resulted in a loss of adsorption with the same phage during subsequent infection cycles (Holloway and Cooper 1962). This was due to alterations in the linkage stereochemistry between B-chains repeat units from $\alpha 1 \rightarrow 4$ to $\beta 1 \rightarrow 4$, resulting in

serotype conversion and a modification of the D-fucose with the addition of an acetyl-group. Lysogenization by D3 also impeded the binding ability of the unrelated LPS-specific phage E79, a PB1-like phage (Kuzio and Kropinski 1983).

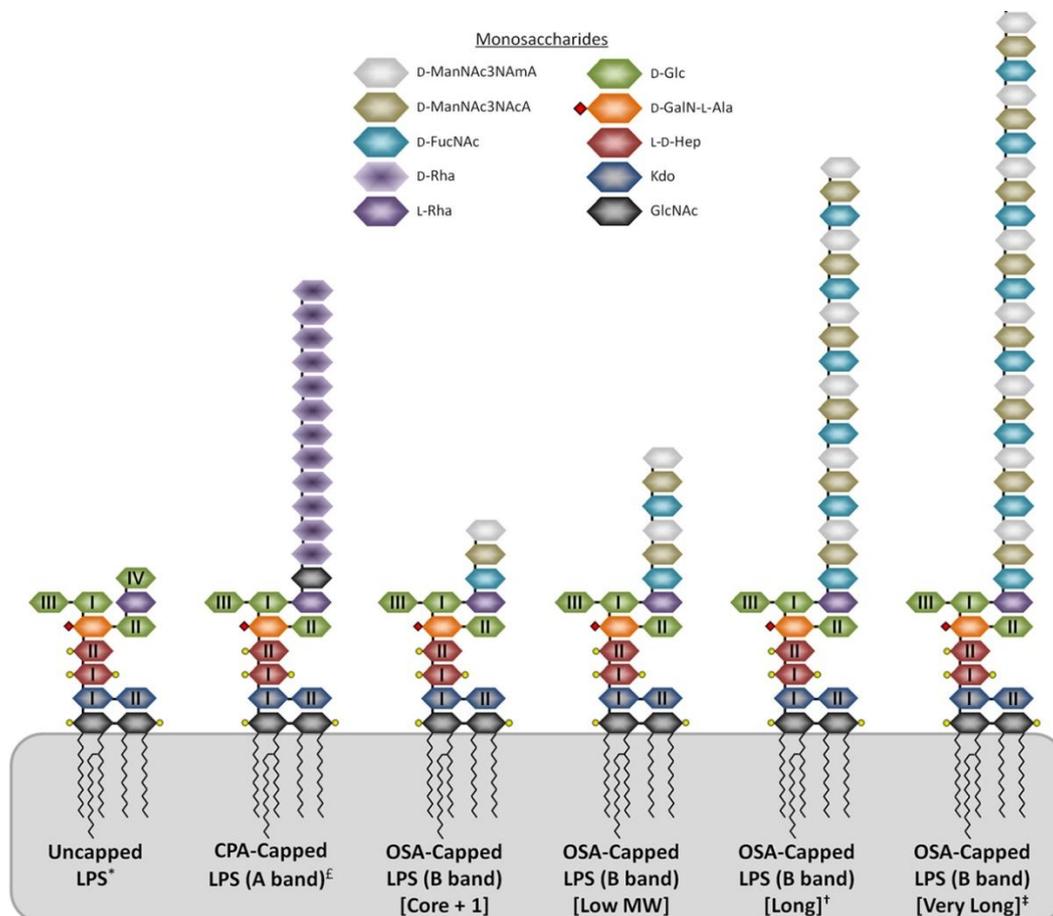


FIGURE 7. DIVERSITY OF SURFACE LIPOPOLYSACCHARIDE GLYCOFORMS IN A SINGLE *P. aeruginosa* CELL. A-chains (or A-bands) are constituted of units of D-rhamnose reported in violet; short, long and very long B-chains (or B-bands) are constituted of repeated trisaccharide units. CPA, common polymeric antigen; OSA, O-specific antigen. Figure extracted and modified from (Lam, Taylor *et al.* 2011).

In *P. aeruginosa* serotype O5 strains such as PAO1, the B-chains are synthesized via the Wzy-dependent pathway, in which several integral inner membrane proteins work together to assemble the mature glycoform in *P. aeruginosa* PAO1 (Burrows, Charter *et al.* 1996). The starting point of LPS biosynthesis is characterized by the addition of the trisaccharide repeat units to the undecaprenyl-pyrophosphate (Und-PP) located in the inner leaflet of the inner membrane; the O-antigen flippase Wzx translocates the Und-PP-trisaccharide linked to the outer leaflet of the inner membrane. Periplasmic polymerization of trisaccharide repeat units

is then mediated by the O-antigen polymerase Wzy and this continues until the chain length regulator proteins Wzz1 and Wzz2 block the polymerization at specific lengths (Figure 8) (Daniels, Griffiths *et al.* 2002). Completed O-antigen chains are ligated to lipid A-core by the O-antigen ligase WaaL to form B-chains LPS (Abeyrathne, Daniels *et al.* 2005).

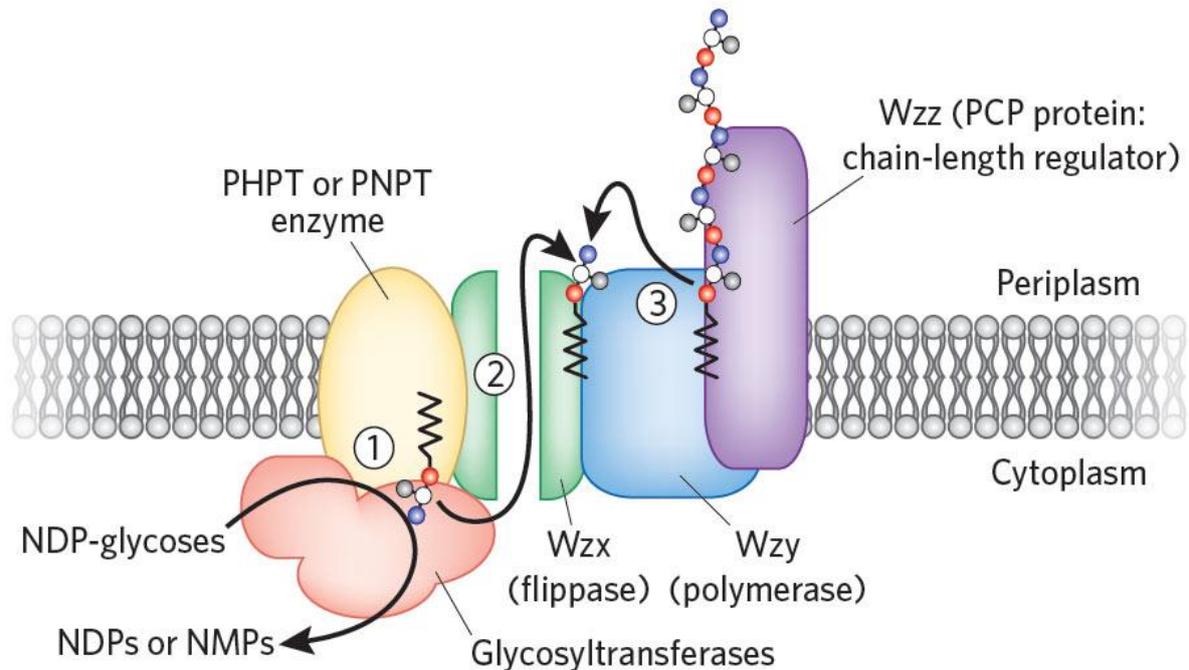


FIGURE 8. SYNTHESIS OF B-CHAINS OF O-ANTIGEN BY THE *Wzy/Wzx* PATHWAY. 1) Biosynthesis of Und-PP by the action of the polyisoprenyl-phosphate hexose-1-phosphate transferase (PHPT) or polyisoprenyl-phosphate *n*-acetylhexosamine-1-phosphate transferase (PNPT) enzyme. The individual repeat unit is completed by glycosyltransferase enzymes. 2) This process is accomplished by Wzx, an integral membrane protein. 3) The polymerization reaction involves the transfer of growing polymer from its Und-PP carrier to the incoming Und-PP-repeat unit; polymerization is catalyzed by Wzy. The final player in the Wzy-dependent pathway is the Wzz protein belonging to the polysaccharide copolymerase family which determines the length of the O-chain. Figure extracted and modified from (Whitfield 2010).

BACTERIAL APPENDAGES: TYPE IV PILI

Pili are one of the most common forms of bacterial surface appendages, involved in adherence and motility, and playing a role in the competence for DNA uptake (Burrows 2012). Four sub-complexes form the major constituents of the type IV pili in *P. aeruginosa* PAO1 (Figure 9). In the outer membrane, a sub-complex composed of the secretin PilQ, an oligomer of 12-14 subunits (Collins, Ford *et al.* 2003; Burkhardt, Vonck *et al.* 2011), and its

pilQ protein PilF, form a pore through which the pilus is extruded (Koo, Burrows *et al.* 2012). In the inner membrane is located a motor constituted of four proteins: PilC forms a platform to which three cytoplasmic ATPases (PilB, PilT and PilU) providing the energy for the extension and retraction of the pilus (Takhar, Kemp *et al.* 2013), are anchored. The inner membrane and outer membrane sub-complexes are connected by the alignment sub-complex constituted of proteins PilM, PilN, PilO and PilP (Sampaleanu, Bonanno *et al.* 2009) and a large protein, FimV possessing a periplasmic and a cytoplasmic domains connected by a transmembrane domain. The outer membrane sub-complex serves as a channel for the extrusion of the fourth sub-complex, the helical pilus fiber composed of the major pilin protein PilA, other minor pilins (FimU, PilV, PilW, PilX and PilE) and the non-pilin protein PilY1 (Nguyen, Sugiman-Marangos *et al.* 2015).

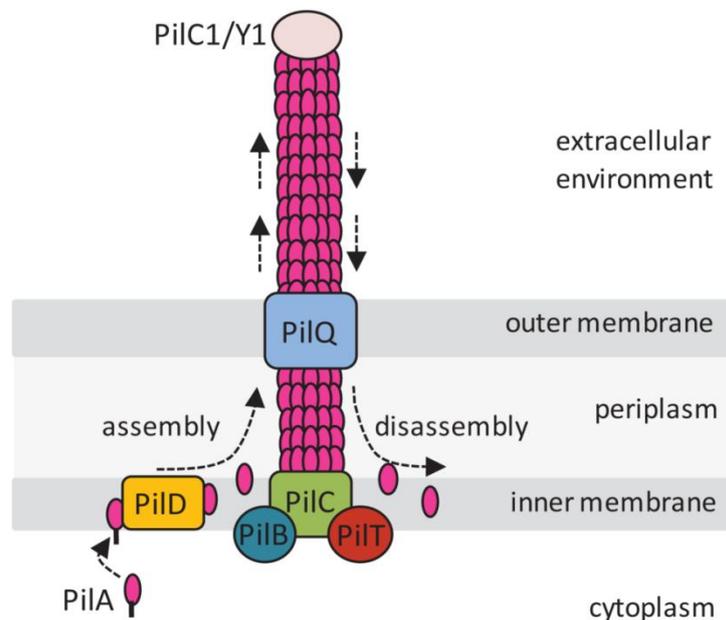


FIGURE 9. TYPE IV PILUS IN *P. aeruginosa*. Only some of the proteins involved in the assembly of the Type IV pili are reported here. Figure extracted and modified from (Burdman, Bahar *et al.* 2011).

Whereas the regulation of the transcription of pilus structural subunits is relatively simple, the mechanism of extension and retraction of the type IV pili is regulated by a complex system similar to that observed for the flagellar chemotaxis system of *E. coli* (Whitchurch, Leech *et*

al. 2004). There are several proteins involved in this regulation including PilJ, localized at the two cellular poles, and involved in the assembly/full extension of the pilus in response to environmental stimuli (DeLange, Collins *et al.* 2007).

Type IV pili have been demonstrated to allow the adherence of *P. aeruginosa* cells to the host epithelium (Bucior, Pielage *et al.* 2012), and this is believed to constitute the first important step of host infection. Type IV pili defective mutants show reduction in the biofilm development as observed for *P. aeruginosa* PAO1 PilA mutants (Klausen, Heydorn *et al.* 2003). It has been shown that type IV pili are also required for a form of surface-associated movement known as twitching motility. Twitching motility is thought to be a consequence of the extension and retraction of type IV pili, which propels the bacteria across a surface (Bradley 1980).

Type IV pilus is known to be the receptor for *Pseudomonas* phages belonging to the ϕ -KMV genus (Chibeu, Ceysens *et al.* 2009) and for some RNA phages, known to induce the pilus retraction after adsorption (Bradley 1972). Mutations in different genes involved in the biogenesis of the type IV pili are shown to affect the bacterial twitching motility but defects in type IV pili are not always associated with inhibition of phage adsorption (Whitchurch and Mattick 1994). The fact that twitching motility is not always associated with phage sensitivity suggests that the latter may not be directly dependent upon fimbrial function but may rather depend on its assembly/disassembly regulation. The *pilT* and *pilU* genes are known to regulate the extension and retraction of the type IV pili, and defective mutants are impaired in twitching on a semisolid surface. PilT and PilU mutants presented different pattern of susceptibility when infected with phages known to adsorb on type IV pilus (Whitchurch and Mattick 1994). Whitchurch and colleagues proposed that this was due to the impossibility for the mutants to retract their pili. The resulting hyperpilated phenotype would allow phage adsorption but not the successive phases of infection, requiring pilus retraction. DeLange and

colleagues made similar observations with PilJ mutants (DeLange, Collins *et al.* 2007). Although impaired in their ability to twitch, the three PilJ mutants were still phage-sensitive. Moreover, the total amount of PilA proteins was comparable with that of the wild-type suggesting that the type IV pili was present but unable to retract (DeLange, Collins *et al.* 2007).

P. AERUGINOSA MUCOID PHENOTYPE

P. aeruginosa is an opportunistic pathogen frequently responsible for mortality in CF patients (Govan and Deretic 1996). Colonization of CF airways by *P. aeruginosa* occurs early in the lifetime of the host and, eventually, a switch to a mucoid phenotype is observed (Mathee, Ciofu *et al.* 1999). This phenotype is caused by overproduction of alginate, an exopolysaccharide consisting of mannuronic and guluronic acid monomers. The expression of the corresponding operon is induced by AlgU, a sigma factor repressed, normally repressed (in non-mucoid conditions), by the MucA protein (Martin, Holloway *et al.* 1993; DeVries and Ohman 1994).

Alginate is thought to have a protective function in a relatively harsh environment in which the bacteria are continually subjected to oxidative stress and attacks by the immune system (Krieg, Helmke *et al.* 1988; Simpson, Smith *et al.* 1988). The switch to a mucoid phenotype is also thought to promote persistence of *P. aeruginosa* in the airways of CF patients, and is usually coincident with a downturn in the prognosis of these patients (Koch and Høiby 1993). Mutations identified in mucoid *P. aeruginosa* isolated from CF patients have been shown to occur in gene *mucA*. The mutations are usually frameshifts that result in the formation of a truncated MucA protein which is no longer able to repress the activity of AlgU (Mathee, Ciofu *et al.* 1999). AlgU is also able to indirectly modulate the twitching motility by controlling the activity of LecB (Bazire, Shioya *et al.* 2010). Sonawane and colleagues

demonstrated that LecB is involved in multiple functions and affects the expression of PilJ (Sonawane, Jyot *et al.* 2006).

The production of exopolysaccharide, including alginate, has been linked to the resistance to phage adsorption in different cases (Ohshima, Schumacher-Perdreau *et al.* 1988; Labrie, Samson *et al.* 2010). Temperate phages, induced from mucoid clinical isolates of *P. aeruginosa*, select for mucoid variants (Miller and Rubero 1984). In another study started as a phage typing experiment, Martin showed that lytic phages could select for mucoid variants growing from the edges of the lytic plaques. The phage-susceptibility of these variants was not completely abolished (Martin 1973). Scanlan and Buckling obtained similar results in a co-evolution experiment using *P. fluorescens* SBW25 and phage $\phi 2$, a lytic phage using LPS as a receptor (Scanlan and Buckling 2012). Garbe and colleagues observed that the *P. aeruginosa* phage JG024, a lytic PB1-like *myovirus* commonly used in cocktail preparation for phage therapy and adsorbing on LPS, infects mucoid *P. aeruginosa* PAO1 mutants with a reduced efficiency (Garbe, Wesche *et al.* 2010).

In all cases the switch from a non-mucoid to a mucoid phenotype provides partial resistance to the phage likely due to the masking and reduction of the amount of LPS receptor available for the phage adsorption.

II.III.II RESTRICTION MODIFICATION SYSTEM

Restriction-modification systems are important components of prokaryotic defense mechanisms against invading genomes. They are generally constituted of two enzymatic activities: a restriction endonuclease and a methyltransferase. The restriction endonuclease recognizes and cleaves foreign DNA sequences at specific sites, while the methyltransferase activity ensures discrimination between self and nonself DNA, by transferring methyl groups to the same specific DNA sequence within the host's genome (Figure 10).

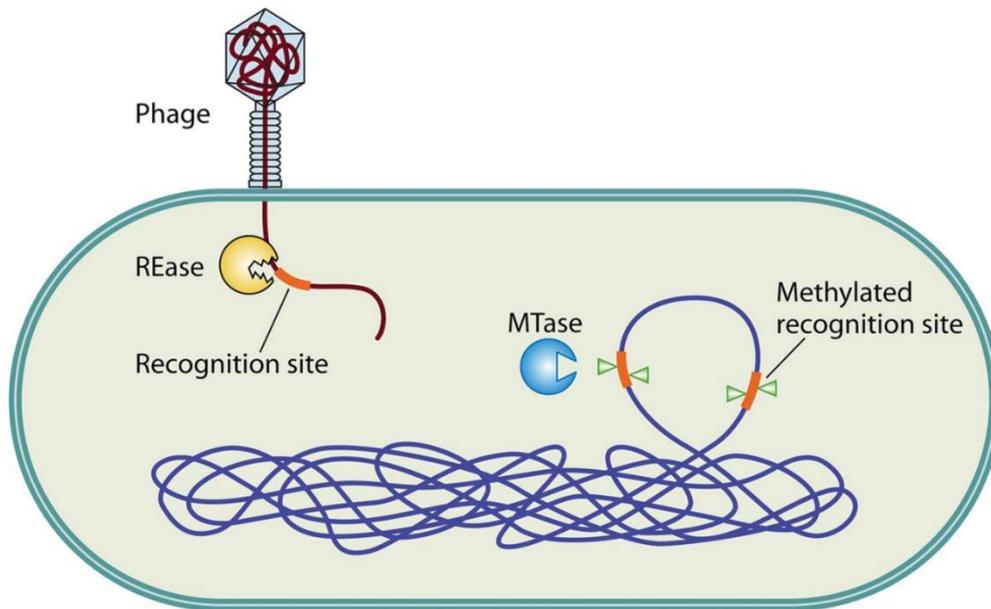


FIGURE 10. RESTRICTION-MODIFICATION SYSTEMS AS DEFENSE MECHANISMS. Restriction-modification systems recognize the methylation status of incoming foreign DNA such as phage genomes. Methylated sequences are recognized as self, while recognition sequences on the incoming DNA lacking methylation are recognized as non-self and are cleaved by the restriction endonuclease (REase). The methylation status at the genomic recognition sites is maintained by the cognate methyltransferase (MTase) of the restriction-modification system. Figure extracted and modified from (Vasu and Nagaraja 2013).

The restriction-modification system could contribute to the colonization by bacteria of a new habitat containing phages (Sneppen, Semsey *et al.* 2015). However, this defense mechanism can be overcome by phages through a number of different kind of anti-restriction systems such as alterations in the orientation of the cleavage site (Meisel, Bickle *et al.* 1992) or incorporation of unusual bases in the DNA (Warren 1980).

II.III.III THE CRISPR-CAS SYSTEM

The prokaryotic system, based on a region of DNA called Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and associated genes called *cas* (Jansen, Embden *et al.* 2002), is capable of targeting DNA as a way of protecting the host against phages and other mobile genetic elements (Fineran and Charpentier 2012; Heler, Marraffini *et al.* 2014; Rath, Amlinger *et al.* 2015). It is a sophisticated “restriction system” which is genetically

programmed and keeps a “memory of past aggressions” (Bolotin, Quinquis *et al.* 2005; Mojica, Díez-Villaseñor *et al.* 2005; Pourcel, Salvignol *et al.* 2005; Barrangou, Fremaux *et al.* 2007; Hale, Zhao *et al.* 2009). The “memory” is ensured by an approximately 30 bp long DNA sequence, called spacer, corresponding to a fragment of the genetic element to be neutralized (Figure 11). New spacers are added to one side of the CRISPR providing a chronological record of the viruses that the bacteria have previously encountered (Pourcel, Salvignol *et al.* 2005). CRISPR activity requires the presence of a set of CRISPR-associated (*cas*) genes, usually found adjacent to the CRISPR (Jansen, Embden *et al.* 2002).

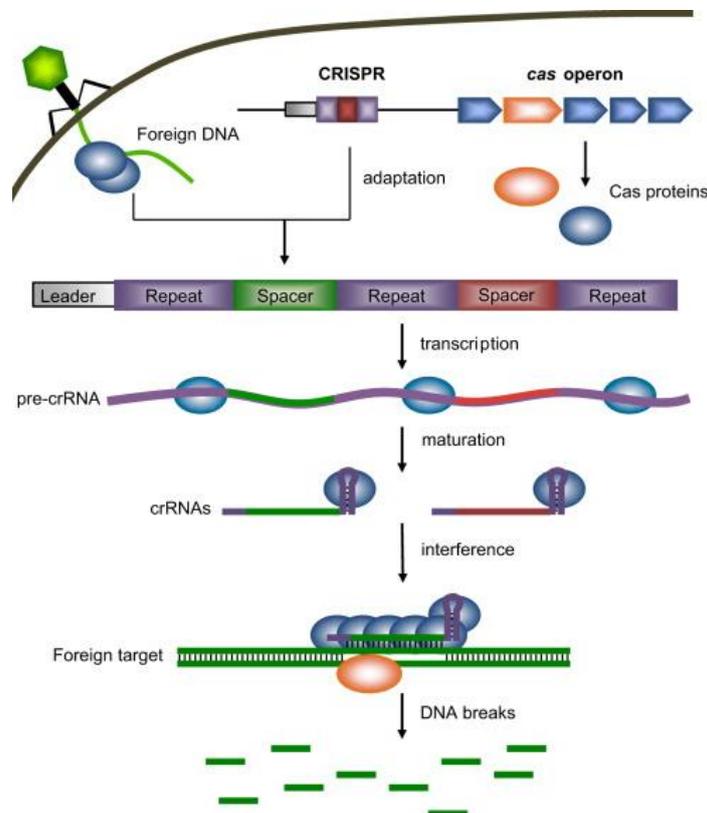


FIGURE 11. OVERVIEW OF CRISPR-Cas IMMUNITY TO VIRUSES OF BACTERIA AND ARCHAEA. During adaptation, the phage genome is recognized by cas proteins and a short sequence of the phage DNA (termed a protospacer) added to the leader end of the CRISPR array, resulting in a new spacer sequence and a duplicated repeat. Transcription of the CRISPR array from a promoter within the leader sequence results in a precursor CRISPR RNA (pre-crRNA) transcript. The pre-crRNA is matured into individual crRNAs by a process involving Cas proteins. The mature crRNAs form a ribonucleoprotein complex, which targets nucleic acids that are complementary to the spacer sequence in the crRNAs. Figure extracted and modified from (Fineran and Charpentier 2012).

The CRISPR-Cas mediated defense process can be divided into three stages: adaptation, characterized by the insertion of new spacers in the CRISPR locus; expression, during which the CRISPR is transcribed into a long precursor CRISPR RNA (pre-crRNA) subsequently processed into mature crRNA by Cas proteins; interference, during which the target nucleic acid is recognized and destroyed by the combined action of crRNA and Cas proteins (Figure 11).

The adaptation phase provides the genetic memory that is a pre-requisite for the subsequent expression and interference phases that neutralize the re-invading nucleic acids. The interference phase is currently the focus of intense technological developments worldwide, as the capacity to cut a genome in a programmable way at multiple places simultaneously opens unprecedented opportunities in terms of genome editing (Jiang and Marraffini 2015).

Two different CRISPR-Cas systems have been observed in *P. aeruginosa* strains. Several studies have shown that in *P. aeruginosa*, CRISPRs carry mostly sequences of temperate phages (Cady, White *et al.* 2011; Essoh, Blouin *et al.* 2013). Essoh and colleagues showed that there was no link between natural resistance to multiple phages and the presence of CRISPR-cas systems (Essoh, Blouin *et al.* 2013)

GOAL OF THE WORK

The main goal of this work was to characterize the resistance mechanisms active in *P. aeruginosa* against virulent bacteriophages and to evaluate the frequency of phage-tolerant variants emergence. We took advantage of the relatively low cost of Next Generation Sequencing (NGS) in order to identify the mutations responsible for the resistance in an experimental design reflecting real-life situation. As previously reviewed, many pathways associated with resistance have already been precisely characterized and resistant transposon mutants could be easily designed or ordered from mutant repositories. In the present work, the natural mutation responsible for resistance was identified by whole genome sequencing, used as an almost first-line assay, with no *a priori* on the underlying mutations.

In the context of phage therapy, only virulent phages can be employed but it is interesting to question the behavior of the bacteria when challenged with different kind of viruses such as temperate phages. Temperate phages are well-known to be able to strongly modify the bacterial phenotype, influencing their virulence against, for example, the human host in the case of human pathogens. This is related to the capacity of these phages to integrate their genome into the bacterial chromosome or to transfer genetic material, and these are two of the main reasons that impede the use of temperate phages in the preparation of therapeutic cocktails.

In order to provide additional evidences corroborating this assumption we performed a study on the selection of phage-tolerant variants by the temperate phage Ab31. The different phenotypes of phage-tolerant bacteria obtained after infection with Ab31 will be described here and detailed results are presented in ANNEX II (Latino, Essoh *et al.* 2014). Phage Ab31 was isolated in Abidjan in the course of the study detailed in the ANNEX I (Essoh, Latino *et al.* 2015). From the same study, other phages were isolated and some of the virulent ones will

be described in the following paragraphs as they were later employed for selecting phage-tolerant variants analysed in the present work and whose published results are reported in the ANNEX III (Latino, Midoux *et al.* 2016).

Globally considered, all the results presented in this work will provide an idea of the complex interactions established between phages and bacteria and how the presence of phages with different behavioral characteristics can similarly shape the bacterial phenotype in a given environment.

RESULTS AND DISCUSSION

Characterization of phage diversity in Abidjan and in-depth analysis of Ab31, a novel chimeric virus

Given the importance of their distribution worldwide, a number of studies have been performed to understand the influence that phages can have on the evolution of microbial communities (Díaz-Muñoz and Koskella 2014). However, few have been devoted to the study of the phage diversity in a given environment.

ANNEX I (Essoh, Latino *et al.* 2015) presents the work we performed in order to analyse the *P. aeruginosa* phage diversity in a given geographical region: several different phages were isolated from sewage water from five locations in the city of Abidjan, (Côte d'Ivoire) over a two-years-period, using a collection of *P. aeruginosa* strains with diverse genotypes. In total 22 phages were isolated and distributed in different phage genera according to their genomic characteristics. Seventeen phages were shown to be virulent and were distributed into seven different genera, while five temperate phages were distributed into three genera. When tested on a panel of different *P. aeruginosa* strains derived from CF patients, virulent phages belonging to the same genus shared a similar host range. In contrast, the more diverse temperate phages showed differences in the host spectrum. This study showed that the phage diversity in a given location can be very high. A few isolation experiments using a large panel of bacterial strains allowed the isolation of virulent phages that belong to genera commonly employed for the constitution of cocktails for therapeutic use and covering a large host spectrum.

Several phages have been included by Kropinski in newly described genera, such as Ab01 (PAKpunavirus), Ab03 (Kpp10virus), Ab28 (Pbunalikevirus), Ab26 (Septima3virus) and Ab18 (Ab18virus).

I SELECTIVE PRESSURE IMPOSED ON *P. AERUGINOSA* BY A

TEMPERATE PHAGE

Among several temperate bacteriophages isolated by Essoh et al., ANNEX I, (Essoh, Latino *et al.* 2015), Ab31 was characterized in more detail because it did not resemble any previously isolated bacteriophage (Latino, Essoh *et al.* 2014).

I.I CHARACTERISTICS OF PHAGE AB31

Electron microscopy performed on phage Ab31 showed that it belongs to the *Podoviridae* family sharing morphological characteristics with a virulent *P. putida* phage, phage AF (Figure 12) (Cornelissen, Ceysens *et al.* 2012).

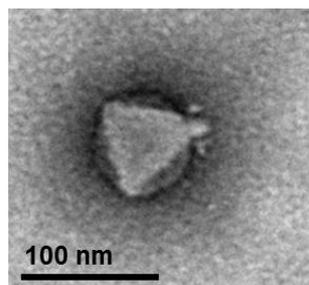


FIGURE 12. ELECTRON MICROSCOPY OF PHAGE Ab31. The phage is constituted of an icosahedral head and a short tail with tail spikes.

The plaques formed by phage AF on *P. putida*, are surrounded by a halo due to an exopolysaccharide-degrading activity carried by the tail spikes and able to cleave the O-antigen of the host LPS (Cornelissen, Ceysens *et al.* 2012). Despite its morphological similarity with phage AF, the plaques formed by phage Ab31 were not surrounded by such a halo when the phage was spotted on *P. aeruginosa* susceptible strains, and sequence alignment of the tail spike protein of both phages showed similarities only in the N-terminal part. Conservation of the N-terminal part is necessary for association of the spikes with the tail structure, whereas the C-terminal part of the spike protein is involved in recognition and binding to the host surface. Thus, it illustrates the high

level of variability of tail spikes in phages belonging to the same genus (Steven, Trus *et al.* 1988; Cornelissen, Ceysens *et al.* 2011) reflecting adaptation to hosts with different surface components.

Looking at the Ab31 genome sequence it was possible to distinguish two main modules (Figure 13).

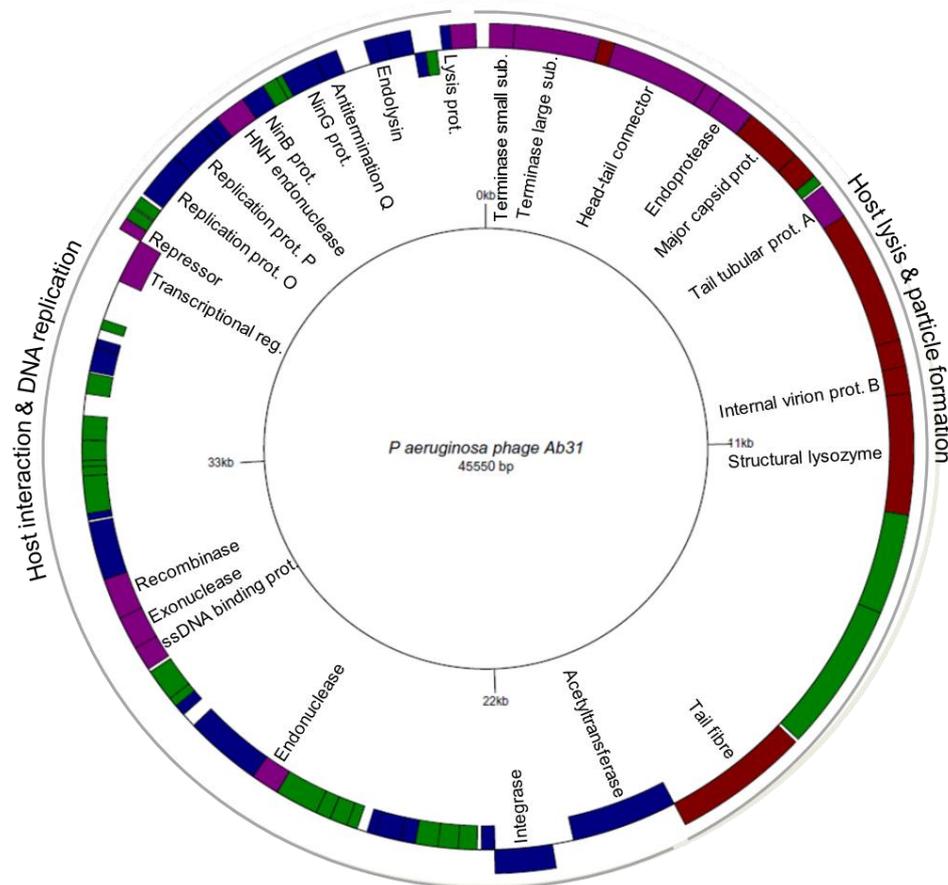


FIGURE 13. ANNOTATION OF THE Ab31 GENOME. The Ab31 hypothetical terminal ends are at the 1 position. The morphogenesis module includes proteins similar to those encoded by phage AF (red), whereas the replication, recombination and lysis modules consist of genes similar to those of phage PAJU2 (blue). Genes encoding hypothetical proteins which have homologies with other phages are shown in purple color. Genes encoding hypothetical proteins of unknown function are shown in green color.

The first region, showing homologies with the AF phage genome, covered the so-called late region and contained sequences encoding the structural proteins of the phage, such as those for capsid, tail-to-head connector, tail and tail spikes. The second Ab31 genomic region encoded proteins involved in recombination and replication of the phage genome, constituting the so-called early/middle region. This region contained several genes that showed similarities with those of PAJU2 (Uchiyama, Rashel *et al.* 2009), a temperate phage of *P. aeruginosa*, suggesting that, although

phage Ab31 showed a morphology similar to the virulent AF *podovirus*, it may behave as a temperate phage capable of lysogenizing *P. aeruginosa* strains.

I.II BACTERIAL RESISTANCE AGAINST PHAGE AB31

Phage Ab31 displayed a very narrow host range, being able to specifically infect the reference strain PA14, and Tr60 and Tr162, two strains genetically related to PA14, isolated from French CF patients in the same hospital. Several other strains possessing the same O-serotype or belonging to the PA14 clonal complex but isolated at other locations were found to be resistant to Ab31.

In order to search for potential lysogens and evaluate the genotypic and phenotypic impact of infection on susceptible *P. aeruginosa* strains, some Tr60 and PA14 phage-tolerant variants were isolated and their genomes sequenced. The parental Tr60 strain was also sequenced and its genome assembled and compared with that of PA14. In Tr60, two genome modifications were identified: a deletion of 11 kbp corresponding to the prophage Pf1 sequence and a second deletion of 22 kbp shown to encode in PA14, among other proteins, a resolvase and a recombinase, commonly known to participate in DNA transfer.

The genomes of two sequenced Ab31-tolerant variants (Tr60-10A and PA14-P1), suspected to contain phage DNA when analysed by PCR, surprisingly did not contain phage reads. A possible explanation could be that the phage genome did not integrate into the bacterial chromosome but was retained in a small proportion of cells after infection, representing what Ackermann and DuBow called a carrier state (Ackermann and DuBow 1987). Indeed, there is evidence that some of the putative lysogenic bacteria lost the phage genome after several replatings. In contrast, Tr60-100B was shown to possess Ab31 genome stably integrated into its chromosome as a true lysogen. The phage DNA integrated through a site-specific recombination process using a shared 64 bp sequence, as shown for PAJU2 (Uchiyama, Rashel *et al.* 2009).

In the course of the same study, mucoid variants of *P. aeruginosa* Tr60 and PA14 were obtained after infection of the host in liquid medium. These variants were stably resistant to the phage, although they were not lysogenic. Miller and colleagues showed that temperate phages with elongated heads and flexible tails (similar to PAJU2 virion), induced from CF-associated *P. aeruginosa* strains, were capable of converting non-mucoid strains to the mucoid phenotype (Miller and Rubero 1984). Sequencing of two Tr60-tolerant variants (Tr60-10A and Tr60-100A) with mucoid phenotype, revealed a deletion of 234 kbp identical in both variants. This region encodes several proteins that could be involved, directly or indirectly, in the regulation of alginate expression responsible for conversion to a mucoid phenotype (Wozniak and Ohman 1994; Qiu, Eisinger *et al.* 2007).

Notably, the large deletion of 234 kbp included genes encoding proteins necessary to assemble a fimbrial organelle. This gene cluster which encodes components of the chaperone-usher pathway and a fimbrial unit, participates in biofilm formation (Ruer, Stender *et al.* 2007; Vallet-Gely, Sharp *et al.* 2007). Fimbriae could be involved in phage adsorption but further investigation is required to test this hypothesis. The deletion also contains two porins, and one member of the LamB/YcsF family protein. Previous studies have shown that an outer membrane porin encoded by the *ompLC* gene in *Edwardsiella ictaluri* is required for phage sensitivity (Hossain, Rahman *et al.* 2012), while LamB is the receptor for *E. coli* bacteriophage λ . LamB was shown to be sufficient to confer λ phage sensitivity upon transformation of the *lamB* gene into bacteria of different species (Randall-Hazelbauer and Schwartz 1973). In conclusion, a number of proteins encoded by this deleted region might be responsible for the adsorption of phage Ab31 on the host surface and more work is necessary to identify the receptor for this phage.

It is surprising that two seemingly independent Tr60-tolerant variants possessing the large deletion were selected after infection with the phage. Two hypotheses could be formulated to explain the origin of the deletion. The first hypothesis is that a variant subpopulation with the 234 kbp deletion preexisted in the Tr60 stock suspension, and that the phage infection led to its selection. Performing

PCR on about 100 isolated colonies or on the total DNA extracted from the Tr60 stock culture did not show any sign of these variants as preexistent. Another possible explanation involves the direct action of the phage on the bacterial chromosome. This hypothesis is supported by the finding that at both ends of the 234 kbp region present in the original Tr60 strain, there are sequences of 10 bp in length (ctcggcatga and ctcggcgatga) that differ by a single nucleotide insertion (Figure 14). Notably a similar sequence (c-cggcatga) was detected in the phage Ab31 genome at the end of the gene encoding an acetyl-transferase, upstream of the phage integrase (Figure 14). The 10 bp sequence “ctcggcgatga” constitutes the junction of the deleted region on the resistant bacterial genome (Figure 14). Moreover, the sequence upstream the 234 kbp region encodes several proteins involved in transposition, including a bacterial transposase. This suggests that the origin of the deletion in Tr60 was a recombination and/or transposition event in which the phage was also involved.

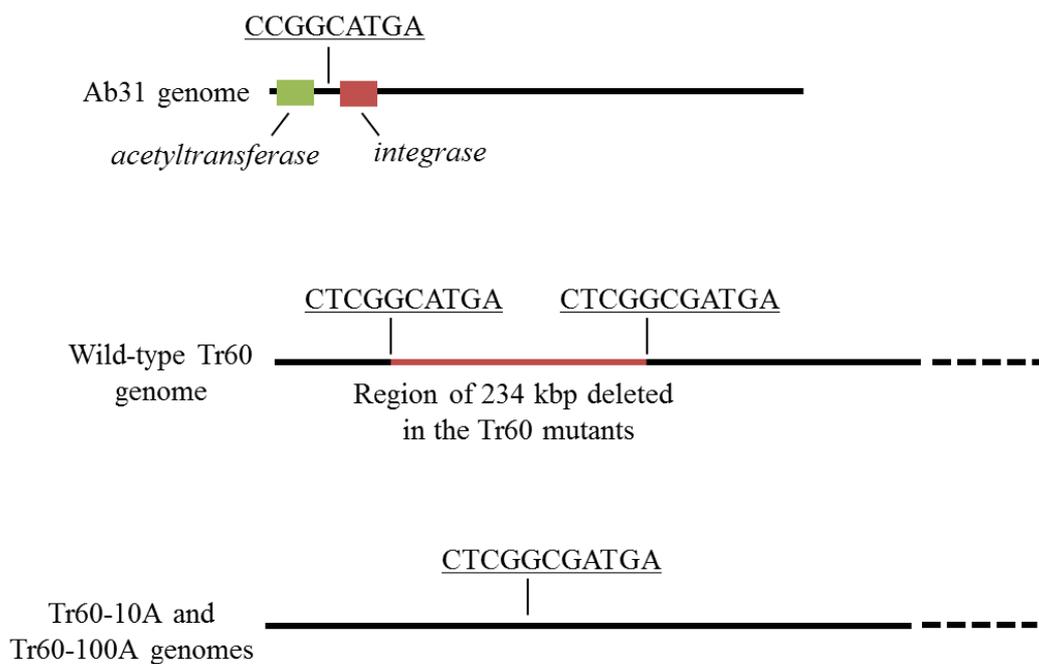


FIGURE 14. SIMILAR SEQUENCE FOUND IN Ab31, Tr60, Tr60-10A AND Tr60-100A GENOMES. In Ab31 genome this sequence is positioned between the acetyltransferase and integrase encoding genes; in Tr60 it could be found at the two flanking ends of the 234 kbp region, deleted in Tr60-10A and Tr60-100A; in the two Ab31-tolerant mutants, Tr60-10A and Tr60-100A a unique sequence was found where the 234 kbp region was missing.

Large genomic deletions have been observed during early stage adaptation of *P. aeruginosa* in CF patients, but none were as large as 234 kbp, which represents about 3.6% of the genome (Cramer,

Klockgether *et al.* 2011; Rau, Marvig *et al.* 2012). Rau and colleagues described a deletion of 148 kbp, encompassing the *cupA* cluster (Rau, Marvig *et al.* 2012). It is not known whether the presence of phages could play a role in the induction of such deletions. Ab31-tolerant strain PA14-P1 showed no deletion corresponding to that which characterized Tr60-10A and Tr60-100A. In contrast, a number of indels were observed in different genes, but at this time it is impossible to know which one is responsible for phage resistance.

I.III AB31 IS A CHIMERIC PHAGE

Phages AF and PAJU2 infect *P. putida* and *P. aeruginosa*, respectively. These bacterial species are closely related, and phage genome exchanges probably occurred during infection of a lysogenic host by a virulent phage similar to AF. As a result of their mosaic structure, some temperate phage genomes can migrate between unrelated bacteria (Krylov, Kropinski *et al.* 2012). Although genetically distant, phages AF and PAJU2 share a lambdoid genome organization which could favor genetic replacement (modular exchanges of gene blocks) (Brüssow and Hendrix 2002; Casjens 2005). Similar events seem to occur between D3112-like phages morphologically identical to phage lambda, and the transposable coliphage Mu belonging to the *Myoviridae* family (Braid, Silhavy *et al.* 2004). Several types of recombination events are thought to build phage genomes. Conserved sequences exist at gene boundaries that could serve to target homologous recombination at these positions, via transposition or site-specific recombination (Hatfull and Hendrix 2011). However a major contributor to phage genome building is illegitimate recombination, or recombination between short conserved sequences (a few bases), coupled with functional selection of genes (Juhala, Ford *et al.* 2000; Hatfull and Hendrix 2011).

CONCLUSION

This work revealed diverse aspects of phage/bacteria and phage/phage interactions and open the way to new investigations. Ab31 receptor is still unknown and it would be interesting to check whether Ab31 can induce large deletions in other experimental contexts.

II TOLERANT VARIANTS SURVIVING INFECTION WITH VIRULENT PHAGES

II.I RATIONALE AND EXPERIMENTAL PROCEDURE

In CF patients, failure of treatments of chronic infections is mainly due to the ability of *P. aeruginosa* to persist in a biofilm on the lung epithelial cell surface. There, the bacteria are embedded in an extracellular matrix constituted of polymers, proteins and DNA, which protects the bacteria not only from the antibiotic treatments, but also from the attack of innate or acquired immune system, (Høiby, Ciofu *et al.* 2010). When phage therapy is used against *P. aeruginosa* infection *in vivo*, the bacteria are adsorbed on a surface and display little movements. In these conditions, low phage titers can be employed thus preventing the sudden killing of a large proportion of bacteria and the release of high amount of toxins, such as LPS, that could induce a severe inflammatory response in the patient, aggravating the prognosis. The amount of phages will be sufficient to allow multiplication in the host, release of new phages and infection of adjacent bacterial cells. Moreover, as previously discussed in the “INTRODUCTION”, the combined use of phages targeting different receptors may help lowering the frequency of resistant variants appearance. Therefore, in this study, we have tried to reproduce the conditions of phage therapy in order to evaluate the frequency and characteristics of bacterial resistance.

II.I.I GENETIC DIVERSITY OF PHAGES USED IN THIS STUDY

In order to evaluate possible differences in the frequency of resistant variants appearance, four different virulent phages displaying various host ranges (Essoh, Latino *et al.* 2015) and using different receptors were used, alone or in combination of two or four.

We selected four phages that shared the ability to grow and kill a common host, *P. aeruginosa* strain PAO1: phages Ab05 (ϕ -KMV-like *podovirus*), Ab09 (LIT1-like *podovirus*), Ab17 (KPP10-like *myovirus*) and Ab27 (PB1-like *myovirus*). Among the other phages we isolated, those belonging to the PAK-P1 genus were not able to grow on PAO1. Therefore, although employed in the preparation of therapeutic cocktail, we could not include a PAK-P1 representative in the study presented in ANNEX III (Latino, Midoux *et al.* 2016).

The genome organization of these four phages resembled that of their respective phage genera.

PHAGE AB05

As the other phages belonging to the ϕ -KMV genera, phage Ab05 presents a well-defined genome organization divided in three main parts: early genes, metabolic genes ending with a gene encoding an RNA polymerase (ORF31) and late genes, constituted of sequences encoding for virion proteins and the lysis cassette. This cassette formed of a pinholin (ORF49), endolysin (ORF50), and spanins (ORFs 51–52) is similar to the one described in other ϕ -KMV-like phages (Briers, Peeters *et al.* 2011). As reported for ϕ -KMV-like phages, the phage RNA polymerase is responsible for the transcription of genes encoded in the late region. The RNA polymerase is preceded by ORF 30 that showed a partial similarity with the bacterial RNA polymerase inhibitor found in ϕ -KMV-like phages by Klimuk *et al.* (Klimuk, Akulenko *et al.* 2013).

PHAGE AB09

Phage Ab09, similarly to other LIT1-like phages, encodes a virion-encapsulated RNA polymerase (ORF66) which allows transcription of early genes, and a second type of T7-like heterodimeric RNA polymerase (ORF18 and ORF19). Like phage LIT1, it carries the

rIIA/rIIB lysis inhibition cassette (ORF38 and ORF39), which is widely distributed among the genomes of phages infecting different host genera (Kushkina, Tovkach *et al.* 2013; Wittmann, Dreiseikelmann *et al.* 2014). This system enables a delay in the host lysis, providing this phage with the ability to produce phage particles in high amount compared to other phages, thus constituting a competitive advantage over the others. If this type of phage is integrated within a therapeutic cocktail product, it could be important to perform propagation separately, otherwise the phage might dominate the phage population. However, the role of such a cassette remains uncertain and several additional roles have been attributed to it. It may be involved in the host resistance mechanism of the abortive infection system, during which the phage can enter the bacterial cell but cannot complete its multiplication cycle (Labrie, Samson *et al.* 2010).

PHAGE AB17

Like all KPP10-like phages isolated in Abidjan in the course of this study, Ab17 possesses three tRNA, and a genome organization similar to phage KPP10. Interestingly, these phages present zones of variability probably due to horizontal genes transfer or recombination interspersed in highly similar genomic segments. Several regions of short insertion/deletions can be observed between the different phages, sometimes resulting in the fusion of two putative ORFs. Phage Ab17 presents the highest level of genome variability. In particular it lacks a 3271 bp region encompassing seven hypothetical ORFs (ORF34 to ORF40 in phage KPP10), and perfectly conserved in the other KPP10-like phages isolated in Abidjan. As a result, a putative RNA ligase (ORF91 in Ab17) is formed by the fusion of the beginning of ORF89 and ORF97.

PHAGE AB27

In keeping with other phages of the PB1 genus (Ceysens, Miroshnikov *et al.* 2009), the Ab27 genome does not encode an RNA polymerase, thus transcription relies entirely on the host machinery. The genome encodes a DNA polymerase III alpha-subunit that catalyses the polymerization reaction of the host DNA polymerase III holoenzyme (Kelman and O'Donnell 1995). The presence of phage-encoded DNA primase and helicase suggests that viral dsDNA elongation is performed by the phage, independently of the host replication machinery. Interestingly between ORF60 and ORF61, Ab27, as other PB1-like phages, carries a 30 bp sequence (GATGCCCCGGCGAACCGGGGCGGGGTGGTT) that is commonly found in several *P. aeruginosa* strains as a spacer in the CRISPR elements. The CRISPR-Cas system plays a role in *P. aeruginosa* resistance to bacteriophages and plasmids (Cady, White *et al.* 2011), but is usually not associated with resistance to virulent phages.

II.I.II CHOICES ABOUT THE INFECTION CONDITIONS

We sought to test bacterial resistance to virulent phages in conditions that mimic the *in vivo* situation, and thus decided to perform phage infections on solid agar plates instead of liquid cultures, more commonly employed in the investigation of bacterial resistance. This should allow us to isolate different resistant variants that do not derive from a single parental clone, a situation commonly observed when isolating resistant variants from infections performed on planktonic cultures. Specifically, a planktonic *P. aeruginosa* PAO1 culture named PAO1_{Or}, where “Or” stands for Orsay, the site where our laboratory is located, was infected at a low MOI of 0.1 (one phage for ten bacteria) and kept for 15 min at room temperature before plating in soft agar on LB solid medium, thus restricting diffusion of bacteria and newly produced phages. PAO1_{Or} was grown from a single PAO1 colony.

II.I.III PHAGES COMBINATIONS

Double infections were designed (Figure 15) by coupling a *myovirus* and a *podovirus* with different receptors and similar plaque morphology, in order to possibly avoid the overgrowing of one phage to the detriment of the other and, thus, the preferential selection of variants resistant to only one phage.

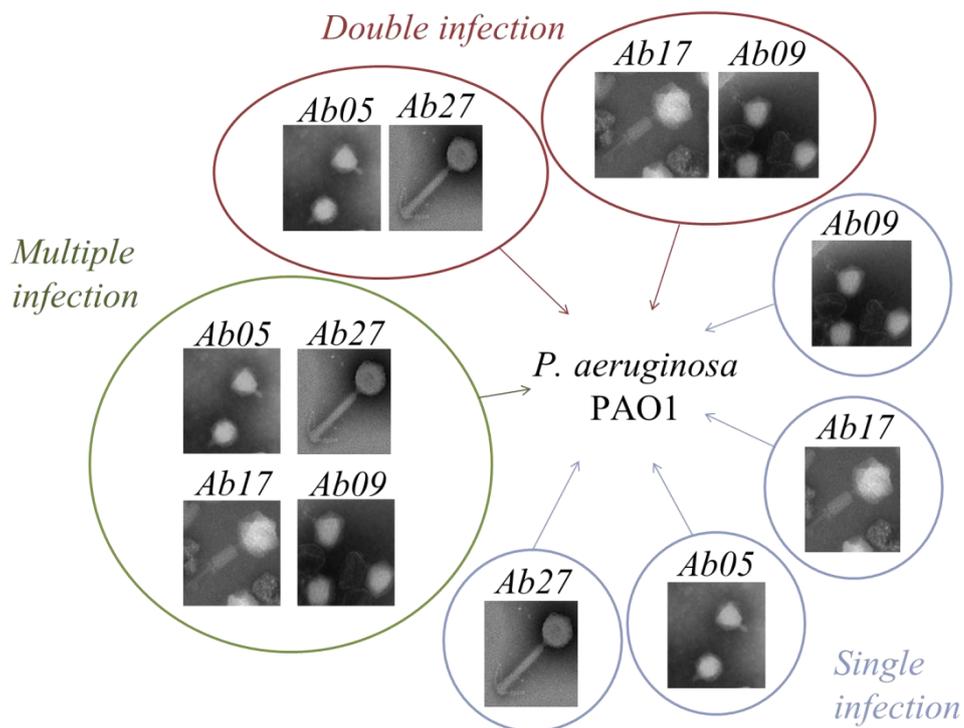


FIGURE 15. DIFFERENT KINDS OF INFECTIONS PERFORMED IN THIS STUDY. Single phage infections (blue circles), double phage infections (red circles) coupling a *myovirus* Ab27 or Ab17 with a *podovirus* Ab05 or Ab09, respectively and multiple phage infection (green circle) performed by mixing the four different phages in a 1:1:1:1 ratio. All the infections were performed at an MOI of 0.1.

Both Ab09 and Ab17 produced clear plaques on PAO1_{Or}, whereas Ab27 gave tiny and turbid plaques, and Ab05 produced more or less clear plaques with a halo (Figure 16).

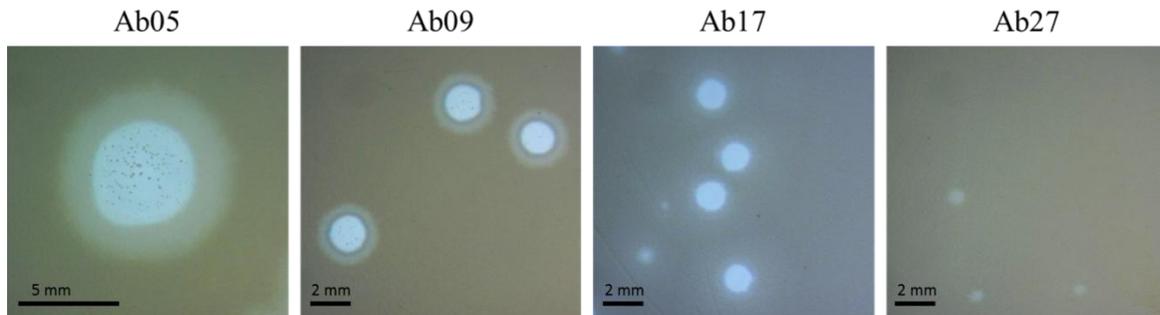


FIGURE 16. PLAQUES PRODUCED ON PAO1_{Or} BY THE DIFFERENT PHAGES USED FOR THIS STUDY.

As discussed in the “INTRODUCTION”, phages belonging to the ϕ -KMV genus, with some exceptions, are known to target the type IV pilus as a receptor (Chibeu, Ceysens *et al.* 2009). In order to confirm that phage Ab05 also requires type IV pili for infection, we tested the phage on a panel of four different type IV pilus PAO1 transposon mutants (PilA, PilR, PilQ and PilY1) and all of them were shown to be completely resistant to the infection performed with this phage.

In contrast, phages Ab09, Ab17 and Ab27 are known to belong to phage genera whose members target LPS as receptor to initiate the first steps of infection. Indeed, we showed that a PAO1 transposon mutant for the *algC* gene, known to encode a key enzyme that provides sugar precursors for the synthesis of alginate, LPS and other exopolysaccharides (Ma, Wang *et al.* 2012), thus completely devoid of LPS, was completely resistant to infection performed with the three phages. We decided to couple phage Ab09 and phage Ab17 on one end and phage Ab05 and phage Ab27 on the other end, to perform the double infections illustrated in Figure 15.

II.II PHENOTYPE OF PHAGE-TOLERANT VARIANTS

Using the approach described in “MATERIAL AND METHODS”, we were able to calculate the frequency of phage-tolerant variants for all the infection experiments we performed. Phage-

tolerant variants arising after single phage infection were recovered at a frequency of 3.2×10^{-5} . The frequency at which phage-tolerant variants were recovered after double or multiple infections was 4×10^{-6} and 3.8×10^{-6} , respectively. We counted the number of phage-tolerant colonies growing on the plate 24 h after infection, divided by the total number of bacteria used for the infection. At this step, named P1 (first plating), we were not able to confirm the stability of the bacterial resistance to the phage. For this reason, we refer to them as tolerant, tolerance being defined as the capacity to survive the phage infection, whatever the mechanism the bacteria use as a defense. In general, we will name our variants phage-tolerant variants until the mechanism of resistance is established. We will refer to tolerant variants as resistant only if the change involves alterations of the bacterial surface, consequently impeding phage adsorption.

The majority of PAO1_{Or} variants recovered after Ab05, Ab09 and Ab17 single, double or multiple infections (32 variants) were tolerant to at least one phage. In contrast, none of the surviving bacteria (ten variants) recovered from infections with Ab27 alone turned out to be stably tolerant to Ab27. To test if phage Ab27 may select more easily for bacteria growing in planktonic culture, we performed liquid infections using different parameters such as temperature (30°C, 37°C, 42°C), MOI (0.1, 0.01, 0.001), infection timing (during bacterial exponential or stationary phase growth) or infecting the same culture with the phage every 24 h, for a total of three infections (72 h culture). All our attempts for isolating Ab27-tolerant variants at P3 stage were unsuccessful.

II.II.I COLONY MORPHOLOGY OF PHAGE-TOLERANT VARIANTS ON DIFFERENT MEDIUM

For further studies we retained and analysed 32 variants shown to be stably tolerant to at least one of the four phages used in this work. In order to get additional information on their growth and metabolism we cultured these variants on a different medium, the Sheep Blood

agar, and looked at the colony morphology. We confirmed the existence of new phenotypic characteristics compared to the parental PAO1_{Or} strain, not only in terms of growth characteristics, but also in terms of β -hemolytic activity. The β -hemolysis is usually tested by plating the bacteria on Sheep Blood agar plates (5%) and observing the edge of the colonies. Various species of streptococci, gram-positive bacteria, have been shown to carry this activity due to the production and secretion of streptolysins, peptides able to lyse the red blood cells (Nagamune, Ohnishi *et al.* 1996). Variants PAO1-02, PAO1-13 and PAO1-17 lacked the hemolytic ability displayed by the wild-type PAO1_{Or} but, interestingly, the colonies of PAO1-13 presented zones of reversion to the wild-type phenotype (Figure 17A).

A mucoid phenotype was stably observed for PAO1-02, PAO1-06 and PAO1-13 (Figure 17B). PAO1-17 was particularly interesting as it continuously produced three types of colonies on solid LB media, some being mucoid, some with a smooth appearance as seen in the control PAO1_{Or} strain, and others with irregular transparent appearance (Figure 17C). When replated, the transparent colonies again produced the three types of colonies, whereas the others stably maintained their phenotype.

In the course of this study, phage Ab09 was shown to select mainly for mutants with an increased capability to produce alginate, thus leading to the mucoid conversion of the parental phenotype.

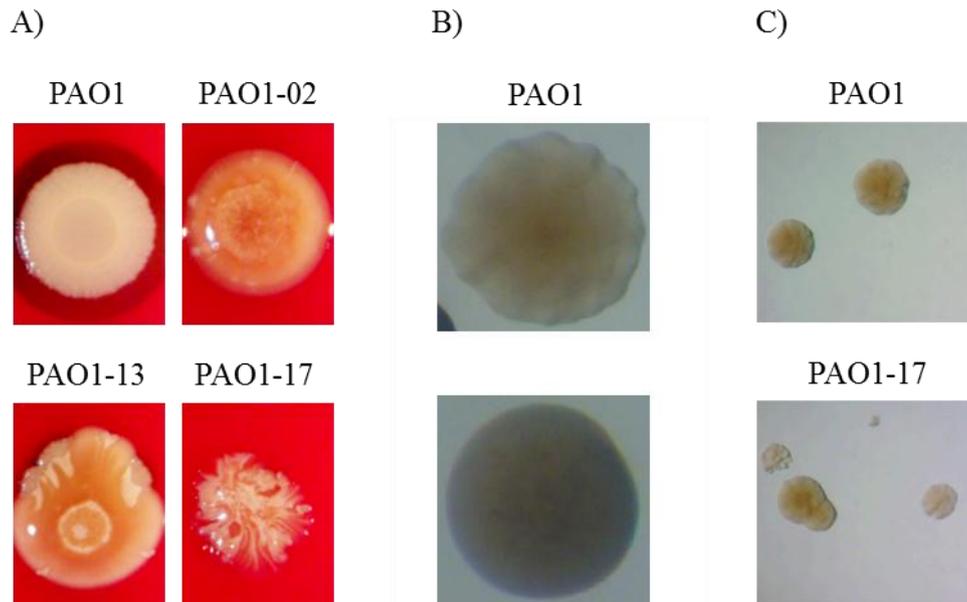


FIGURE 17. A) PLATING OF SOME PHAGE-TOLERANT VARIANTS ON SHEEP BLOOD AGAR 5%. B) MUCOID PHENOTYPE OF PAO1-06. C) IRREGULAR MORPHOLOGY OF PAO1-17 COLONIES. In the picture are shown colonies with morphology similar to PAO1_{or} and others with a transparent appearance.

II.II.II CLUSTERING OF THE THIRTY-TWO VARIANTS ACCORDING TO THEIR PHAGE-TOLERANCE PATTERN

The 32 variants, shown to be stably tolerant to at least one phage at the P3 purification step, were clustered in five different groups according to their pattern of susceptibility to the four phages employed in this study, evaluated by plating efficiency (Table 1).

The Group 1 contained all the variants showed to be susceptible to phage Ab05 and more or less tolerant to the phages Ab09, Ab17 and Ab27. In terms of plating efficiency, Group 1 variants were shown to be completely susceptible to phage Ab05, although, on some of them, the phage produced turbid plaques smaller than those observed on the initial PAO1_{or}. Complete resistance to Ab17 and Ab27 was seen in all the variants. Only half of them resisted Ab09, while the other half displayed a reduction in the efficiency of plating of phage Ab09 and the formation of smaller and less clear plaques. These variants were isolated mostly from

infection performed with phages Ab09, Ab17 or double infection with both of them. They displayed cross-resistance to phages using LPS as a receptor.

Six Group 2 variants, of which three were mucoid, showed intermediate susceptibility patterns to the different phages. Although the mucoid phenotype seems to provide the bacteria with a strong protective barrier against several external factors (Hentzer, Teitzel *et al.* 2001; Leid, Willson *et al.* 2005), the alginate matrix does not totally inhibit phage adsorption, as turbid plaques can be observed for all phages. It may prevent entrance of phage DNA or release of phage particles as well as of extracellular toxins, responsible, for example, for the hemolytic activity displayed by the wild-type PAO1_{Or} strain.

The five Group 3 variants were resistant only to phage Ab05, whereas Group 4 variants displayed full resistance to phages Ab05 and Ab27, and reduced susceptibility to phage Ab17 and Ab09, characterized by the production of small plaques instead of large, clear ones. Variant PAO1-30 was quite particular since, although completely resistant to phage Ab05, it displayed complete susceptibility to phage Ab09 and reduced efficiency of plating for phage Ab17; in contrast to the other variants of this group, PAO1-30 seemed not to completely inhibit the infection by phage Ab27; indeed, even with lower efficiency of plating and producing smaller plaques, phage Ab27 was able to productively infect this variant.

Group 5 was characterized by variants able to resist infection by all phages used in this study.

	Infection	PAO1 _{Or} variant	Phage resistance								Phage DNA [‡]
			Ab05		Ab09		Ab17		Ab27		
			PM	EOP	PM	EOP	PM	EOP	PM	EOP	
Group 1	Ab09, Ab17	01	N	100	-	0	-	0	-	0	-
	Ab09, Ab17	04	N	100	-	0	-	0	-	0	-
	Ab09, Ab17	05	N	100	-	0	-	0	-	0	-
	Ab09	07	N	100	-	0	-	0	-	0	-
	Ab17	09	N	100	-	0	-	0	-	0	-
	Ab17	12	N	100	-	0	-	0	-	0	-
	Ab17	14	s	100	s	100	-	0	-	0	-
	Ab09	15	s	100	s	2	-	0	-	0	-
	Ab17	18	s	100	s	20	-	0	-	0	-
	Ab09, Ab17	19	N	100	s	8	-	0	-	0	Ab09
	Cocktail*	21	s	100	s	20	-	0	-	0	-
	Ab05, Ab27	32	N	100	-	0	-	0	-	0	Ab27
Ab05, Ab27	34	s	100	s	33	-	0	-	0	Ab27	
Group 2	Ab09, Ab17	02 [†]	s	100	s	100	N	100	-	0	-
	Ab09, Ab17	03	N	100	s	20	N	100	N	33	-
	Ab09, Ab17	06 [†]	N	100	s	43	N	100	N	100	-
	Ab17	10	s	100	s	100	s	100	N	73	-
	Ab09	13 [†]	s	100	s	100	N	100	s	100	-
	Ab09	17	s	100	-	0	N	100	-	0	Ab09
Group 3	Cocktail*	25	-	0	N	100	N	100	s	100	Ab05
	Ab05	26	-	0	N	100	N	100	N	100	Ab05
	Ab05	27	-	0	N	100	N	100	N	100	Ab05
	Ab05	28	-	0	N	100	N	100	N	100	-
	Ab05	29	-	0	N	100	N	100	N	100	Ab05
Group 4	Cocktail*	24	-	0	s	100	s	100	-	0	Ab27
	Ab05	30	-	0	N	100	N	50	s	10	Ab05
	Ab05, Ab27	36	-	0	s	100	s	100	-	0	Ab27
	Ab05, Ab27	37	-	0	s	100	s	100	-	0	Ab05
Group 5	Cocktail*	20	-	0	-	0	-	0	-	0	Ab17
	Cocktail*	22	-	0	-	0	-	0	-	0	Ab17
	Ab05, Ab27	33	-	0	-	0	-	0	-	0	Ab27
	Ab05, Ab27	35	-	0	-	0	-	0	-	0	-

TABLE 1 CLUSTERING OF P3 PHAGE-TOLERANT VARIANTS ACCORDING TO THEIR TOLERANCE PATTERN AGAINST FOUR PHAGES USED IN THIS STUDY. PM, plaque morphology; EOP, efficiency of plating; N, normal plaque morphology compared to that observed on the control PAO1_{Or}; s, small and turbid plaques; -, no plaques observed; [†] mucoid variant; [‡] detection by PCR; * the four phages were used for the infection in a form of a cocktail.

In general, the plaque enlargement occurs as a consequence of two distinct phenomena: a “diffusion” step that directly contributes to the growth of a plaque’s diameter, and an “infection” step, strictly dependent on the phage latent period and burst size (Abedon 2008). A change in plaque size not coupled with a decrease in the efficiency of plating as observed, for example, for phage Ab05, Ab09 and Ab27 when infecting the variant PAO1-13, could reflect a decrease in the diffusion ability of phages. This could be caused for instance by the production of a denser extracellular matrix such as alginate in mucoids. With some phages infecting, for example, the mucoid variants, we also observed the formation of more turbid plaques. Plaque turbidity could be due to the slowing down of the infection in terms of number of cells infected in a certain period of time (increase of latent period length, decrease of burst size).

Unexpectedly, PCR amplification showed that phage DNA could still be detected at the P3 re-isolation step in 15 of the 32 variants (Table 1), even when we treated them with a virucide in order to exclude the presence of phages bound to the bacterial surface. All these data suggested the existence of a pseudolysogenic stage (discussed later) as these phages are not temperate.

We hypothesized that the resistance of phage-tolerant variants not containing phage DNA could have been due to modifications of the bacterial extracellular surface, altering or masking the phage receptor, thus inhibiting their binding. We performed adsorption assay on phage-free variants and evaluated the amount of unbound phages expressed as percentage of the initial amount of phages used for the assay. The results correlated well with the phage-tolerance pattern of the mutants suggesting that phage infection could be impaired at the level of phage adsorption to its receptor (Figure 18). For instance, group 3 variant PAO1-28 and group 5 variant PAO1-35 which were tolerant to Ab05 did not significantly adsorb Ab05. Variant PAO1-28 adsorbed the three other phages and was susceptible to their infection, whereas variant PAO1-35, fully tolerant to phages Ab09, Ab17 and Ab27, adsorbed none of them.

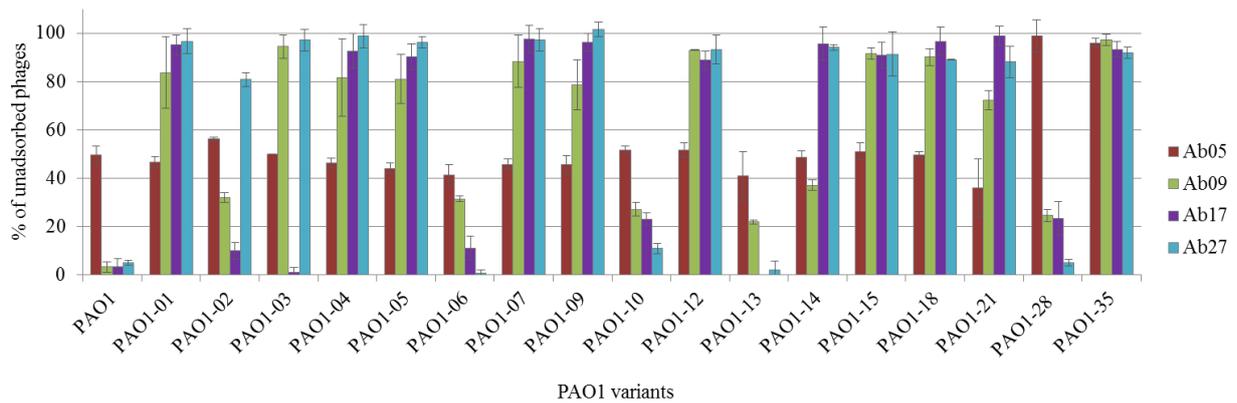


FIGURE 18. PHAGE ABSORPTION ON SEVENTEEN VARIANTS DEVOID OF PHAGE DNA. Y-axis, percentage of unadsorbed phages at 16 min after infection. The standard deviation is the result of three independent assays.

The different assays show that mutants display a large variety of phenotypic changes depending on the bacteriophage used. In order to identify the mutations conferring resistance, and to investigate in more details the presence of phage DNA, we performed draft whole genome sequencing on DNA extracted from 23 variants at the P3 purification step, distributed into the different groups and in addition used a candidate gene approach to find mutations in the mucoid variant PAO1-02.

II.III A WIDE RANGE OF CHROMOSOMAL MUTATIONS IS SELECTED BY PHAGES

To identify *de novo* mutations, we first sequenced the genome of the parental PAO1_{Or} strain, prepared from the culture used to derive phage-tolerant variants. The PAO1_{Or} sequencing reads were mapped against the sequence of the reference PAO1-UW (NC_002516) strain, allowing the assembly of the full genome and identification of differences (Figure 19).

Compared to PAO1-UW (Stover, Pham *et al.* 2000), PAO1_{Or} possessed a large inversion between rRNA sequences at position at position 727255 to 4788575 (Figure 19).

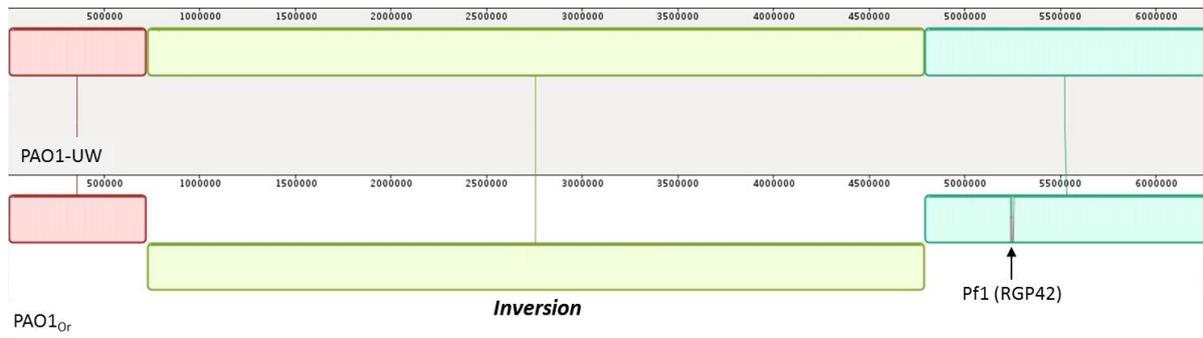


FIGURE 19. ALIGNMENT OF PAO1-UW AND PAO1_{Or} GENOME SEQUENCES. The central block (light green) represents the inverted region in PAO1_{Or} compared to PAO1-UW strain.

Klockgether and colleagues showed that PAO1-UW possess a single copy of the Pf1 prophage and that two of the PAO1-derived reference substrains, MPAO1 and PAO1-DSM, possess a second additional copy in their genome (Klockgether, Munder *et al.* 2010). We found that PAO1_{Or} also possess the two copies of the prophage Pf1 and the second copy, named RGP42 in MPAO1, is located at position 5 242 103 to 5 254 164 (Figure 19). PAO1_{Or} possesses 63 SNPs or short indels events compared to PAO1-UW. As expected, some of these differences, including the inversion and the presence of an additional copy of the filamentous prophage Pf1, were previously reported by Klockgether and colleagues for both substrains MPAO1 and PAO1-DSM, while others were specific to the PAO1_{Or} substrain.

The sequencing reads from each of the 23 sequenced phage-tolerant variants were mapped against the PAO1_{Or} genome showing a uniform distribution with a mean coverage from 50 up to 200 fold.

Fourteen variants showed a single mutation in their genome, while six variants were shown to be double mutants. Two variants, PAO1-14 and PAO1-21, were shown to possess the same mutation, although isolated from two independent experiments. No mutations could be detected in variants PAO1-30, PAO1-32 and PAO1-34. An additional variant with a mucoid phenotype, PAO1-02, was analyzed by a candidate gene approach and this allowed to find a frameshift

mutation in the *mucA* gene. These results show that the approach we employed allowed us to characterize a large diversity of independent variants.

Table 2 displays the position and nature of the mutations, as well as the percentage of sequencing reads containing a particular mutation. In twelve out of fourteen variants with a single mutation, the mutation was present in more than 98% of the reads, and usually 100%. Among these, more than 20% of reads were of phage origin in variants PAO1-19 and PAO1-20. No phage reads were detected in the ten others. The ratio of mutated reads was only 47% and 81% in single mutation variants PAO1-17 and PAO1-26 respectively. Phage DNA was detected in both variants. In four (variants PAO1-22, PAO1-24, PAO1-33, PAO1-37) among the six variants with two mutations, one of the mutations was present only in a fraction of the population. Group 5 variant PAO1-33 showed two mutations at the same position in *wzy*, in equal proportion, the A(7) to A(8) variant and a A(7) to (6) variant in addition to a *pilYI* deletion. Curiously, both PAO1-33 and PAO1-37 were *wzy-pilYI* double mutants. In these four cases, phage reads were detected. No phage reads were detected in the last two double mutants, variants PAO1-04 and PAO1-35.

	PAO1 variant	Phage resistance*	Position on PAO1 _{Or}	Mutation	Locus tag	Protein alteration [‡]	% of reads representing		
							Mutation	Phage [§]	Bacteria
Group 1	01	S R R R	1 976 849	A(7) → A(8)	<i>wzy</i>	74/438 aa	100	0	98
	04	S R R R	1 977 338	G(6) → G(5)	<i>wzy</i>	224/438 aa	100	0	99
			4 736 958	G → A	<i>migA</i>	Arg → His	100		
	07	S R R R	1 977 338	G(6) → G(5)	<i>wzy</i>	224/438 aa	98	0	98
	14	s s R R	1 976 849	A(7) → A(8)	<i>wzy</i>	74/438 aa	100	0	98
	15	s R R R	1 986 619	G(9) → G(8)	<i>wbpL</i>	88/339 aa	100	0	99
	18	s I R R	5 634 829	C → A	<i>wapH</i>	Arg → Leu	99	0	99
	19	S I R R	5 632 885	G → C	<i>dnpA</i>	67/472 aa	100	24 [Ab09]	74
	21	s I R R	1 976 849	A(7) → A(8)	<i>wzy</i>	74/438 aa	100	0	99
	32	S R R R	-	-	-	-	0	85 [Ab27]	13
34	s I R R	-	-	-	-	0	73 [Ab27]	25	
Group 2	02	s s S R	4 683 540	G(3) → G(2)	<i>mucA</i>	146/194 aa			
	03	S I S I	4 487 654	C(5) → C(6)	<i>wzz2</i>	228/443 aa	100	0	98
	06	S I S S	4 683 359	T(4) → T(3)	<i>mucA</i>	253/194 aa	100	0	99
	10	s s s I	5 327 357	A → C	<i>pgi</i>	Thr → Pro	99	0	99
	13	s s S s	4 683 943	T → C	<i>mucA...algU</i>	-	100	0	99
	17	s R s R	1 977 343	C → A	<i>wzy</i>	220/438 aa	47	2 [Ab09]	97
Group 3	26	R S S S	5 689 432	19 bp	<i>pilQ</i>	180/714 aa	81	2 [Ab05]	97
Group 4	24	R s s R	1 976 849	A(7) → A(8)	<i>wzy</i>	74/438 aa	40	24 [Ab27]	72
			5 095 901	G → C	<i>pilR</i>	Arg → Pro	98		
	30	R S I I	-	-	-	-	0	40 [Ab05]	57
	37	R s s R	5 103 099	10 bp	<i>pilY1</i>	816/1161 aa	67	3 [Ab05]	96
1 977 570			A → G	<i>wzy</i>	Asp → Gly	100			
Group 5	20	R R R R	5 688 665	555 bp	<i>pilQ</i>	529/714 aa	100	35 [Ab17]	63
	22	R R R R	5 095 650	C(2) → C(1)	<i>pilR</i>	334/445 aa	97	11 [Ab17]	88
			6 005 075	213 bp	<i>algC</i>	797/868 aa	63		
	33	R R R R	1 976 849	A(7) → A(8)	<i>wzy</i>	74/438 aa	49	2 [Ab27]	98
			1 976 849	A(7) → A(6)	<i>wzy</i>	54/438 aa	49		
			5 102 164	109 bp	<i>pilY1</i>	501/1161 aa	100		
	35	R R R R	451 455	11 bp	<i>pilJ</i>	751/682 aa	100	0	98
1 976 849			A(7) → A(8)	<i>wzy</i>	74/438 aa	100			

TABLE 2 TWENTY-EIGHT MUTATIONS IDENTIFIED IN P3 PHAGE-TOLERANT VARIANTS BY COMPARISON WITH THE REFERENCE PAO1_{Or}. *Phage resistance pattern is reported in the order Ab05, Ab09, Ab17 and Ab27. S, completely susceptible; R, completely resistant; I, reduced efficiency of plating; s, normal efficiency of plating but small and turbid plaques. Number of repeated nucleotides indicated in parentheses. [‡]Length of the mutated protein/wild-type. [§]Phage found by sequencing indicated in square brackets. ^{||}Mutation found by PCR and Sanger sequencing of the *mucA* gene. Intergenic region (variant 13, group 2) indicated by ellipsis (...).

Three kinds of genetic alterations were found in the phage-tolerant variants: phase variation, deletions of more than 10 bp, and nucleotide substitutions. In total, thirteen genes (*algC*, *dnpA*, *migA*, *mucA*, *pgi*, *pilJ*, *pilQ*, *pilR*, *pilY1*, *wzy*, *wzz2*, *wbpL*, *wapH*) and the *muA...algU* intergenic region were affected in the 28 mutations detected (Table 2).

II.III.I PHASE VARIATION MUTATIONS ARE REVERSIBLE

Mutations in *wzy* were observed in 10 variants. In two instances, variants PAO1-17 and PAO1-24, slightly less than half of the bacteria were mutated. In both cases, one of the phages used in the infection was still present. In eight variants the *wzy* mutations occurred in an homopolymeric tract. The last two cases were point mutations. Mutations A(7) to A(8) at position 1 976 849 as well as A(7) to A(6) at the same position in variant PAO1-33, and G(6) to G(5) at position 1 977 338, were found in six, one and two instances, respectively (variant PAO1-33 showed both the A(7) to A(8) and A(7) to A(6) mutations, in equal amount). Single nucleotide indels were observed in *pilR* (C2 to C1), *mucA* (T4 to T3 and G3 to G2), *wzz2* (C5 to C6) and *wbpL* (G9 to G8) inducing a frameshift. This resulted in early termination of protein synthesis except for the T4 to T3 mutation in *mucA* which suppressed normal termination of translation resulting in the production of a longer MucA protein fused with the beginning of MucB. Natural mutations of *mucA* previously observed in strains isolated from CF patients were phase variation mutations (Spencer, Kas *et al.* 2003) or other frameshift mutations (Pulcrano, Iula *et al.* 2012), resulting in the production of truncated proteins as seen in PAO1-02.

In total, 13 among the 28 mutations identified in the present study are predicted to be reversible at a high rate. Most of the mutations identified in this study occurred in genes (*wzy*, *wzz2*, *wbpL*, *wapH*, *dnpA*, *migA*, *pgi* and *algC*) involved in the LPS biosynthesis pathway. The mutant PAO1-01, PAO1-14, PAO1-21, PAO1-24, PAO1-33 and PAO1-35 and PAO1-04 and PAO1-07 have the same kind of phase variation mutation in *wzy* gene, PAO1-15

possesses a phase variation mutation in the *wbpL* gene and PAO1-03 has a phase variation mutation in the *wzz2* gene. Phase variation mutations in genes involved in the O-antigen production can temporarily confer to the mutants the resistance against the phages at a cost of losing the A- and/or B-chains of the O-antigen, that, however, *in vitro* and in absence of the human host cells, are not necessary.

II.III.II DELETIONS

Multiple components are involved in the type IV pilus biogenesis (Kim, Oh *et al.* 2006). We observed seven mutations affecting *pilJ*, *pilR*, *pilQ* and *pilY1*. Deletions were found in three structural genes. *pilJ* was missing 11 bp in variant PAO1-35. *pilQ* was missing 19 bp in variant PAO1-26 and 555 bp in variant PAO1-20. The *pilY1* gene lacked 10 and 109 bp in variants PAO1-37 and PAO1-33 respectively. These deletions caused a frameshift and the creation of a premature stop codon, or suppressed some internal domains. The regulatory gene *pilR* lost one C in variant PAO1-22 and contained a missense mutation in variant PAO1-24.

We also identified a deletion of 213 bp in the *algC* gene, in variant PAO1-22. Mutations in *algC* affect the biosynthesis of alginate, LPS and rhamnolipids, biosurfactants necessary for bacterial swarming motility and biofilm formation (Olvera, Goldberg *et al.* 1999).

II.III.III NUCLEOTIDE SUBSTITUTIONS

Eight variants showed a single nucleotide substitution. Two of them, PAO1-17 and PAO1-19, had non-sense mutations converting a Tyrosine in a stop codon and producing a shorter Wzy and DnpA proteins, respectively. Two variants showed non-synonymous mutations that changed the positively charged Arginine into the nonpolar Leucine in PAO1-18 WapH, or into the uncharged polar Proline in PAO1-24 PilR. A third non-synonymous mutation was observed in the double mutant PAO-37, affecting *wzy* and changing the negatively charged

Aspartate in the nonpolar aminoacid Glycine. PAO1-13 possessed a transversion of T into C in the promoter region of *mucA*, presumably affecting its expression. PAO1-10 and the double mutant PAO1-04 were shown to have a nucleotide substitution that led to the replacement of the positively charged Arginine and the uncharged Threonine with the positively charged Histidine and the uncharged Proline in Wzy and Pgi, respectively. In this case the aminoacid charge or polarity did not change but the substitution of this aminoacid could lead to conformational changes in the proteins, thus affecting their interactions with other proteins.

The different mutations potentially affected the biosynthesis of membrane structures that participate in binding of phages to their receptor. Mutations in the gene cluster regulating the production of alginate were selected by Ab09, and could reduce the efficiency of infection of all the phages. The *wzy*, *wzz2* or *wbpL* genes, are members of the heteropolymeric O-specific antigen biosynthesis cluster in PAO1 (Lam, Taylor *et al.* 2011). The *migA* gene encodes a rhamnosyltransferase involved in the LPS core capping, whereas *dnpA* is a de-N-acetylase, and *pgi* and *wapH* encode glycosyltransferases. Overall, the phage susceptibility pattern of each mutant correlated well with the nature of the mutated genes. Infection with Ab09, Ab17 and Ab27 mainly selected mutations in genes regulating LPS biosynthesis, while Ab05 selected mutations in genes involved in type IV pili synthesis.

II.IV PERSISTENCE OF PHAGE DNA CONFERS IMMUNITY

In eleven variants, phage DNA represented 2 to 85% of the total sequencing reads, and there was no evidence of insertion of the phage genome in any of the samples, thus confirming the pseudolysogenic nature of the variants. Interestingly, in three phage-tolerant variants (PAO1-30, PAO1-32 and PAO1-34) no chromosomal mutation could be found but they possessed large amounts of phage DNA. PAO1-32 and PAO1-34 immune to Ab09, Ab17 and Ab27

contained Ab27 DNA whereas PAO1-30 immune to Ab05, Ab17 and Ab27 contained high levels of Ab05 DNA.

II.IV.I PSEUDOLYSOGENIC PHAGES WITH TAIL FIBER MUTATIONS

In the sequenced samples in which high numbers of phage DNA reads were present, it was possible to assemble the phage genome sequence. This led to the identification of several single nucleotide differences in tail fiber genes, as compared to the parental genotype. To check whether these mutations were already present in the phages used to infect bacteria, we analysed the affected regions by PCR and sequencing. In three pseudolysogens obtained independently, an Ab05 tail fiber gene displayed two SNPs, also observed in a fraction of the phages used to select for resistant bacteria (Figure 20).

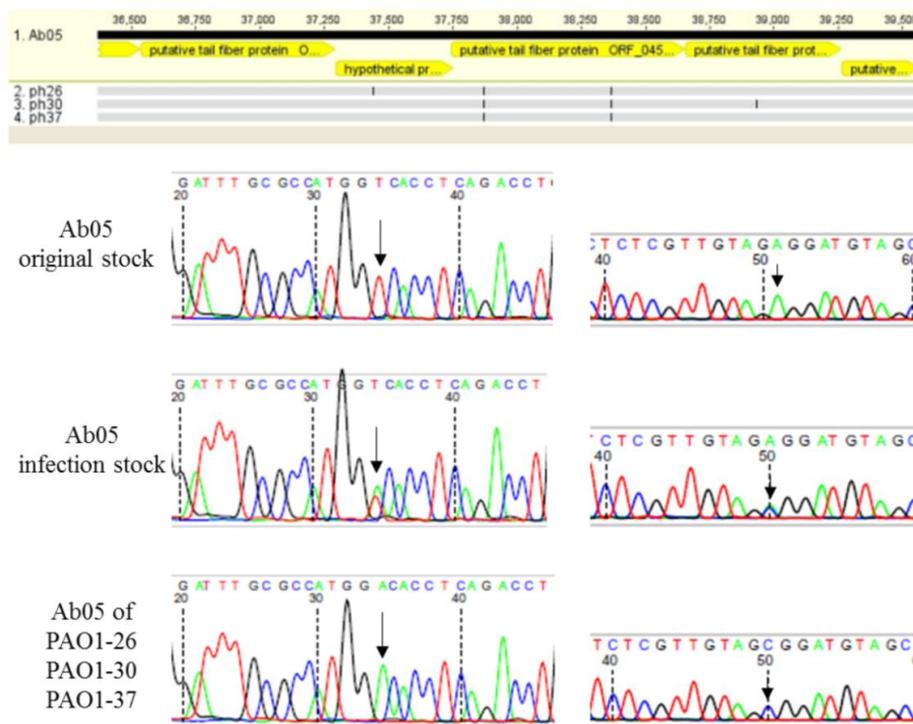


FIGURE 20. NEW MUTATIONS IN TAIL FIBER GENES OF PSEUDOLYSOGENIC PHAGES. The phage recovered from variants PAO1-26, PAO1-30 and PAO1-37 share mutations T→A at position 37873 and A→C at position 38368. The mutations were already present in the phage stock used for infections at almost equal frequency. The third shared mutation at position 1076 is not shown. Phage recovered from PAO1-26 has an additional mutation at position 37443. A fraction of phages recovered from both PAO1-26 and PAO1-30 show an additional mutation at position 35108 and 38934, respectively (Table 3).

Similarly a single SNP was observed in an Ab17 tail fiber gene from variants PAO1-19, PAO1-20 and PAO1-22, and in the ribosome binding site of an Ab27 tail gene from variants PAO1-24, PAO1-32, PAO1-33 and PAO1-34 (Table 3).

Variant	Phage	Position on phage genome	Mutated reads (%)	Gene	Product	Mutation	Protein effect
PAO1-26	Ab05	1076	99.4%			A→G	
		35108	66.0%	ORF42	putative internal (core) protein	G→A	V→I
		37443	97.9%	ORF44	hypothetical protein	A→C	H→P
		37873	97.2%	ORF45	putative tail fiber protein	T→A	V→D
		38368	97.5%	ORF45	putative tail fiber protein	T→G	L→R
PAO1-30		1076	98.0%			A→G	
		37873	96.4%	ORF45	putative tail fiber protein	T→A	V→D
		38368	97.5%	ORF45	putative tail fiber protein	T→G	L→R
		38934	70.8%	ORF46	putative tail fiber protein	A→G	N→D
PAO1-35		1076	97.4%			A→G	
		37873	98.4%	ORF45	putative tail fiber protein	T→A	V→D
		38368	100.0%	ORF45	putative tail fiber protein	T→G	L→R
PAO1-37		1076	97.7%			A→G	
		37873	97.8%	ORF45	putative tail fiber protein	T→A	V→D
		38368	99.0%	ORF45	putative tail fiber protein	T→G	L→R
PAO1-19	Ab17	47129	98.2%	ORF80	putative tail fiber protein	C→T	R→W
PAO1-20		47129	99.1%	ORF80	putative tail fiber protein	C→T	R→W
PAO1-22		44765	65.1%	ORF78	putative baseplate protein	T→G	D→E
		47129	98.3%	ORF80	putative tail fiber protein	C→T	R→W
PAO1-24	Ab27	36370	36.0%			G(9)→G(10)	
PAO1-32		26899	97.8%	ORF48	hypothetical protein	A→G	T→A
		36370	35.3%			G(9)→G(10)	
PAO1-33		26899	99.5%	ORF48	hypothetical protein	A→G	T→A
		36370	24.2%			G(9)→G(10)	
		36370	47.4%			G(9)→G(11)	
PAO1-34		41863	91.4%	ORF63	putative tail fiber protein	C→T	T→I
		26899	31.3%	ORF48	hypothetical protein	A→G	T→A
		36370	41.4%			G(9)→G(10)	

TABLE 3 MUTATIONS IDENTIFIED IN THE PHAGE GENOME RE-ASSEMBLED IN THE PSEUDOLYSOGEN VARIANTS.

The presence of two or more phage populations in the stock prepared starting from the Ab05 original homogeneous stock suggests that the mutations had spontaneously occurred during phage amplification. The presence of a fraction of the population with mutations in genes encoding tail fibers could explain why phage Ab05, in this specific case, did not adsorb completely on the bacterial surface even after 16 min as reported in (Figure 18). Heterogeneity in the adsorption characteristics of a bacteriophage population has been widely reported. For example, with the coliphage T4, it has been observed that a subset of the population had extremely slow adsorption kinetics or failed to adsorb at all (Storms and Sauvageau 2014).

II.IV.II STABILITY OF THE PSEUDOLYSOGENIC STAGE

We found that viable phages were released by pseudolysogens, sometimes at high titers, during overnight culture in LB medium. This demonstrated that a portion of the bacterial population could achieve a productive viral cycle, whereas the others resisted infection. To evaluate the dynamics inside pseudolysogen colonies, we measured the percentage of bacteria containing phage DNA and producing viable phages and evaluate for how long phage DNA was maintained. For this purpose, 1 μ l of bacteria from the frozen P3 stock was spread on LB agar (P3₀), and 52 colonies were picked-up and deposited both on LB agar plates, and on LB agar plates covered with a lawn of soft agar containing PAO1_{Or} (see PAO1-30 replatings as an example in Figure 21). After incubation at 37°C for 24 h, a lysis zone could be seen around phage-producing colonies on the lawn of PAO1_{Or}. One phage-producing colony from the LB agar plate was then streaked onto a new LB plate and the procedure was repeated. It allowed the quantification of the fraction of pseudolysogenic cells contained in a single colony and the assessment of the stability of the pseudolysogenic state. The percentage of phage-producing colonies for each replating experiment until no more phage-producing colonies were detected, varied among the tested variants (Table 4).

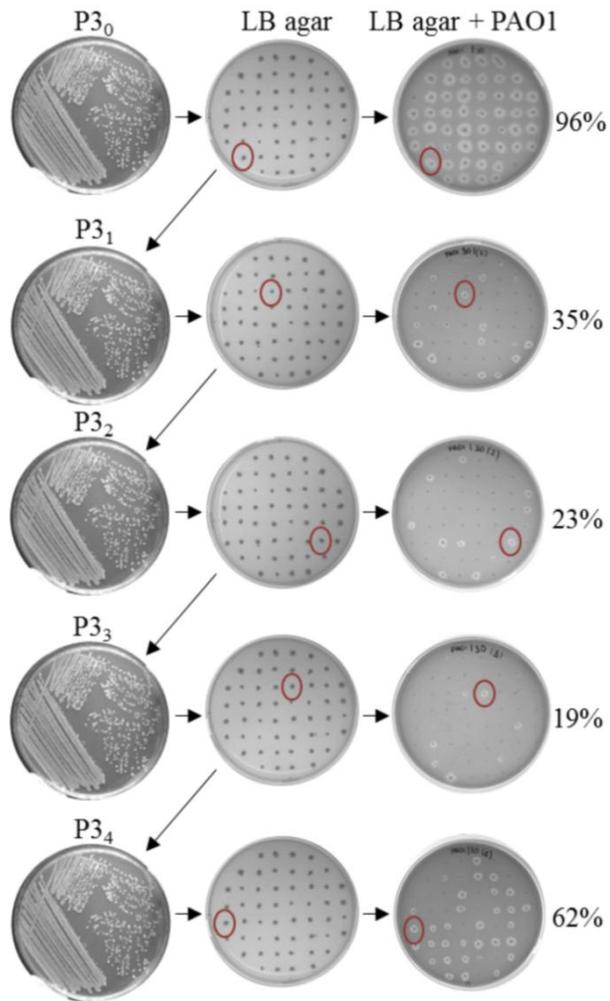


FIGURE 21. REPLATINGS OF PHAGE-TOLERANT VARIANT PAO1-30.

In addition, hybridization with phage DNA probes in a colony lift experiment showed that bacteria not releasing phages were devoid of phage DNA, allowing us to exclude the presence of colonies able to contain phage DNA without releasing functional phage particles. The presence of phage DNA and phage particles in important amounts, in variants following five up to ten colony replatings, and of bacteria devoid of phages, implied that there was a continuous lysis of some infected cells, production of cured progeny which became susceptible to phage infections, and amplification of phages by infection of these bacteria.

PAO1 _{Or} variant	Replating										
	P3 ₀	P3 ₁	P3 ₂	P3 ₃	P3 ₄	P3 ₅	P3 ₆	P3 ₇	P3 ₈	P3 ₉	P3 ₁₀
19	4	0	-	-	-	-	-	-	-	-	-
20	6	0	-	-	-	-	-	-	-	-	-
22	44	0	-	-	-	-	-	-	-	-	-
24	100	0	-	-	-	-	-	-	-	-	-
26	54	0	-	-	-	-	-	-	-	-	-
36	100	0	-	-	-	-	-	-	-	-	-
37	46	0	-	-	-	-	-	-	-	-	-
25	4	8	0	-	-	-	-	-	-	-	-
34	83	6	33	0	-	-	-	-	-	-	-
33	40	12	62	0							
17	98	56	92	0	-	-	-	-	-	-	-
32	38	31	44	69	38	0	-	-	-	-	-
30	96	35	23	19	62	25	100	100	88	2	0

TABLE 4 PERCENTAGE OF PHAGE-PRODUCING COLONIES DURING REPLATINGS OF PAO1_{Or} VARIANTS CONTAINING PHAGE DNA.

Interestingly, PAO1-30, which kept phage-producing cells for the longest time, showed peaks of abundance, reflecting a particular equilibrium between phage production and bacteria predation similarly to what was previously seen in liquid medium by Ripp and Miller (Ripp and Miller 1998).

II.IV.III HIGH FREQUENCY OF DOUBLE MUTANTS IS DUE TO PERSISTENCE OF PHAGE DNA

The mixture of wild-type and mutant reads in some samples after three purification steps, always accompanied by phage DNA, and the high frequency of double mutants, suggested that immunity provided by the phage in a pseudolysogenic state allowed survival and subsequent emergence of mutations. To investigate this hypothesis, we tested whether new mutations would appear in response to the pressure imposed by phages. We went back to the -80°C stocks of variants (P3), isolated new colonies and tested them for the presence of phage DNA by PCR, until a colony devoid of phage DNA was found. Phage susceptibility was re-tested, and the mutations previously identified by whole genome sequencing were searched. Different situations existed when phage DNA was no longer present. The 19 bp PAO1-26 *pilQ* microdeletion present in 81% of P3 bacteria (Table 2) was found in about two thirds of the colonies re-isolated after -80°C storage, and it was associated with resistance to Ab05. Similarly, upon re-isolation of PAO1-37, about 50% of colonies were stable *wzy-pilY1* double mutants, devoid of Ab05 and displaying resistance to Ab05 and Ab27. In other variants, the phage susceptibility profile changed when additional colony re-isolation steps were performed, and new mutations could be found upon sequencing. All the variants re-isolated from the original frozen stock are listed in Table 5, with their resistance pattern and the identified mutations.

PAO1 _{Or} variant	Resistance pattern*	Sequencing method	Position on PAO1 _{Or}	Mutation ^a	Mutation event	Locus tag	Protein alteration [‡]
17_1	I R R R	PCR	4 683 508	C(5)→C(4)	F	<i>mucA</i>	146/194 aa
		candidate gene approach	1 977 343	<u>C→A</u>	<u>TV</u>	<u>wzy</u>	<u>220/438 aa</u>
20_1	R R R R	Illumina	1 976 837	C→T	TS	<i>wzy</i>	Ser → Phe
			5 688 664	<u>555 bp</u>	<u>D</u>	<u>pilQ</u>	<u>529/714 aa</u>
22_1	R R R R	Illumina	1 976 848	A(7)→A(8)	F	<i>wzy</i>	74/438 aa
			5 095 649	<u>C(2)→C(1)</u>	<u>F</u>	<u>pilR</u>	<u>334/445 aa</u>
24_1	R R R R	PCR	1 976 848	A(7)→A(6)	F	<i>wzy</i>	54/438 aa
		candidate gene approach	5 095 901	<u>G→C</u>	<u>TV</u>	<u>pilR</u>	<u>Arg → Pro</u>
25_1	R S S S	Illumina	5 096 064	A→C	TV	<i>pilR</i>	Thr → Pro
30_1	R S S S	PCR	5 688 968	T→G	TV	<i>pilQ</i>	Thr → Pro
		candidate gene approach					
36_1	R R R R	Illumina	1 976 848	A(7)→A(6)	F	<i>wzy</i>	54/438 aa
			5 071 804	G→A	TS	<i>pilC</i>	Arg → His

TABLE 5 PHAGE-TOLERANCE PATTERN AND MUTATIONS IN SECONDARY ISOLATED VARIANTS. *Phage resistance pattern is reported in the order Ab05, Ab09, Ab17 and Ab27. S, completely susceptible; R, completely resistant; I, reduced efficiency of plating; s, normal efficiency of plating but small and turbid plaques. ^aThe number of repeated nucleotides is indicated in parentheses. [‡]Length of the mutated protein/wild-type. F, frameshift; TV, transversion; TS, transition; D, deletion. Underlined: previously seen mutation, as reported in TABLE 2.

In the mucoid variant PAO1-17_1 devoid of phage Ab09, the *mucA* sequenced PCR product showed superimposition of two sequencing profiles indicating the presence of a new *mucA* frameshift mutation (a deletion of a single C in a stretch of five Cs present in the wild-type strain) in about half of the bacteria (Figure 22A). PAO1-20_1 acquired a mutation in *wzy*, providing resistance to LPS-dependent phages. By the same way, PAO1-22_1 showed a new mutation in *wzy* gene, losing the deletion in *algC*, previously identified in PAO1-22. For PAO1-24, after several steps of re-isolation, a colony, named PAO1-24_1, devoid of Ab27 DNA was shown to resist all four phages. The *pilR* mutation was confirmed through PCR and DNA sequencing. Surprisingly, sequencing of a *wzy* PCR amplicon showed that the original

insertion of an additional A (A8) in a stretch of seven As residues in the wild-type *wzy* gene was rare and largely replaced by a deletion of one A (A6). The sequencing profile showed the superimposition of the A8 and A6 profiles (Figure 22B).

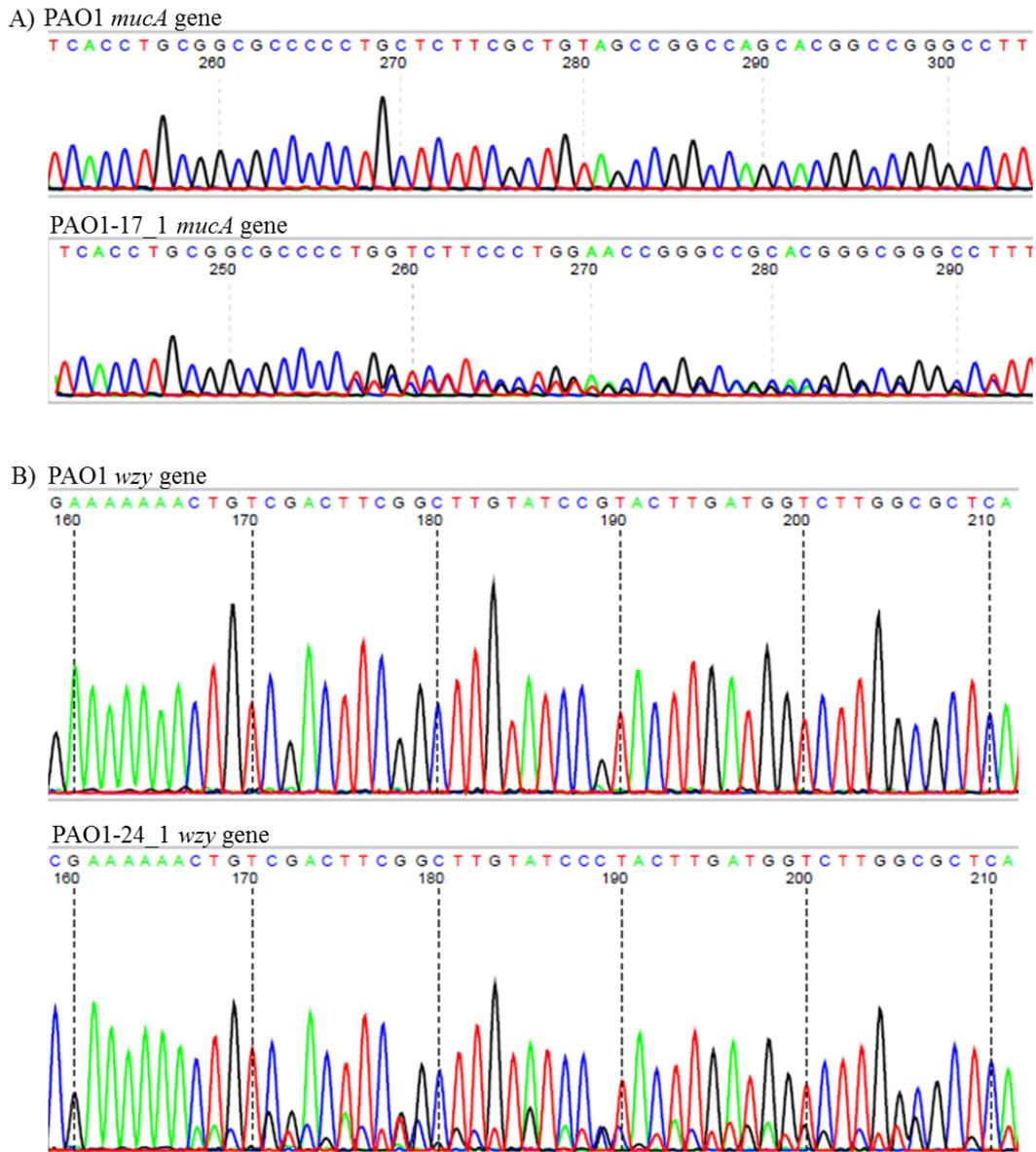


FIGURE 22. SUPERIMPOSITION OF SANGER SEQUENCING PROFILES OF PAO1-17_1 *mucA* GENE (A) AND PAO1-24_1 *wzy* GENE (B).

We also sequenced the genome of two variants re-isolated from PAO1-25 and PAO1-36 and devoid of phage DNA (PAO1-25_1 and PAO1-36_1), and mutations were found in *pilR*, and *wzy* and *pilC*, respectively.

All the mutations we identify by Illumina were confirmed by Sanger sequencing of the PCR amplification products.

Adsorption assay was performed with all the new mutants and showed that the ability of phages to adsorb on their surface was impaired (Figure 23).

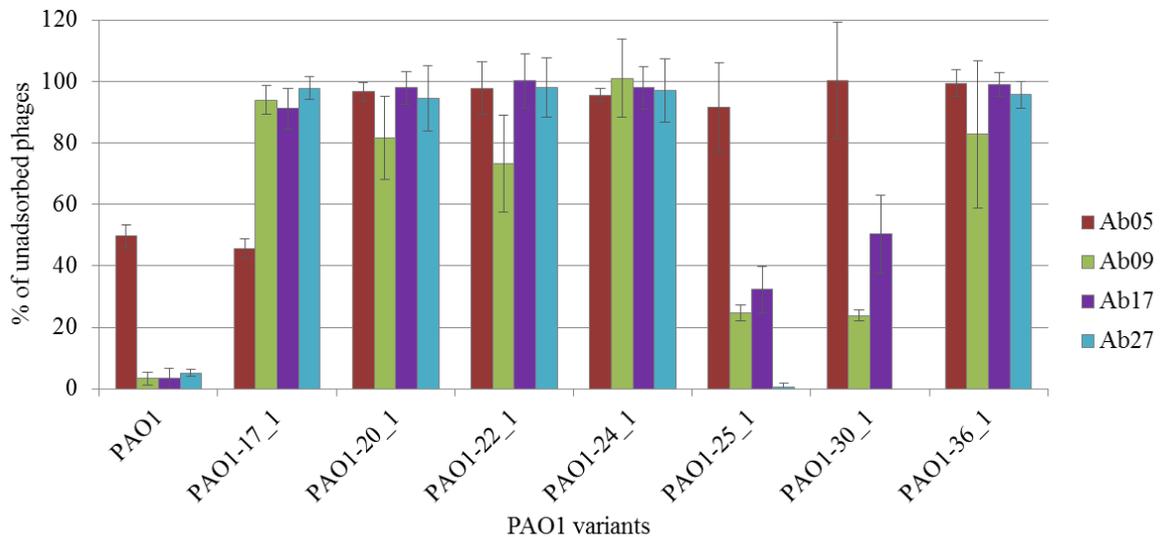


FIGURE 23. ADSORPTION EXPERIMENT PERFORMED ON SECONDARY ISOLATED MUTANTS.

On the y-axis is reported the percentage of unadsorbed phages at 16 min after infection. The standard deviation is the result of three independent assays. PAO1 is SSSS, PAO1-17_1 is IRRR, PAO1-25_1 and PAO1-30_1 are RSSS, PAO1-20_1, PAO1-22_1, PAO1-24_1 and PAO1_36 are RRRR (TABLE 5), where resistance is reported against phages Ab05, Ab09, Ab17 and Ab27, respectively.

All secondary-isolated variants possessing mutations in genes involved in the biogenesis of type IV pili were tested for their ability to twitch on LB agar plates. The twitching motility of all these variants was strongly defective when compared to the PAO1_{Or} control (Figure 24).

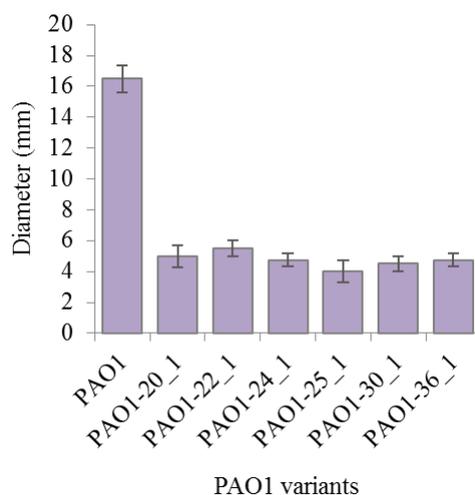


FIGURE 24. TWITCHING MOTILITY ASSAY ON SECONDARY MUTANTS. Bacterial motility is expressed as the diameter (mm) of the growth zone at the bottom of the agar plate. The standard deviation is the result of three independent assays.

In summary, it appears that a pseudolysogenic colony continuously evolves suggesting that the constant production of new functional phage particles eventually selects for new phage-resistant variants.

II.IV.IV PSEUDOLYSOGENY CONFERS IMMUNITY TO PHAGES

Colony re-isolation was also performed for the three pseudolysogens for which no chromosomal mutation could be observed. A PAO1-30 colony devoid of Ab05 DNA and called PAO1-30_1 was isolated and still resisted Ab05. The *pilQ*, *pilR* and *pilY1* genes involved in type IV pilus assembly were PCR-analysed in a candidate gene approach, and a new *pilQ* mutation was identified showing a substitution of a T by a G (Table 5). In contrast, PAO1-32 and PAO1-34 colonies devoid of Ab27 DNA recovered full susceptibility to all phages. This indicates that Ab27 conferred the observed superinfection exclusion. Interestingly, for these two variants, new mutants were not obtained during re-isolation of single colonies, which is compatible with the observation that it was not possible to isolate

variants selected by phage Ab27 in a single infection experiment, both on solid and liquid media.

Given the fact that PAO1-30, although completely resistant to phage Ab05, was also partially resistant to phage Ab17 and Ab27, we hypothesized that the presence of the phage could have influenced somehow the structure of the receptor for these two phages, LPS in this specific case.

II.V MUTATIONS AFFECT PHAGE RECEPTORS

To confirm that the observed mutations were responsible for affecting the bacteriophage receptor, we investigated the phenotype of the three classes of mutants affected in (i) type IV pilus, (ii) alginate biosynthesis or (iii) LPS and complemented the mutated genes for some of them restoring the phage susceptibility.

II.V.I PILUS TYPE IV MUTANTS ARE DEFECTIVE IN TWITCHING MOTILITY AND BIOFILM FORMATION

The motility of all the variants was evaluated by performing a twitching assay on semisolid agar. Compared to the PAO1_{Or} control, the diameter of the twitching zone was significantly reduced in all variants, but the strongest effect was observed with Ab05-tolerant variants (groups 3 to 5 in Table 1, i.e. variants PAO1-20, PAO1-22, PAO1-24 to PAO1-30, PAO1-33, PAO1-35 to PAO1-37), and with PAO1-32 and PAO1-34 (Figure 25).

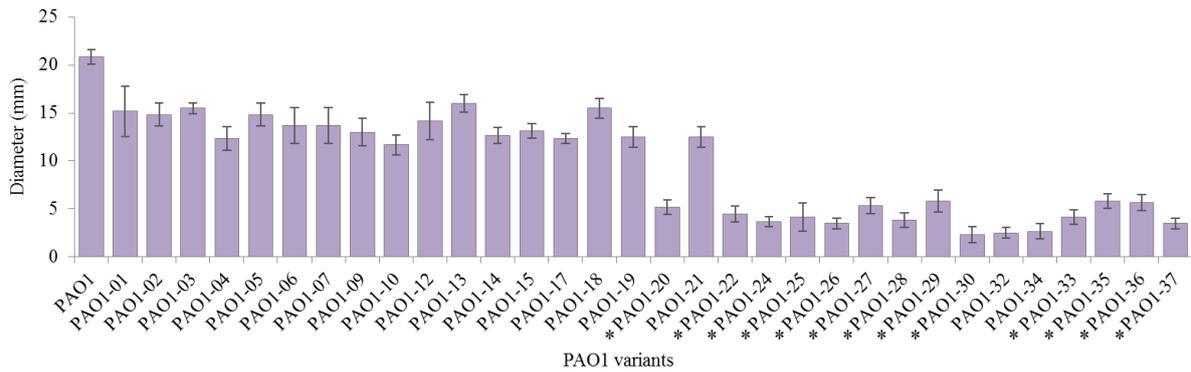


FIGURE 25. TWITCHING MOTILITY OF PHAGE-TOLERANT VARIANTS AT P3. Ab05-tolerant variants are marked by an asterisk. Bacterial motility is expressed as the diameter (mm) of the growth zone at the bottom of the agar plate. The standard deviation is the result of three independent assays.

We observed that the inhibition of twitching of Ab05-tolerant variants was accompanied by a decrease in biofilm formation, except for PAO1-20 and PAO1-33 (Figure 26). These variants presented deletions in *pilQ* and *pilY1* genes, respectively. It might be possible that these mutations can impair the functions of the type IV pilus but not the development of biofilm. A decrease in the biofilm production was also observed for the mucoid variant PAO1-06. In contrast, PAO1-18, PAO1-19 and PAO1-32 were shown to produce biofilm in a significantly higher amount compared to wild-type PAO1_{Or}.

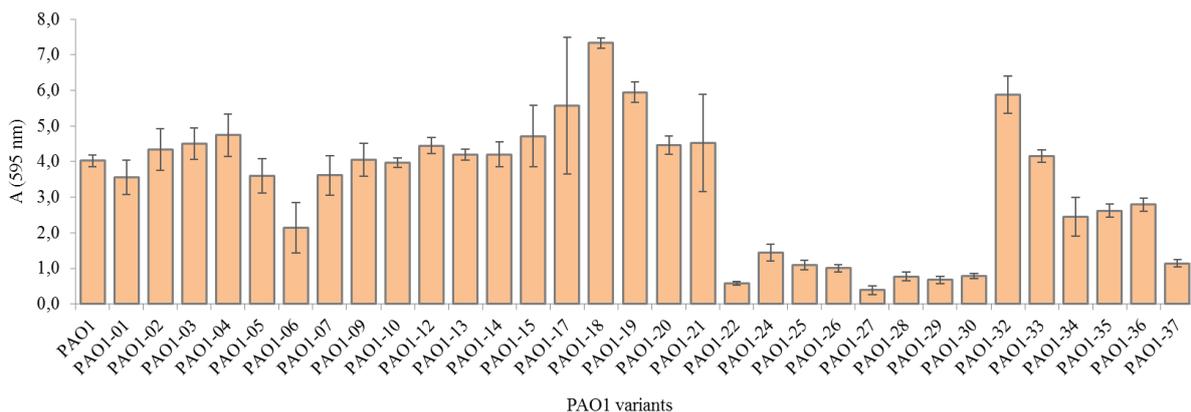


FIGURE 26. BIOFILM FORMATION ASSAY OF ORIGINAL (P3) PHAGE-TOLERANT VARIANTS. The amount of bacteria bound to the wells was evaluated by measuring the A₅₉₅ of crystal violet resuspended in ethanol. The standard deviation is the result of three independent assays.

As previously indicated, the diameter of the twitching zone was significantly reduced in all variants compared to the PAO1_{Or} control, but the strongest effect was observed with those bearing a mutation in Pil genes and/or resisting Ab05 infection (Figure 25). PAO1-32 and PAO1-34 were also affected in twitching although no Pil mutations could be observed. We speculate that this behavior may be related to a continuous cell death due to phage production. Indeed upon culture in LB broth the cells lysed totally after reaching an absorbance at 600 nm (A_{600}) of 0.8.

In order to prove that mutations in type IV pili were actually responsible for the bacterial resistance to phage Ab05, complementation assays were performed. Variants PAO1-24_1 and PAO1-25_1, *pilR* mutants, and variants PAO1-20_1, PAO1-26_1 and PAO1-30_1, *pilQ* mutants, were successfully complemented using respectively the full *pilR* or *pilQ* gene, and their susceptibility to phage Ab05 was restored.

II.V.II THE MUCOID PHENOTYPE COULD BE REVERTED THROUGH COMPLEMENTATION

To confirm that the observed *mucA* mutations were responsible for the mucoid phenotype, we tested whether the mutants could be complemented by the wild-type gene. A full *mucA* amplicon was cloned into an expression vector which was then introduced into PAO1-02, PAO1-06 and PAO1-13. In the three cases the transformants were no longer showing a mucoid appearance, whereas the vector alone was not reversing the mucoid phenotype. In addition, the *mucA* transformants recovered normal susceptibility to all phages.

II.V.III LIPOPOLYSACCHARIDE IS MODIFIED IN AB09-, AB17- AND AB27-TOLERANT VARIANTS

As already mentioned in the “INTRODUCTION”, in strain PAO1, the O-antigen is produced via the *wzy/wzx*-dependent assembly pathway, allowing the production of B-chains (Islam and Lam 2014). The undecaprenyl-pyrophosphate-linked repeat units are translocated from the inner to the outer leaflet of the inner membrane by flippase Wzx (Marolda, Vicarioli *et al.* 2004), where they are polymerized by Wzy. O-unit addition occurs at the reducing terminus of the growing chain, the length of which is regulated by the polysaccharide copolymerase Wzz, resulting in organism-specific preferred modal lengths. This polymerized glycan is then anchored to lipid A-core oligosaccharide by WaaL in the case of O-antigen to form a mature LPS molecule (Ruan, Loyola *et al.* 2012). The LPS A-chains consist in a succession of tri-D-rhamnose units, added on the L-rhamnose bound to the β -glucose residue and WbpL is responsible for this reaction (Lam, Taylor *et al.* 2011). The same enzyme allows the formation of the acceptor for the consequent addition of the trisaccharide units by the Wzy enzyme ensuring the formation of the B-chains (Figure 27). A large number of genes, including *wapH*, *dnpA* and *pgi*, as explained above, are thought to play a role in the biosynthesis of the different glycoforms necessary to insure the production of LPS but their precise site of action has not been clarified yet.

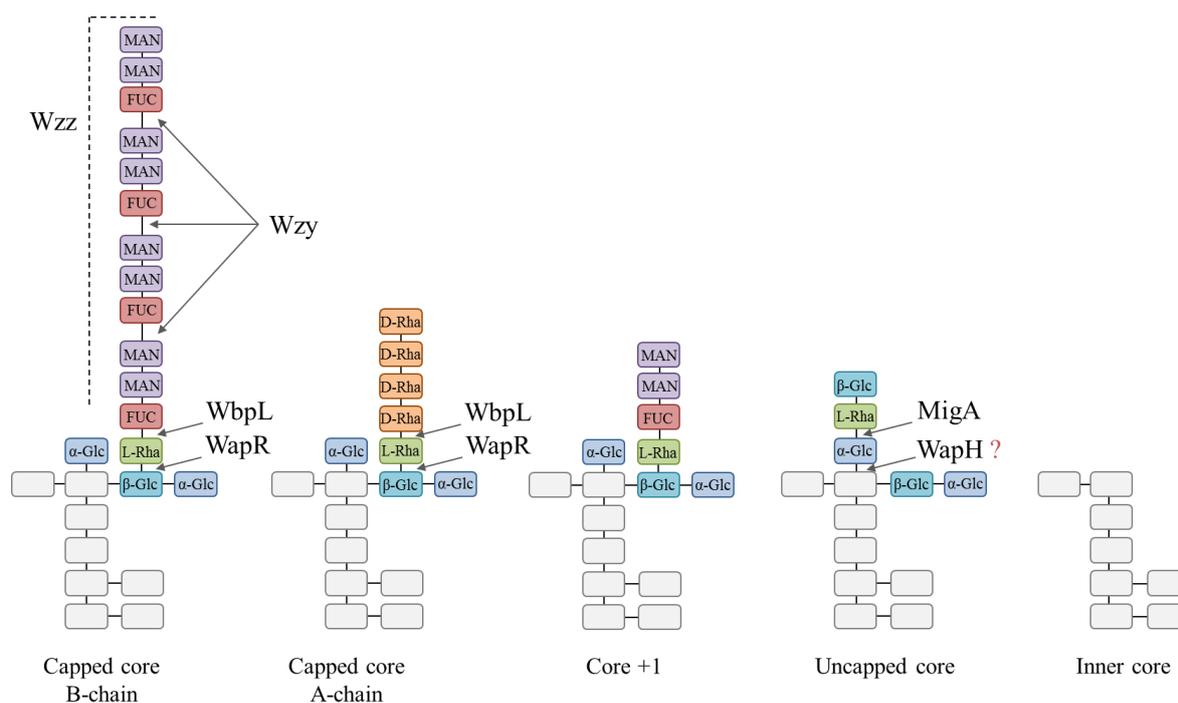


FIGURE 27. SITE OF ACTION OF DIFFERENT PROTEINS KNOWN TO BE INVOLVED IN THE LIPOLYSACCHARIDE SYNTHESIS. The capped core form carrying the B-chains or A-chains and the uncapped core form of LPS are simultaneously present on the surface of *P. aeruginosa* PAO1. The role of WapH is hypothetical (Kocíncová, Ostler *et al.* 2012).

In order to better assess the effect of the different mutations we observed in phage-resistant mutants, the LPS of eleven variants representative of each different mutation was extracted and then analyzed by SDS-PAGE (Figure 28). All the samples used for this analysis were prepared from P3 mutants (PAO1-04, PAO1-07, PAO1-01, PAO1-03, PAO1-10, PAO1-18 and PAO1-15) or, for those that were shown to contain phage DNA (PAO1-37 and PAO1-19), re-isolated colonies possessing the same mutation as the original P3 mutant but devoid of phage DNA (confirmed by PCR and sequencing). PAO1-20_1 and PAO1-36_1 were secondary mutants whose genome characteristics are reported in Table 5.

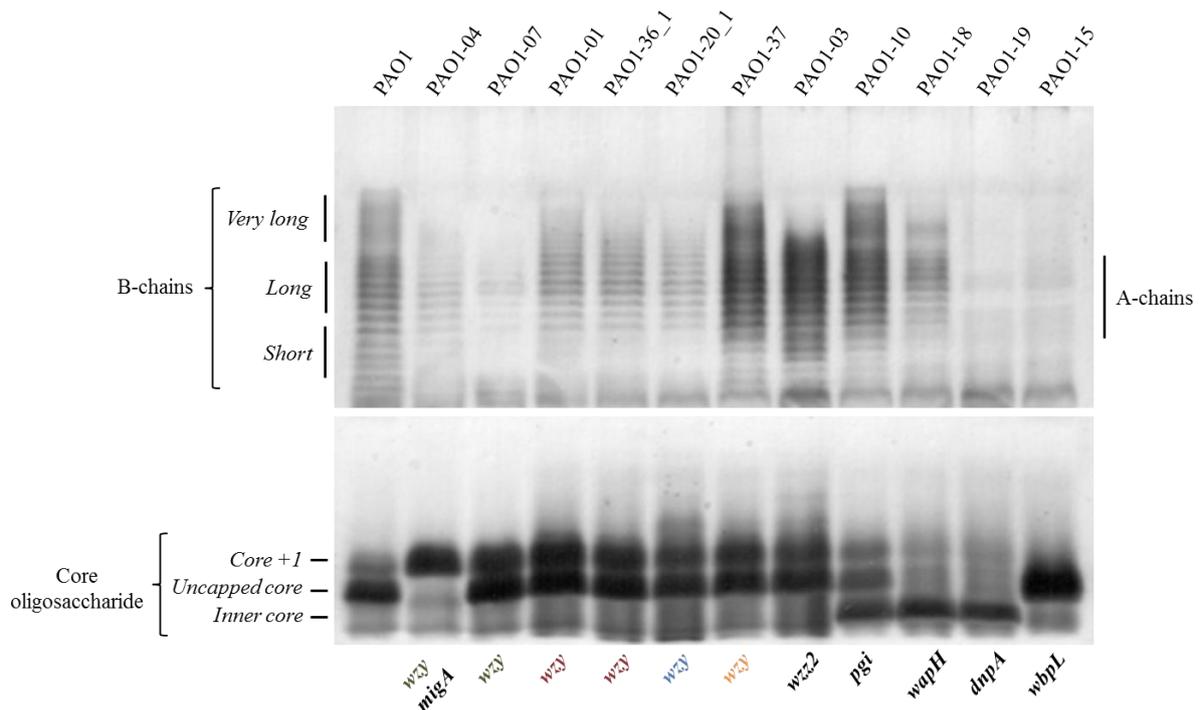


FIGURE 28. SDS-PAGE OF LIPOPOLYSACCHARIDE EXTRACTED FROM DIFFERENT MUTANTS. The mutated gene is reported in the bottom part of the picture. For *wzy* gene, the color represents a specific kind of mutation. Different amounts of samples were loaded on the two gels in order to discriminate the different bands: 12.5 μ g of resuspended lyophilized bacteria were loaded on the top gel and 2.5 μ g on the bottom one. WT, wild-type.

THE WZY MUTANTS

PAO1-04 and PAO1-07 possessing the same *wzy* mutation showed the same banding pattern of the O-antigen characterized by absence of B-chains. PAO1-04, with an additional mutation in *migA* gene, appeared to have converted all the core oligosaccharide in core +1 form. The absence of B-chains would explain why these bacteria completely resist phages Ab09, Ab17 and Ab27. Accordingly, complementation of PAO1-04 and PAO1-07 for the *wzy* gene restored the susceptibility to the three phages.

The LPS of PAO1-01 and PAO1-36_1 appeared to possess A-chains, but to be completely defective in B-chains. In both variants, mutations existed in the homopolymeric tracts of As located at the beginning of the coding sequence of *wzy* gene. These mutations induce a

frameshift producing a truncated protein of 74 and 54 aminoacids in PAO1-01 and PAO1-36_1, respectively. Both possessed the same banding pattern concerning the core oligosaccharide but, compared to the wild-type PAO1_{Or}, the core +1 form is present in higher amount. Both mutants could be successfully complemented by the wild-type *wzy* gene.

The LPS of PAO1-20_1 presented a similar banding pattern for both O-antigen and core oligosaccharide compared to PAO1-01 and PAO1-36_1. Interestingly, the mutation in *wzy* involved a nucleotide substitution producing a Wzy protein with an aminoacid substitution at position 42 (Ser → Phe). The modification of the Wzy protein allowed the bacteria to be completely resistant to phages Ab09, Ab17 and Ab27.

Islam and colleagues (Islam, Huszczyński *et al.* 2013) performed systematic site-directed mutagenesis of 83 periplasmic and cytoplasmic residue positions spanning the length of the Wzy protein of *P. aeruginosa* PAO1 in order to identify residues of functional importance for the polymerization of O-antigen. They did not obtain a mutant similar to mutant PAO1-20_1. As previously said, the mutation in PAO1-20_1 completely abolished the B-chains synthesis of O-antigen suggesting that the Serine, a polar residue, if exchanged with a hydrophobic residue as the Phenylalanine, can induce conformational change that alters the activity of the protein.

An aminoacid substitution was also identified in PAO1-37 Wzy protein. In this case, an Aspartate residue (position 286) was replaced by a Glycine. The presence of this mutation seemed not to impede the production of the O-antigen and core oligosaccharide. Only a slight increase in the amount of core +1 form, A-chains and long and very long B-chains, could be observed compared to the wild-type PAO1_{Or}. It is possible that the mutated protein directly plays a role in the bacterial resistance to phages. Indeed PAO1-37 appeared to be completely resistant to phage Ab27 and less susceptible to phages Ab09 and Ab17. Mutations of this

particular aminoacid have been described by Islam and colleagues (Islam, Huszczyński *et al.* 2013). They found that a mutant Asp → Ala at position 286 could only partially restore B-chains biosynthesis when used in the complementation assay in a mutant PAO1 lacking *wzy* gene. The defect could be fully complemented by the like-charge substitution Asp → Glu, resulting in a Wzy variant that recovered the wild-type activity.

Mutants PAO1-20_1 and PAO1-37 could not be complemented by the wild-type *wzy* gene.

THE WZZ2 MUTANT

PAO1-03, possessing a frameshift mutation in *wzz2* gene, presented a normal banding pattern concerning A-chains, short and long B-chains but lacked the very long B-chains (Figure 28). The core +1 form, as well as all the chains of the O-antigen possessed by this variant, seemed to be present in higher amount compared to the wild-type PAO1_{Or}. The involvement of the Wzz2 in the synthesis of very long B-chains has been reported by Daniels and colleagues (Daniels, Griffiths *et al.* 2002). The lack of very long chains seemed to affect the infection by phages Ab09 and Ab27 reducing their efficiency of plating.

THE PGI, WAPH, DNPA AND WBPL MUTANTS

An aminoacid substitution replacing a Threonine with a Proline in the *pgi* gene of PAO1-10 seemed to partially affect the production of short A- and B-chains, whereas long and very long chains were not affected. The amount of core +1 form was comparable with that of the wild-type PAO1_{Or}. The uncapped core band was less intense whereas the band corresponding to the inner core is much more intense. The ability to produce clear plaques by phages Ab09 and Ab17 was impaired. These phages produced turbid plaques but with the same efficiency with which they produced clear plaques on the wild-type PAO1_{Or}. In contrast, the efficiency of plating was reduced for phage Ab27. The *pgi* gene has been shown to encode the

phosphoglucose isomerase enzyme involved in the reversible conversion of glucose-6-phosphate in fructose-6-phosphate, and this, together with the activity of other enzymes, has been proposed to be one of the fundamental reactions necessary for the production of the monomers that constitute the LPS (Ye, Zielinski *et al.* 1994).

PAO1-18 was shown to be completely resistant to phages Ab17 and Ab27, and to be partially resistant (reduced efficiency of plating) to phage Ab09. It possessed a mutation in *wapH* resulting in an aminoacid substitution (Arg → Leu). The LPS O-antigen banding pattern appeared to be somewhat similar to that of PAO1-01, PAO1-36_1 and PAO1-20_1, presenting A-chains, some long B-chains, but lacking the short ones. The core oligosaccharide was reduced to the inner core form and neither the uncapped core nor the core +1 forms could be detected. The role of WapH, a glucosyltransferase, has not been described yet, but Kocíncová and colleagues hypothesized that it could be involved in the transfer of α -glucose (II) to the inner core of the LPS (Kocíncová, Ostler *et al.* 2012).

The inner core oligosaccharide was the only form present in the LPS of PAO1-19. The *dnpA* gene of this mutant produces a 67 aminoacids protein instead of the wild-type 472 aminoacids protein, leading to a complete lack of A- and B-chains of the O-antigen. This made it resistant to phages Ab17 and Ab27, and partially resistant to phage Ab09, in a way similar to PAO1-18. DnpA, a de-N-acetylase enzyme, has been shown to play a role in non-inherited *P. aeruginosa* fluoroquinolone tolerance and to allow the increase of the persistence fraction of *P. aeruginosa* both in planktonic culture and in a biofilm model (Liebens, Defraigne *et al.* 2014). DnpA is part of the conserved core oligosaccharide biosynthesis gene cluster but Liebens and colleagues failed to identify the alterations in the core oligosaccharide when they analysed the LPS of a transposon mutant for this protein (Liebens, Defraigne *et al.* 2014). Here, the PAO1-19 profile shown on Figure 28 is unique.

In contrast, PAO1-15, although lacking A- and B-chains, possessed a wild-type uncapped core oligosaccharide form. We identified a mutation in the *wbpL* gene (the corresponding protein is 88 aminoacids in length instead of 339 aminoacids), known to be required for the initiation of both A- and B-chains synthesis (Rocchetta, Burrows *et al.* 1998). This variant was totally resistant to phages Ab09, Ab17 and Ab27.

Taking in consideration these different observations, we conclude that Ab09, Ab17 and Ab27 require the presence of B-chains to infect PAO1_{Or}. More specifically we think that Ab17 necessitates the presence of short B-chains since it is not at all able to infect variants that lack them (PAO1-04, PAO1-07, PAO1-36_1, PAO1-01, PAO1-20_1, PAO1-18, PAO1-19 and PAO1-15) but it can infect PAO1-37 and PAO1-10 with the same efficiency, although producing smaller and more turbid plaques. In these two mutants the short B-chains are present even if in less amount compared to the wild-type PAO1_{Or}. Another observation that can support this hypothesis is given by the fact that mutant PAO1-03 possessing an amount of short B-chains similar to that of the wild-type PAO1_{Or} is normally susceptible to phage Ab17 infection.

To go further in the identification of the phage receptors, we tested the susceptibility of a PAO1 *migA* transposon mutant to the three phages: the mutant was completely resistant to Ab09, partially resistant to Ab27 and completely susceptible to Ab17. MigA is a rhamnosyltransferase, an enzyme able to link L-rhamnose to the α -glucose (II) of the core oligosaccharide (Figure 27). Defects in or absence of this enzyme, results in the lack of uncapped oligosaccharide form on the bacterial surface, without interfering with the addition of one O-antigen unit or the complete A- and B-chains owing to the action of WapR (Figure 27) (Kocíncová, Ostler *et al.* 2012).

It seems that, like phage Ab17, phage Ab09 also requires short B-chains for the initiation of the infection since is able to infect with different efficiencies mutants PAO1-03, PAO1-10 and PAO1-37. However, the infection can occur even if the short B-chains are absent but only if the inner core is directly accessible on the bacterial surface, thus in total absence of the O-antigen, like for the mutants PAO1-18 and PAO1-19. All the other mutants are completely resistant to phage Ab09 lacking completely the short B-chains and inner core form (PAO1-04, PAO1-07, PAO1-36_1, PAO1-01, PAO1-20_1 and PAO1-15). It is possible that Ab09 needs to bind to the inner core for close association with the membrane and injection of the DNA. The fact that it is a *podovirus* (short tail) may explain the difference in comparison to Ab17 and Ab27, two *myoviruses*.

II.V.IV THE LIPOPOLYSACCHARIDE OF PSEUDOLYSOGENS

In order to investigate the basis for resistance of pseudolysogens to different phages, we analysed their LPS. The three pseudolysogens devoid of detectable mutations seemed to have lost the very long and part of the long B-chains of the O-antigen. Although not as well separated as in the control wild-type PAO1_{Or}, faint aggregations of bands could still be observed for all the samples at the level of short B-chains. The pattern is close to that of Wzy mutants, but it is difficult to understand how the presence of Ab27 or Ab05 DNA might lead to such an inhibition.

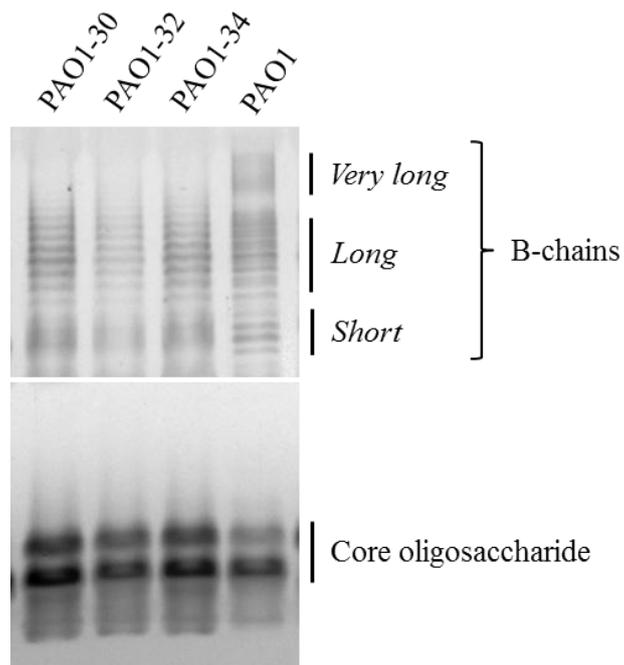


FIGURE 29 SDS-PAGE ANALYSIS OF THREE LPS EXTRACTED FROM PSEUDOLYSOGENS COMPARED WITH THE WT PAO1_{Or}. Different amounts of samples were loaded on the two gels in order to discriminate the different bands: 12.5 μ g of lyophilized bacteria were loaded on the gel shown on top and 2.5 μ g on the bottom one.

GLOBAL DISCUSSION

I PSEUDOLYSOGENY ALLOWS SELECTION OF MUTANTS

In our experimental model, pseudolysogeny appears to be a frequent outcome of infection by the four virulent phages, providing immunity to the bacteria, and allowing emergence of mutations in genes involved in receptor synthesis. In the present investigation, we might even be underestimating the frequency of pseudolysogeny as we started the analyses after three replatings for purification purposes. The frequency of single mutants was on the order of one per 10^5 plated bacteria but, surprisingly, we observed that double mutants could be recovered at a frequency of 10^{-6} , which is far higher than expected if these were present at the onset of infection. We show that selection of a second mutation takes place in pseudolysogenic colonies that can constitute a reservoir for bacteriophages exerting a permanent pressure on the bacteria.

I.I CONDITIONS FAVORING THE APPEARANCE OF PSEUDOLYSOGENS

Five sectors have been identified in a phage plaque. Layer V, the more external, is constituted by uninfected bacterial cells replicating normally and benefiting of the fresh media surrounding them. In layer IV, more internal, bacteria replicate, phages are present and diffuse through the extracellular environment. Layer III is constituted of infected cells that are not lysed yet and layer II is the turbid one, where most of the infected cells are lysed. Layer I in the middle of the plaque contains no more infected or susceptible bacteria, but rather phage particles and resistant variants (Abedon 2008). It is possible that in the middle of a plaque, the medium has lost most of its nutritive components used by the bacteria to replicate and allow phage amplification.

In order to isolate phage-tolerant variants, we performed infections on plates using the approach described in ANNEX III: phages and bacteria are mixed and plated on the agar layer, phage-infected cells allow phage multiplication and plaques formations, the infection propagates and the plaques enlarge until all the susceptible bacteria present on the plate, with the exception of those that are phage-tolerant, are lysed. Thus we can imagine that bacteria on the plate passed through all the steps of plaques formation being subjected to different medium composition and variable amount of phages. In this situation, the only surviving bacterial cells are those that are phage-tolerant because they possess mutations in phage receptor and/or have been pseudolysogenized by the phage. We cannot predict whether the pseudolysogens appear early during the plaque formation, thus in the layer I, or during the plaque enlargement, for example in layers II or III. However, it would be plausible to think that pseudolysogeny occurs when the amount of phages and resistant mutants is higher than the total amount of susceptible bacteria allowing phages to be protected against extinction.

I.II PSEUDOLYSOGENY CONTRIBUTES TO THE SELECTION OF SINGLE/DOUBLE MUTANTS

In this study we observed that the selection of single and/or double mutants could be influenced by the appearance of pseudolysogenic cells. We propose a model in which a pseudolysogenic cell, containing several phage genome copies, forms, after several rounds of division, a colony containing bacteria cured of the phage and bacteria in which the phage lytic cycle is resumed, producing new phages (Figure 30). The cured bacteria become prey for further amplification and production of new pseudolysogens in which phage growth is stalled. However, in the present study the phage/host equilibrium is not stable. The relative efficiency of reactivation of the phage cycle and production of cured bacteria determines the duration of the pseudolysogeny stage.

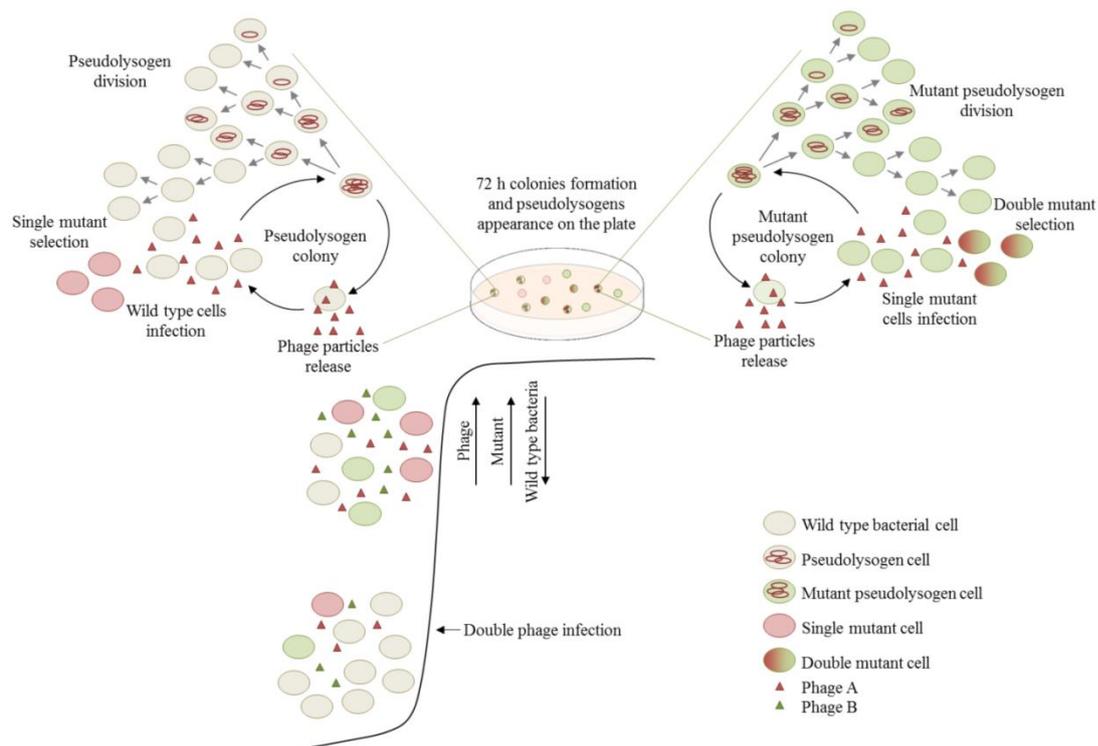


FIGURE 30. MODEL OF PSEUDOLYSOGENY EVOLUTION. Continuous production of cured bacteria and release of phages from reactivated lytic cycle in pseudolysogenic cells leads to the emergence of mutations. Single mutants (left) or double mutants (right) selected by bacteriophage (phage A) resulted from the activation of a lytic cycle in a wild-type pseudolysogen or in a pseudolysogen already containing a mutation, previously selected by another phage using a different receptor for infection (phage B), respectively.

I.III IMMUNITY PROVIDED BY THE PSEUDOLYSOGENIC PHAGE

Temperate phages are able, through lysogenization, to provide the host with immunity to superinfection mediated by immunity genes and this characteristic is supposed to differentiate true lysogeny from pseudolysogeny (Wommack and Colwell 2000). The present pseudolysogens demonstrate inhibition of superinfection by the same phage and, more interestingly, by phages of different genera, which bind to different receptors. Performing LPS analysis on three pseudolysogens in which we did not detect mutations, PAO1-30, PAO1-32 and PAO1-34, we found that the structure of their O-antigen is altered when compared to wild-type PAO1_{Or}.

There are often several genes located in the early region of the phage genome, whose function is unknown and that may play an important role during the first steps of infection modulating the bacterial metabolic machinery. In the case of our pseudolysogenic phages, these genes might alter the bacterial LPS biosynthetic pathway making the receptor temporarily unavailable, and consequently allowing the infected cells to become resistant to superinfection by the same phage or other phages. Particularly interesting is the case of the variant PAO1-30. This variant is pseudolysogen for the phage Ab05 and it demonstrates superinfection inhibition for the same phage but also for phages Ab17 and Ab27.

In general, in phages belonging to the same genus, the early genes region can present high level of divergence as seen, for example, in ϕ -KMV-like phages (Klimuk, Akulenko *et al.* 2013). One interesting example is provided by the *P. aeruginosa* LKA1 phage, whose early region encodes 18 small proteins with currently no similarity to other gene products in the GenBank database. In the same way, phage Ab05 possesses an early region encoding several proteins with unknown function. It is tempting to speculate that they might play a role in the regulation of the maintenance of pseudolysogeny in the host population and more generally allow the bacteria to switch between one phage life strategy and another one according to the environmental conditions. They might also modulate the production of LPS protecting the bacteria from the infection with the other phages.

II RED QUEEN DYNAMICS/ARM RACE CO-EVOLUTION

Studies performed in chemostats have addressed the co-evolution dynamics of phage and bacteria in controlled growth conditions (Buckling and Rainey 2002; Betts, Kaltz *et al.* 2014). Generally, when preys and predators are left to evolve for a long time two possible outcomes are predictable: the Arms race, the fittest genotype survives and this limits the diversity, or the Red Queen dynamics, where the frequency-dependent selection leads to constant production of new mutants, thus maintaining diversity (Dennehy 2012).

In our assay, which takes place in a micro community, after several rounds of co-evolution, the population of free phages fluctuates, to the extent that they may seem to almost disappear within the colony. A large diversity of resistant mutants is selected, and eventually the colony will be phage-free. Reversion to wild-type phenotype is observed for alginate and LPS mutants so that new preys will emerge.

II.I FITNESS COST LINKED TO ACQUISITION OF PHAGE-RESISTANCE

A large proportion of the phage-tolerant variants that we isolated possessed frameshift mutations known as phase variation (Henderson, Owen *et al.* 1999). Frameshift mutations, allow bacteria to adapt to different kind of environments and are reversible when the selective pressure is no longer applied (Segura, Hurtado *et al.* 2004).

Frameshift mutations that we identified occurred in the *wzy/wzx*-dependent pathway responsible for the synthesis of O-antigen (Islam and Lam 2014) and in genes involved in the alginate biosynthesis regulation. Both wild-type and mutant forms of *wzy* and *mucA* genes were simultaneously found in the presently described mutants, suggesting that the mutation can reverse at a high rate. Constant variations in LPS and alginate biosynthesis pathways may help *P. aeruginosa* face aggressions or environmental changes. This might be one explanation

for the “colonial dissociation” frequently observed with *P. aeruginosa*, characterized by colonial differences of a single strain (Zierdt and Schmidt 1964).

It may be worth to mention that, with one exception (PAO1-22), all the mutations occurring in the type IV pilus biosynthetic genes were deletions not likely to revert back to the wild-type genotype. Most probably the lack of type IV pili, even in absence of phages targeting the pilus as a receptor, does not affect the surviving and the normal growth of the bacteria. In contrast, the mutations occurring in LPS biosynthesis genes could seriously impact the surviving of the bacteria and can require a high fitness cost that induces the bacteria to revert back to the wild-type phenotype as soon as the selective pressure imposed by the phage is no longer present.

The failure to isolate stable Ab27-tolerant variants could be due to high fitness cost associated with the acquisition of phage-tolerance through alteration of the phage receptor. The selection of small-colony phage-tolerant variants has been documented with phages E79 (Hosseinidoust, Tufenkji *et al.* 2013), KTN6 and KT28 (Danis-Włodarczyk, Olszak *et al.* 2015), and PB1 (Lim, Phang *et al.* 2016) all belonging to the PB1-like genus. The small colony variants isolated during the course of those studies showed impaired biofilm formation, decreased twitching motility, reduced elastase and pyocyanin production, higher susceptibility to the antibiotic ciprofloxacin and exhibited higher surface hydrophobicity than the wild-type strain, indicative of changes in the LPS (Lim, Phang *et al.* 2016). Although all these alterations could allow the bacteria to resist the phage, they could impair the growth and survival of the bacteria in absence of the applied selective pressure (phage), or they could be highly reversible mutations that, when the phage is no longer present (P3 stage), allow the bacteria to switch back to the wild-type phenotype.

II.II PSEUDOLYSOGENIC MUTANT PHAGES

We observed, with three phages, the presence of new phage genotypes in pseudolysogens, all three showing one or two SNPs in a tail fiber gene. The mutations were present in a subpopulation of phages used to derive the resistant mutants, and may have been selected during co-evolution of phages and bacteria. No particular behavior of these phages as compared to the parental ones, such as plaque morphology, and growth characteristics could be demonstrated. However, it is possible that these phages are capable of inducing a pseudolysogenic stage at a higher frequency as compared to the ancestral phage. Our results confirm that success in infection is not sufficient for phage survival, as phages are dependent upon the survival of their host population (Chaturongakul and Ounjai 2014), and therefore phage-host relationships can be seen as not merely parasitic but as mutualistic (Williams 2013).

It is argued that in an environment of homogeneous mixing, such as a liquid culture, a phage should evolve toward a high adsorption rate to maximize its fitness (Shao and Wang 2008). But not all hosts are equally worth infecting. Unlike phage T7, whose productive infection is independent of host physiological state (Schrader, Schrader *et al.* 1997), most phages rely on the exponentially growing bacterial cells for productive infections (Adams 1959). Because natural bacterial populations are mostly in stationary phase or follow an alternation of exponential and stationary phases, it is easy to imagine that phages regularly have to cope with bacterial hosts in various physiological states, constituting uncertain environments.

By definition, strictly lytic phages cannot become prophages. Therefore, it is not clear how these viruses survive an adverse environment like the stationary phase. At least three scenarios are possible. (1) Pseudolysogeny, in which the infecting phage genome is not integrated into the bacterial genome but stays in the cytoplasm as an episome (Ripp and

Miller 1998). (2) The production of virions with different adsorption rates by a single genotype (diversified bet-hedging). Such phenomenon has already been observed in early phage literature: Schlesinger (Schlesinger 1932) reported the existence of a small subpopulation of phages with very low adsorption rates, referred to as the “residual fraction” and measured its proportion (0.3%). Those phages could be seen as a form of dormancy that allows phages to survive harsh seasons like stationary phase. (3) Selection of a phage genotype with an intermediate adsorption rate (conservative bet-hedging). As reported by Gallet and colleagues (Gallet, Lenormand *et al.* 2012) high adsorption rate would be favorable for phage multiplication during bacterial exponential growth, while lower adsorption rate will provide phages an advantage in presence of stationary phase host. Since during bacterial growth the bacterial cells are not synchronized one could imagine that mixed phage population with different adsorption rates could constitute an advantage allowing the phages to be protected from extinction.

We were able to find, in the case of Ab05, only the mutant phage in the pseudolysogens that were analysed (PAO1-26, PAO1-30 and PAO1-37), thus we could hypothesize that this constitute the slowly adsorbing phage faction and that this characteristic can somehow favor the appearance of pseudolysogen allowing phage to be protected from extinction. Additional experiments are needed to verify this hypothesis, as the mutant phage appears to produce normal plaques on PAO1.

CONCLUSIONS

Phage therapy is considered as a promising complementary approach to fight antibiotic resistant strains (Abedon, Kuhl *et al.* 2011). Either readymade cocktails or "sur-measure" phages will be used to treat patients, similarly to what is still done in several countries of Eastern Europe. It is important to investigate the risks linked to the use of phages, particularly in the selection of bacterial mutants that could show deleterious characteristics (Hosseini-doust, Tufenkji *et al.* 2013), or drive the expression of undesirable bacterial virulence factors (Olszak, Zarnowiec *et al.* 2015).

The different phenotypic assays that we performed showed that, depending on the phage used, the selected mutants, obtained at a high frequency, display a large variety of phenotypic changes related to membrane permeability and cell motility. Hosseini-doust *et al.* (Hosseini-doust, Tufenkji *et al.* 2013) described such phenotypes induced by two phages which use type IV pilus and LPS as receptors, but in the course of their study they could not identify the mutations (Hosseini-doust, Tufenkji *et al.* 2013). We took advantage of Illumina sequencing technology to identify bacterial mutations at a relatively low cost and were able to perform an original work going further on the analysis of phenotypic and genotypic traits selected by the phages.

Phenotypic changes have been shown to alter bacterial virulence (Lyczak, Cannon *et al.* 2000). In the same way, the phage-resistant phenotypes selected by phages used in this study could seriously affect the outcome of treatment in the context of phage therapy. Indeed, we showed that phage Ab09 often selects for mutants with a mucoid phenotype, related to an increased capability to produce alginates. In the context of CF infection, mucoidy has been shown to favor the formation of protected colonies with increased resistance to opsonization,

phagocytosis and destruction by antibiotics (Pritt, O'Brien *et al.* 2007). This suggests that Ab09-like phages should be used with caution.

In the course of the same study, we isolated reversible phase variation mutations mainly in genes encoding enzymes for the LPS synthesis, and irreversible deletions in genes for the type IV pilus biogenesis. As previously mentioned, the adherence step of *P. aeruginosa* cells to the epithelium appears to be the most important and critical stage of the infection process. Since the pilus is responsible for the initial contact between the bacterium and the epithelial cell surface (Bucior, Pielage *et al.* 2012), successful blocking of bacterial adherence, through mutations in genes regulating the expression of the type IV pilus, could result in prevention of infection (Hahn 1997), thus making the ϕ -KMV phages good candidates for the constitution of phage cocktails for phage therapy treatment. In contrast, the high reversibility of phase variation mutations that we identified in most of the genes involved in LPS biosynthesis, selected by phages targeting LPS as a receptor, make such phages less suitable for phage therapy. Moreover, it has been reported that alterations of a single chain or both A- and B-chains of the O-antigen of *P. aeruginosa* PAO1 can give rise to mutants with increased cytotoxicity mediated by the type III secretion system (TTSS) (Augustin, Song *et al.* 2007). In addition, changes in O-antigen expression in PAO1 affects the size and protein content of outer membrane vesicles and favors the formation of a robust biofilm (Murphy, Park *et al.* 2014).

The approach we used allowed us to demonstrate the central role played by pseudolysogeny during lytic phage infections. This role had previously been underestimated for a number of reasons and now it opens up a new area of phage research at a time when our appreciation of the impacts of microorganisms on ecosystem function and biological processes is rising.

In summary, in a phage therapy context, it is very important to study the effect each phage can have on the bacterial strain causing a disease, alone or in combination with other phages in the form of a cocktail. It is also necessary to consider that pseudolysogeny plays an important role in the persistence of phages in the bacterial population, acting in the selection of phage-tolerant variants and allowing phage-bacteria co-evolution.

All these evidences support the idea that it is better not to abandon the use of antibiotics, as it has already been proposed (Torres-Barceló and Hochberg 2016), since together with phages they can complement each other in the killing of the pathogenic bacterial strain.

PERSPECTIVES

NEW INSIGHTS INTO PSEUDOLYSOGENY

The tremendous impact of bacteriophages, the biosphere's most numerous organisms, on the ecology and evolution of bacteria is increasingly recognized, but most knowledge is limited to a few well-studied model organisms in highly controlled and restricted laboratory conditions. Hence, the lytic and lysogenic life cycles of organisms, such as phage λ or phage T4, have been exceptionally well-characterized. However, since the 1920s, other phage lifestyles were suspected to exist, namely pseudolysogeny, where phage DNA is neither transcribed nor integrated into the host genome following infection. In addition, the number and diversity of newly isolated phages let imagine that multiple types of interactions may exist with their host.

Pseudolysogeny is poorly studied because it is technically difficult to study at the single cell level and is believed to be a consequence of host starvation. Recent studies, however, show that pseudolysogeny is probably widespread, and can occur even in healthy, actively growing hosts. It is likely that pseudolysogeny, like lysogeny, is a bet hedging strategy against host and phage extinction. Pseudolysogens are resistant to phage attack, but can be cured of infection, thus providing additional sensitive hosts on which phage infections can be sustained. As such, pseudolysogeny can drive bacterial co-evolution and facilitate horizontal gene transfer of genes increasing bacterial fitness.

The recent development of new technologies such as fluorescent time-lapse imaging and RNA-Seq provide new tools to overcome the difficulties associated with studying pseudolysogeny. Herein two hypotheses are proposed regarding the induction of pseudolysogeny: i) the frequency of pseudolysogeny depends on the multiplicity of infection (i.e., ratio of phage:host) and ii) expression of a phage transcription inhibitor induces and maintains pseudolysogeny. A model is introduced where a phage transcription inhibitor inducing pseudolysogeny is rapidly degraded in healthy cells

but accumulates in slowly growing and/or multiplying infected cells. This inhibitor, either alone or in conjunction with a bacterial surface-receptor altering protein, provides superinfection immunity to pseudolysogenic cells. Pseudolysogeny in this manner is an evolved bacteriophage strategy that results in i) phage persistence; ii) bacterial host persistence; and iii) bacterial host diversification and co-evolution rather than a side-effect of host starvation. We are developing a project aiming to establish the conditions inducing pseudolysogeny and to analyse the molecular players, either bacterial or phagic, that act to maintain this stage. These studies would open up a new area of phage research and clarify aspects of pseudolysogeny that could play crucial roles in shaping microbial communities in various environments.

PHAGE-RESISTANCE IN ANOTHER *P. AERUGINOSA* STRAIN

In order to assess if the same virulent phages used to select *P. aeruginosa* PAO1 phage-resistant variants, are able to select similar variants in another *P. aeruginosa* strain, we chose PcyII-10 strain isolated from a burned patient of the Percy Hospital (Clamart, France) and showing high susceptibility to many phages. We performed infections at different MOI and isolated a collection of about 40 different variants tolerant at least to one phage used for the infection. We started the phenotypic characterization and we selected 10 phage-tolerant variants that are being presently sequenced. Preliminary results showed that, similarly to what was observed for Ab05-infected PAO1_{Or} variants, phage Ab05 was able to pseudolysogenize PcyII-10 at high frequency and select for variants with reduced efficiency of biofilm production and twitching motility. Concerning the infection performed with the phage Ab09, mucoid variants could be selected; surprisingly, other variants selected by this phage were able to tolerate also the infection with phage Ab05 suggesting that these variant may possess alterations of the pilus type IV structure, other than those allowing the tolerance to phage Ab09. We hypothesized that the phage may have selected for variants with large genomic deletions, similar to those selected by the temperate phage Ab31, encompassing genes for the biosynthesis of receptor for both phages. Preliminary PCR analysis performed on one

of these variant showed that as a matter of fact it could present a big deletion, but in order to have reliable evidences to prove our hypothesis, complete genome sequencing data are necessary.

PSEUDOLYSOGENY AND CRISPR-CAS SYSTEM

The strain PcyII-10 was chosen because it possesses three type I-F CRISPR elements and a set of *cas* genes, potentially capable of participating in resistance to phages (Bikard and Marraffini 2012; Cady, Bondy-Denomy *et al.* 2012). We think that pseudolysogeny might give time and opportunity to the bacteria to acquire new spacers.

THE LIPOPOLYSACCHARIDE AS A PHAGE RECEPTOR

The *P. aeruginosa* mutants with divergent LPS synthesis following the acquisition of host resistance are presently being investigated in detail in order to identify the precise phage receptor. LPSs are purified and analyzed by PAGE. In addition, some PAO1_{Or} phage-tolerant variants are being analysed by mass spectrometry. This work, in conjunction with purified-LPS phage-adsorption assays, will help understand the exact site of adsorption used by the phage during the first steps of infection.

LINK BETWEEN PHAGE AND ANTIBIOTIC RESISTANCE

Using virulent phages we selected for some resistant variants possessing alterations in the structure of the phage receptor and, more in general, of their extracellular surface. For instance, performing double infection with phage Ab09 and Ab17, we were able to isolate a *dnpA* mutant, the variant PAO1-19, known from literature to possess reduced tolerance to fluoroquinolone treatment (Liebens, Defraigne *et al.* 2014). It would be interesting to perform an antibiotic sensitivity test on all the variants we isolated in order to look whether their antibiogram has changed compared to that of the wild-type PAO1_{Or} strain.

MATERIAL AND METHODS

I PHAGES

Two *podoviruses*, vB_PaeP_PAO1_Ab05 (Ab05) and vB_PaeP_C2-10_Ab09 (Ab09), and two *myoviruses*, vB_PaeM_PAO1_Ab17 (Ab17) and vB_PaeM_PAO1_Ab27 (Ab27), were used in this study, alone, or combining a *podovirus* with a *myovirus*, or in a cocktail of four phages as described in “II.I RATIONALE AND EXPERIMENTAL PROCEDURE”. Ab05 is a ϕ -KMV like phage, Ab09 is a LIT1-like phage, Ab17 is a KPP10-like phage and Ab27 is a PB1-like phage. They have been isolated in Abidjan (Côte d’Ivoire) and have been described in detail in (Essoh, Latino *et al.* 2015). On PAO1_{Or}, phage Ab05 produced clear plaques with a halo, phage Ab09 and phage Ab17 produced clear plaques of medium size. In contrast, phage Ab27 produced turbid tiny plaques.

I.I PHAGE AMPLIFICATION

Phage stock was prepared by mixing a single plaque with 100 μ l of fresh overnight PAO1_{Or} culture, keeping the mixture for 15 min at room temperature and then pouring it on a freshly prepared LB (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 15 g/l agar) plate using 4 ml of soft agar (10 g/l tryptone, 5 g/l yeast extract, 10 g/l NaCl and 7 g/l agar). The plate was incubated for 6-8 h at 37°C until all the bacteria were completely lysed. Then, the top agar containing the amplified phage particles was recovered and resuspended in 5 ml of SMG buffer (NaCl at 5.8 g/l, MgSO₄ at 2 g/l, 1 M Tris-HCl, and gelatin at 0.1 g/l [pH 8.0]). The suspension was then centrifuged at 4000 rpm for 10 min at 4°C to separate bacterial debris and agar, and the supernatant fraction containing the phages was recovered and filtered using a 0.22 μ m filter.

I.II ELECTRON MICROSCOPY

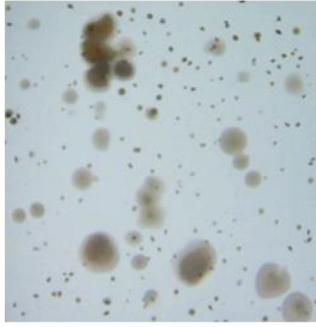
The phage stock was treated overnight at 4°C with 10% PEG 8000, and then centrifuged at 10 000 rpm for 20 min. The pellet of PEG-phages was resuspended in 1 ml of SMG buffer and one volume of chloroform was added. The suspension was centrifuged at 10 000 for 10 min at 4°C and the supernatant was recovered, filtered using 0.22 µm filter and ultracentrifuged at 30 000 rpm for 2 h at 10°C. The phage pellet was resuspended in 50 µl of SMG buffer. Five µl of the sample were stained with 2% potassium phosphotungstate (pH 7) and visualized using an EM208S transmission electron microscope (FEI, Eindhoven, The Netherlands) operating at 80 kV.

II RESISTANT-VARIANT COLLECTION PREPARATION

II.I ISOLATION OF PHAGE-RESISTANT VARIANTS

P. aeruginosa PAO1, a reference strain originating from a patient (Stover, Pham *et al.* 2000), was inoculated into 5 ml of LB medium and grown (37°C, 180 rpm) to an optical density at 600 nm (A_{600}) of 0.2. All the infections were performed at an MOI of 0.1. The majority of infections were performed on solid medium. For this a 10 µl inoculum of the bacterial culture (2×10^6 CFU) was mixed with either 10 µl containing 10^5 PFU of a single phage (Ab09, Ab17, Ab27 or Ab05), or of a 1:1 mixture of Ab09 and Ab17 or of Ab05 and Ab27, or of a 1:1:1:1 mixture of Ab09, Ab17, Ab05 and Ab27 or 10 µl of SMG phage buffer as a control. The mixture was kept for 15 min at room temperature and then poured on a fresh LB agar plate using 4 ml of soft agar pre-warmed at 45°C and the plates were incubated at 37°C for 3 days. For phage Ab27 alone or associated with Ab05, liquid infection was performed by infecting the bacteria during the log phase (A_{600} of 0.6) at an MOI of 0.001 each 24 h for a total of 3 infections. Thereafter the surviving bacteria were plated onto LB agar plates.

Phage-tolerant colonies
appearing after 72 h
from infection



Three replatings

P3



Stock -80°C

DNA sequencing

Phenotypic assays

FIGURE 31. ISOLATION OF PHAGE-TOLERANT VARIANTS. Colonies surviving phage infection after 72 h on LB agar plates were replated three times (P1, P2 and P3) before the P3 culture was made and stored at -80°C.

For each experiment, around 8 colonies were picked and re-isolated three times before being tested for resistance to phages. The 32 samples, as described in Table 1, were finally grown in 3 ml of LB medium ($A_{600} = 0.8$ to 1), and used to prepare frozen stocks (Figure 31). From the same bacterial culture, DNA was extracted, and used for whole genome sequencing.

II.II CALCULATION OF THE FREQUENCY OF RESISTANCE

Overnight culture of *P. aeruginosa* PAO1_{Or} was used to inoculate a fresh medium to an A_{600} of 0.1. When the bacterial culture reached the *log* phase (A_{600} of ≈ 1), 10-fold dilutions were performed. 100 μ l of each dilution were then mixed with 10 μ l ($\approx 10^6$ PFU) of single phage suspension or mixture of two or four phages as described above, kept for 15 min at room temperature and then poured on fresh agar plates using 4 ml of soft agar. Plates were inverted and incubated at 37°C for 24 h. The frequency of resistance was calculated considering that all the colonies growing on the plates after 24 h of incubation were resistant to phages used for the infection.

II.III PHAGE SUSCEPTIBILITY ASSAY

Aliquots (500 μ l) from the liquid culture of each variant ($A_{600} = 0.8$ to 1.2) were mixed with 6 ml of 0.7% LB agar and poured onto a squared 1.5% LB agar plates. Five dilutions (10^{10} , 10^9 , 10^8 , 10^7 , 10^6 PFU/ml) of the ancestral forms of each phage were spotted (10 μ l) onto the soft agar layer, incubated at 37°C overnight, and inspected for plaque formation. The resistance of the variants to the phage was expressed as EOP taking PAO1_{Or} as a control.

III PHENOTYPIC CHARACTERIZATION

A planktonic culture of the progenitor *P. aeruginosa* PAO1_{Or} prepared from a single colony of a fresh LB agar plate was used in all characterization experiments as a reference. Overnight culture (10 μ l) of all PAO1_{Or} phage-resistant mutants was spotted on LB medium or Sheep blood (5%). Plates were incubated for 24 h at 37°C.

III.I ADSORPTION ASSAY

An overnight bacterial culture was diluted to an A_{600} of 0.1-0.6 and let to equilibrate at 37°C. Approximately 10^6 phages were added to 1 ml of the diluted bacterial culture (1×10^8 to 6×10^8 bacteria) and at fixed time point 50 μ l of the mixture were transferred to an Eppendorf tube containing 940 μ l of LB medium and 10 μ l of chloroform. The suspension was vortexed for 5 sec in order to allow the chloroform to bind and precipitate the phages adsorbed on the bacterial surface. 10 μ l of the unadsorbed phage fraction was plated using spot assay. The plates were inverted and incubated at 37°C overnight and the number of plaques was counted. The phage-adsorption was expressed as percentage of the initial amount of phage employed for the infection that did not absorb on the bacterial surface at 16 min after infection.

III.II TWITCHING MOTILITY ASSAY

One μl of an overnight bacterial culture was inoculated between the agar and the plastic surface of a 1.5% LB agar plate. Plates were incubated at 37°C and the diameter of the motility zone around the point of inoculation was measured after 24 h of incubation.

III.III PLANKTONIC GROWTH RATE AND BIOFILM FORMATION

The bacteria were inoculated into 3 ml of LB medium in a glass tube and incubated for 7 h at 37°C (180 rpm). The A_{600} was monitored with a microplate reader (CHAMELEON) and recorded every hour. The population growth rate ($A_{600}/\Delta t$ maximal rate of change of A_{600} during *log* phase) and final yield (value of A_{600} after 24 h) were recorded as growth determinants.

Ninety-six-wells plaques (Greiner) were inoculated with a diluted overnight bacterial culture (A_{600} of ≈ 0.1) and incubated for 48 h at 37°C . A_{600} was recorded before proceeding with biofilm quantification. The wells were washed three times with PBS, 200 μl of 0.1% w/v crystal violet was added and the plate was kept for 30 min at RT. The unattached crystal violet was washed three times with PBS and then the remaining biomass was quantified by re-suspending the crystal violet in 200 μl of absolute ethanol and measuring the A_{600} that was then divided by the A_{600} value measured for planktonic bacteria in each well to account for the difference in growth rates of the variants.

III.IV VIRUCIDE ASSAY

Fifty μl of frozen pseudolysogen stock were used to inoculate 50 ml of LB media and the culture was grown overnight at 37°C . Virucide was prepared according to the method described in (de Siqueira, Dodd *et al.* 2006). Briefly, bacterial overnight culture was centrifuged at 4000 rpm for 10 min at 4°C and the pellet was washed once with 5 ml of PBS and finally resuspended in 500 μl of PBS. To this, 1.5 ml of virucide was added, the mixture was kept at room temperature for 10 min and the virucide activity was then blocked by diluting the suspension to a final volume of 15 ml.

Bacteria were pelleted, washed with PBS and then treated with 10 µg/ml DNase I for 30 min at 37°C. The sample was washed again and then the bacteria were streaked onto a fresh agar plate and used for total DNA extraction.

III.V LIPOPOLYSACCHARIDE ANALYSIS

LPS was extracted according to the method used by Hitchcock and Brown (Hitchcock and Brown 1983). Bacteria were grown overnight in LB media at 37°C (180 rpm) and then treated overnight at 4°C with formaldehyde 2% followed by three washings with PBS. In order to normalize the samples for the subsequent gel analysis, a similar amount of lyophilized bacteria was disrupted in lysis buffer (Tris 1M, 2% SDS, 4% β-mercaptoethanol and 10% glycerol), prior to proteinase K treatment and LPS extraction. The LPS were resolved by electrophoresis on a 15% SDS-polyacrylamide gel, and the band pattern was visualized using the silver staining method (Fomsgaard, Freudenberg *et al.* 1990).

IV PSEUDOLYSOGENS ANALYSIS

IV.I COLONY TRANSFER AND HYBRIDIZATION

About 100 colony-forming units (cfu) were plated on LB agar and incubated overnight at 37°C. A photograph was taken before applying on the colonies a circular Nylon N+ membrane (Nytran). After 5 min the membrane was lifted using forceps and treated for 2 min with NaOH 0.4 N twice, Tris 1M pH 7.5 twice, 2XSSC twice. The membranes were put on Whatman filter paper, dried and kept at 20°C until use.

Pre-hybridization was performed at 65°C for 4h with 2 ml of hybridization buffer (Church and Gilbert 1984) per membrane. The probe was labeled using the Megaprime™ kit (GE Healthcare Amersham) and hybridization was performed overnight at 65°C in hybridization buffer. Washes were done successively with 2XSSC and 0.1% SDS, 0,5XSSC and 0.1% SDS, 0.2XSSC and 0.1% SDS.

IV.II STABILITY OF PHAGE PERSISTENCE IN PSEUDOLYSOGENS

One loop of frozen bacterial stock was streaked onto a fresh LB agar plate and incubated overnight at 37°C. Starting from this plate, 52 colonies were picked-up with sterile toothpicks and successively inoculated onto two plates, one fresh LB agar plate and one with a lawn of susceptible strain, PAO1_{Or}. The lawn plate was prepared by inoculating 4 ml of pre-warmed soft agar with 100 µl of PAO1_{Or} overnight culture. Both plates were then incubated at 37°C overnight and the phage-producing colonies, producing a lysis zone on the plate covered by the PAO1_{Or} lawn, were counted and expressed in percentage. A positive phage-producing colony was then chosen, picked-up by a sterile loop from the LB agar plate, and re-streaked in order to have again 52 colonies with which to repeat the same experiment. Each passage was repeated until there were no more phage-producing colonies in the pseudolysogen population.

IV.III DNA EXTRACTION, PCR AND SEQUENCING

PCR was performed on thermolysates or pure DNA using primers listed in Table 6. Thermolysates were produced by diluting 10 µl of overnight culture in 200 µl of water and heating at 95°C for 5 min. For DNA purification, bacteria were lysed in lysis buffer (Tris 10 mM, EDTA 10 mM, NaCl 10 mM, SDS 0.5%), treated with proteinase K at 50 µg/ml for 2 hours at 50°C, followed by one phenol and one chloroform extraction, and ethanol precipitation. The isolates were verified for contamination from other *P. aeruginosa* strains, commonly used in our laboratory, using PCR with primers directed against VNTRs ms216 and ms217 as previously described (Vu-Thien, Corbineau *et al.* 2007). The isolates were also screened for the presence of phage DNA by PCR performed on thermolysates using specific phage primers listed in Table 6.

Name	Sequence (5' → 3')
Ab17-F1	AAGGTGTACCGGAAACGGAC
Ab17-R1	TACCTACGGCTTCCTGCTCT
Ab09-F1	GACGAAGCATCGTTGTACGC
Ab09-R1	AACAACAATCGCAACTGGGC
Ab05-F1	TGAGCGCATGGCTCTACGGT
Ab05-R1	AACCGGAATCGCTGGTCATC
Ab27-F1	CATGAACGGCCGGTTCAAAGT
Ab27-R1	AAGAAGCCGTGCTGGTCGAG
ms216L	ACTACTACGTCGAACACGCCA
ms216R	GATCGAAGACAAGAACCTCG
ms217L	ACAGGACGTGCTGATCCATGA
ms217R	GTGCGGCAAGTGCAACAGCAT
wzy-F1	AGCCTACCCATGCATCCCTA
wzy-R1	AGGCACGCCAGCCCATGGT
wzy-R2	AATCGTAGCGAACGATAGCC
mucA-F1	AAACTGTTCTGGCGATCGGC
mucA-R1	ATATCGCCACCGTGATGCAG
wbpL-F1	ACATTCTTCGCCAGAGGGAG
wbpL-R1	TCCTACCGCCAAGAGGCAAA
wzz2-F1	GACATTGACAGCCTCCAGGG
wzz2-R1	CTACCGATGATTTTCGTTTCGT
algC_F1	GCGTAGAGCAGGTCGACATC
algC_R1	CTTGAAGAACACGTGGCCGC
dnpA_F1	AGCACCGAGGGTTCGAGGAA
dnpA_R1	CAAGCGTCATCGACGCAACA
migA_F1	GATTGCCGTTCTGCCGCTTG
migA_R1	GCTGGAGCAGTTGCAGAAAG
pgi_F1	CGAGCACAAGGTCTACGTAC
pgi_R1	CTCCGCAAGGTGTCGAGAGA
pilC_F1	ACCATGATGGGCGCTGGCGTT
pilC_R1	TTCCAAGGCGCCCGATTGCT
pilJ_F1	TCGCAGCGGATCGCGAAGAA
pilJ_R1	CTCCGCAAGGTGTCGAGAGA
pilQ_F1	TAACCGACGTTGGCCTCGAC
pilQ_R1	ACCCGACCTTGAGCCCGGAT
pilQ_F2	GTGGCGAAGTCCTTGACGTC
pilQ_R2	AACCTGACCGCGCTGTCGTC
pilR_F1	GCCTGATCCACGAGCAGGGG
pilR_R1	TCGCGGACGTTGCCCGGGAA
pilY1_F1	CCAACATCAAGCTGCTCTGG
pilY1_R1	TAGTCGGCTACGCCGTCGCT
pilY1_F2	TCGCGCGCAACCAGACCAACA
pilY1_R2	GCTCCACAGCTTGGTCTGCA
wapH_F1	GGACACCGCCAGCAGGCTCA
wapH_R1	GTACGCCAGCCTGTTCGCCG

TABLE 6 OLIGONUCLEOTIDES USED IN THIS STUDY.

IV.IV GENE CLONING AND EXPRESSION

PCR amplicons were cloned into the pUCP24 plasmid, a generous gift of Dr Schweizer (West, Schweizer *et al.* 1994). This is a shuttle vector which replicates in *E. coli* and in *P. aeruginosa*, and contains a multiple cloning site downstream from *lacZα*. The PAO1 *mucA* gene was PCR-amplified using oligonucleotides *mucA_Clon_F_Bam* (5'-TGGGATCCCGAGAAGCCTGACACAGC-3') and *mucA_Clon_R_Hind* (5'-GAAAGCTTACCGCCATCAGGCTGCCA-3'), which included restriction sites for *Bam*HI and *Hind*III. The amplicons were digested with *Bam*HI/ *Hind*III, ligated into the similarly digested vector and transformed into *E. coli*, in which replication of pUCP24 is optimal (West, Schweizer *et al.* 1994). A selected recombinant was then used to transform *P. aeruginosa* strains by electroporation using the fast protocol described by Choi and colleagues (Choi, Kumar *et al.* 2006). Transformants were selected using gentamycin 10 µg/ml, and the presence of the plasmid was verified by PCR amplification using a *mucA* forward oligonucleotide *mucA-int_F* (5'-ACGCAGGTAGATCGGCAGAC-3') and a plasmid reverse oligonucleotide *pUCP24_MCS_R* (5'-GGCCTCCTTCGCTATTACGCC-3'). The colony aspect was observed under the stereomicroscope (Motic DM143). The transformants were then tested for their susceptibility to the four phages.

IV.V WHOLE-GENOME SEQUENCING

Purified bacterial DNA (10 µg) was sent for draft whole-genome Illumina sequencing to the IMAGIF platform (CNRS, Gif-sur-Yvette, France). Libraries were made from sheared fragments of DNA with a mean size of 900bp and paired-end reads of 250 bp were produced. Between one and five million reads were obtained corresponding to a 40- to 200-fold mean coverage. The mutations were identified by comparison with the genome of the PAO1_{Or} sequence using native Geneious R9 tools default parameters (Biomatters). The Geneious mapper with the 'Medium-Low Sensitivity/Fast' parameter option was used to map the reads of each variant against the PAO1_{Or} genome. The 'Find Variations/SNPs' analysis was used with the parameter 'Minimum Variant Frequency' set to 0.25. When an SNP or an indel was identified, sequencing reads mapping in the

mutated gene plus 1 kb on both sides were recovered, reassembled and the contig was aligned with the PAO1_{or} genome. This allowed the precise localization of short deletions. Mutations were confirmed by PCR amplification of the affected gene and Sanger sequencing (Beckman-Cogenics) using primers listed in Table 6.

De novo assembly of phage reads was done with the Geneious R9 native assembler using the 'Medium-Low Sensitivity/Fast parameter'.

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ANNEX I

Investigation of a large collection of *Pseudomonas aeruginosa* bacteriophages
collected from a single environmental source in Abidjan, Côte d'Ivoire

RESEARCH ARTICLE

Investigation of a Large Collection of *Pseudomonas aeruginosa* Bacteriophages Collected from a Single Environmental Source in Abidjan, Côte d'Ivoire

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Abstract

Twenty two distinct bacteriophages were isolated from sewage water from five locations in the city of Abidjan, Côte d'Ivoire over a two-year period, using a collection of *Pseudomonas aeruginosa* strains with diverse genotypes. The phages were characterized by their virulence spectrum on a panel of selected *P. aeruginosa* strains from cystic fibrosis patients and by whole genome sequencing. Twelve virions representing the observed diversity were visualised by electron microscopy. The combined observations showed that 17 phages, distributed into seven genera, were virulent, and that five phages were related to temperate phages belonging to three genera. Some showed similarity with known phages only at the protein level. The vast majority of the genetic variations among virulent phages from the same genus resulted from seemingly non-random horizontal transfer events, inside a population of *P. aeruginosa* phages with limited diversity. This suggests the existence of a single environmental reservoir or ecotype in which continuous selection is taking place. In contrast, mostly point mutations were observed among phages potentially capable of lysogenisation. This is the first study of *P. aeruginosa* phage diversity in an African city and it shows that a large variety of phage species can be recovered in a limited geographical site at least when different bacterial strains are used. The relative temporal and spatial stability of the Abidjan phage population might reflect equilibrium in the microbial community from which they are released.

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Introduction

Bacteriophages, viruses that infect bacteria, are the most numerous of all viruses in the biosphere and are estimated to be globally more numerous than bacteria (reviewed in [1,2,3]). This abundance plays important roles in the evolution of bacterial communities and may influence global biogeochemical cycles [4]. A limited number of studies have investigated phage distribution in natural environments, in contrast to the vast knowledge on bacterial ecology [5]. Man-made environments such as waste water systems are rich in microbial communities due to diversified sources of microbes, presence of elevated level of nutrients and the multiple surfaces on which biofilms can form [6]. Different studies have revealed that such ecosystems can be reservoir for human pathogens and associated bacteriophages [7,8].

Evolution of phages, driven by resistance of bacterial hosts, can affect multiple genes, and this is reflected by the large genomic diversity inside phage species [9,10]. Comparison of genomes reveals important levels of mosaicism resulting from recombination, as well as acquisition or loss of genetic material and point mutations [11]. It has been speculated that the recombination functions encoded by many phages are, in addition to their role for phage replication, responsible for the creation and the maintenance of mosaicism. Their activity would re-create different modules with a comparable selective fitness [12]. Viruses constitute a huge reservoir of genetic diversity that is frequently revealed by the discovery of novel genes especially in newly sequenced phage genomes [13]. Metagenomic data from aquatic and human environments show that most viral diversity remains uncharacterized [14,15].

Ubiquitous in the environment, *P. aeruginosa* is one of the major life-threatening opportunistic bacteria responsible for nosocomial infections in immunocompromised people, and for persistent respiratory infections in cystic fibrosis (CF) patients [16,17]. Its ability to adapt to different niches and to develop resistance to classical antibiotic-based therapy has inspired a renewed interest in its bacteriophages. Phage cocktails are used to treat *P. aeruginosa* infections in different countries but clinical assays are needed to evaluate their efficiency and safety. The composition of therapeutic cocktails such as the widely used Pyo-Phage [18] remains empirical, and there is no clear and published rules on how many phages should be included, and what would be the criteria for selecting them.

Considering the large genetic diversity of the *P. aeruginosa* species, it is important for future therapeutic usage to evaluate the virulence spectrum of lytic phages [19,20]. Numerous lytic and temperate *P. aeruginosa* phages have been isolated, mostly from man-made environments of European and Asian countries, and new ones are continuously being described [21,22]. Ceysens et al. analyzing morphologic features and genome organization of a large collection of phages, estimated that most evolved within 12 genera gathering 21 species [10]. Two additional genera of lytic phages were subsequently described. The PAK-P1-like phages include JG004, vB_PaeM_C2-10_Ab1 (alias Ab01) and PaP1 [23,24,25,26], whereas KPP10 and PAK-P3 form a second genus [27,28]. More recently six new species belonging to the *Siphoviridae* family were reported [7], as well as a giant phage [29]. The large majority of siphoviruses of *P. aeruginosa* has been shown to be temperate phages distributed into three different genera, implying the possibility to undergo a lytic or a lysogenic interaction with their host [30]. Such phages often show a very narrow host range. The recently isolated lytic siphovirus PA1 ϕ , a D3112-like phage lacking lysogeny gene modules, displayed a broad bacteria spectrum [31]. Among podoviruses, the Abidjan phage Ab31, a chimera between a temperate and a lytic phage, was shown to be temperate [26]. To understand the mechanisms that shape bacteriophages populations and allow the emergence of new species by mutations and genome exchanges, it is necessary to perform studies resampling the same environment. We previously described a large panel of clinical *P. aeruginosa* isolates that showed a wide variety of

susceptibility to lytic and temperate phages [26]. Here we used a selection of these strains to isolate and characterize 22 novel *P. aeruginosa* phages from sewage water in Abidjan (Ivory Coast) in 2010 and 2011. Comparison of their genomes to those of published phages reveals a large diversity and points to a continuous evolution in a limited time and space scale.

Materials and Methods

Ethics statement

The present project is in compliance with the Helsinki Declaration (Ethical Principles for Medical Research Involving Human Subjects). Bacterial strains were collected as part of the patients' usual care, without any additional sampling, as previously reported [32,33,34]. The ethic committee "Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédicale (CCPPRB) Ile-de-France", who was consulted, specifically approved this study, and declared that patient informed consent was not needed.

Strains and media

The *P. aeruginosa* strains used for enrichment and amplification of phages were from CF patients (Table 1) [32,33,34]. The reference strains PAO1 and PA14 were purchased from the Institut Pasteur Collection (CIP, Paris, France). C50, a strain from clone C [35] was a gift from Ute Römling. Twelve *P. aeruginosa* strains were used for phage isolation, of which six were resistant to Pyo-Phage (a therapeutic cocktail of phages prepared in Tbilisi, Georgia [18]), and to additional phages from Russia and USA ([26] and unpublished results). Five bacterial strains were used for subsequent amplification of the purified phages. Phages p1-14_{pyo}, p8-13_{pyo}, p1-15_{pyo} and p2-10_{pyo} were previously isolated from Pyo-Phage and were used in the present study as controls [26]. Luria broth (LB) medium supplemented with 2 mM CaCl₂ was used for bacterial growth and phage titration. Saline magnesium (SM) buffer (50 mM Tris-HCl pH7.5, 100 mM NaCl, 8.1 mM MgSO₄, 0.01% gelatin) was used to preserve purified phages at 4°C.

Phage isolation, characterization and genome sequencing

Samples were collected in five public sites: four in the Cocody area (La Baie, latitude 5.333704 longitude: -4.015846, Carrefour de la Vie, lat. 5.348505 long. -4.002178, Carrefour de l'Indénié, lat. 5.341143 long -4.017788, Cocody hospital, lat. 5.344389 long. -3.994072) and one in the Treichville area (Treichville hospital, lat. 5.293541, long. -4.003898). No authorisation was required for sampling in the first three sites. Authorisation was obtained for sampling in the Cocody and Treichville hospitals. The sites corresponded to open-air or closed sewage systems. Two of these were from geographically distant hospitals waste water purification stations. The different steps of phage isolation were described previously [26]. Briefly, filtrated water was incubated overnight in LB medium with enrichment bacteria, and, after centrifugation, the supernatant was spotted onto different bacterial strains including the enrichment strain. A total of 22 and nine phages were isolated in October 2010 and November 2011 respectively. Phage Ab12 which formed plaques on strain C5-2 in the first isolation step could not be further maintained in any tested strain. After two rounds of purification using a single plaque, phages were amplified on solid plate by infecting 200–400 µl of a 2x10¹⁰ cfu/ml bacterial suspension with one 24 hours old lysis plaque. One of the phages, Ab01, was previously described [26], and detailed analysis of Ab31 has been reported recently [36]. Phages were given names as recommended by Kropinski *et al.* [37].

Table 1. List of bacteriophages isolated in Abidjan, Côte d'Ivoire.

Phage ID	Alias	Site ^a	Date	Enrichment strain ^b
vB_PaeM_C2-10_Ab1	Ab01 ^c	CV	oct-10	C2-10 (+)
vB_PaeM_C2-10_Ab02	Ab02	Ind	nov-11	C7-6 (-)
vB_PaeM_PAO1_Ab03	Ab03	CoH	nov-11	PAO1 (+)
vB_PaeM_PAO1_Ab04	Ab04	Ind	nov-11	C5-2 (-)
vB_PaeP_PAO1_Ab05	Ab05	CoH	nov-11	PAO1 (+)
vB_PaeM_PAO1_Ab06	Ab06	TrH	nov-11	PAO1 (+)
vB_PaeM_C2-10_Ab07	Ab07	CV	oct-10	C9-11 (+)
vB_PaeM_C2-10_Ab08	Ab08	Ind	oct-10	C9-11 (+)
vB_PaeP_C2-10_Ab09	Ab09	Ind	oct-10	SCH (+)
vB_PaeM_C2-10_Ab10	Ab10	CV	oct-10	SCH (+)
vB_PaeM_PAO1_Ab11	Ab11	CV	oct-10	PAO1 (+)
vB_PaeP_C5-2_Ab12 ^d	Ab12	Ind	nov-11	C5-2 (-)
vB_PaeM_C2-10_Ab13	Ab13	Ba	oct-10	C2-10 (+)
vB_PaeM_C2-10_Ab14	Ab14	Ba	oct-10	C2-10 (+)
vB_PaeM_C2-10_Ab15	Ab15	CoH	oct-10	C9-11 (+)
vB_PaeM_C2-10_Ab16	Ab16	CV	oct-10	C9-11 (+)
vB_PaeM_PAO1_Ab17	Ab17	Ind	nov-11	PAO1 (+)
vB_PaeS_PAO1_Ab18	Ab18	CV	oct-10	PAO1 (+)
vB_PaeS_PAO1_Ab19	Ab19	CV	oct-10	C50 (-)
vB_PaeS_PAO1_Ab20	Ab20	Ind	oct-10	PAO1 (+)
vB_PaeS_PAO1_Ab21	Ab21	Ind	oct-10	PAO1 (+)
vB_PaeP_C2-10_Ab22	Ab22	Ind	nov-11	C8-20 (-)
vB_PaeM_C2-10_Ab23	Ab23	CV	oct-10	C8-20 (-)
vB_PaeM_C2-10_Ab24	Ab24	Ba	oct-10	C8-14 (-)
vB_PaeM_C2-10_Ab25	Ab25	CV	oct-10	SCH (+)
vB_PaeS_SCH_Ab26	Ab26	CV	oct-10	SCH (+)
vB_PaeM_PAO1_Ab27	Ab27	CoH	oct-10	C8-14 (-)
vB_PaeM_PAO1_Ab28	Ab28	Ind	oct-10	C8-14 (-)
vB_PaeM_PAO1_Ab29	Ab29	Ind	oct-10	C3-16 (-)
vB_PaeS_PAO1_Ab30	Ab30	CV	oct-10	C3-16 (-)
vB_PaeP_Tr60_Ab31 ^e	Ab31	TrH	nov-11	PA14 (+)

^a CV, Carrefour de la Vie; Ind, indénié; CoH, Cocody Hospital; TrH, Treichville hospital; Ba, La Baie

^b in parentheses is indicated the Pyo-Phage-susceptibility

^c published in [26]

^d Ab12 was subsequently lost

^e published in [36]

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Host-range spectrum

Phage host range was determined by spotting 10 µl of purified phages with high titer (at least 10⁹ pfu/ml) on stationary *P. aeruginosa* cells. Presence or absence of a lysis zone was appreciated after overnight incubation at 37°C. Phage sensitivity was then confirmed using plaque assay. Briefly, 10 µl of undiluted phage suspension were directly streaked on LB agar plate, and 4 ml of molten top agar (0.7%) containing 2x10⁹ bacteria were poured over the phages. Characteristics of isolated plaques were recorded.

Electron microscopy

Purified and concentrated phages were prepared and visualized by staining 5 μ l of phage suspension with 2% potassium phosphotungstate (pH 7.0) as previously described [26].

DNA purification and enzymatic digestion

DNA preparation was performed using a rapid DNA purification assay as previously described [38]. Briefly, 4 ml of amplified phage suspension were treated with 50 μ g/ml RNase A for 30 min at room temperature, then 0.2 ml 2M Tris-HCl pH 7.5, 0.4 ml 0.5M EDTA, 0.2 ml 10% SDS and 10 μ l diethylpyrocarbonate (1.1 g/ml, Sigma-Aldrich) were added. Following incubation at 65°C for 30 min, 1 ml 5M KOH was added, and the samples were left for 1 h on ice. Centrifugation was performed at 25,000 g for 20 min at 4°C, and the supernatant was precipitated with two volumes of ethanol. DNA was pelleted by centrifugation and washed twice with 70% ethanol then dissolved in 0.4 ml TE (10 mM Tris-HCl (pH7.5), 1 mM EDTA). Enzymatic digestion was carried out on 8 μ l DNA in a final volume of 12 μ l according to the manufacturer's recommendations (Thermo Fisher Scientific, France), and analysed on a 0.8% agarose gel.

Genome sequencing and analysis

Illumina paired-end sequencing was performed by BaseClear (Leiden, The Netherlands) or IMAGIF (Gif sur Yvette, France). The coverage was on the order of 1000X. The 100 bp reads were *de novo* assembled using Velvet [39] and Ray [40] as embedded in BioNumerics version 7.5 (Applied-Maths, Ghent, Belgium). Sub-samples of the initial read files were used, with 5000 to 100,000 reads, and several values of the k-mer length were tested in order to try and obtain a single contig. Once a single circular contig was obtained, the totality of reads was mapped against the sequence in order to identify regions with particular coverage, or the existence of ambiguity at some nucleotides, and to detect reads that did not map to the phage genome. In several phages, the presence of a high peak of reads which all stopped at the same position indicated the end point of a linear genome, as previously observed [24,26]. The genome starting point and the position of a putative Direct Terminal Repeat (DTR) were revealed by a particular distribution of the sequencing reads or by comparison with closely related phages. Multiple alignments were performed using the Geneious 8.1 software platform (Biomatters Ltd, Auckland, New Zealand) running MAFFT [41]. Annotation of open reading frames (ORF) was performed with RAST [42], using a 0.1 blastp E-value cut-off, and ARAGORN was applied to localize tRNA genes [43]. SNPs identification, Minimum spanning tree, and dN/dS analyses were performed in BioNumerics version 7.5 using the full bacteriophage sequences. Phage genome assemblies have been deposited to EBI/EMBL: Ab02 LN610572, Ab03 LN610573, Ab04 LN610581, Ab05 LN610574, Ab06 LN610582, Ab08 LN610575, Ab10 LN610586, Ab11 LN610583, Ab15 LN610587, Ab17 LN610576, Ab18 LN610577, Ab19 LN610584, Ab20 LN610585, Ab22 LN610578, Ab27 LN610579, Ab28 LN610589, Ab29 LN610588, Ab30 LN610590, 1-15_{pyo} LN610580.

Results and Discussion

Isolation of phages from sewage water

In order to favour the isolation of a variety of phages, we selected, in addition to the reference strains PAO1 and PA14, six clinical strains (C3-16, C7-6, C5-2, C8-14, C8-20 and C50) that were previously shown to be resistant to a large collection of phages of different genera (including those of the Pyo-Phage batch tested), and three clinical strains (C9-11, C2-10, SCH) with large susceptibility to these phages [26]. A spot assay was performed with the supernatant, and

phages present in lysis zones were purified by several rounds of single plaque isolation. When different plaque morphologies were observed with a single sample, they were recovered and purified. Thereafter, a limited number of amplification strains were chosen for the good level of phage growth, PAO1 and C2-10 being most frequently used. The water sample origin, the date of isolation and the bacterial strains used to isolate and amplify new phages are summarized in [Table 1](#). Interestingly, phage Ab22 produced both clear and turbid plaques on strain C2-10, in high density zones, and this phenotype was seen even after replating of a clear or a turbid plaque ([S1 Fig](#)). A similar phenotype was previously observed with phage p2-10_{or}, a LUZ24-like phage isolated in Orsay, France [26], and with other phages of this genus [44]. Phage Ab05 produced clear plaques on PA14 and PA01, and a halo was seen on PA14. Phage 1-15_{pyo}, a ϕ KMV-like phage isolated from Pyo-Phage and used here as a control, made similar plaques with a halo on PAO1 [26].

All phage genomes were digested with different restriction enzymes, showing that the banding pattern were identical or very similar in some phages, whereas others had specific profiles (data not shown). Combining the results of different enzymatic digestions, phages Ab05, Ab09, Ab22, Ab26, Ab30 and Ab31 appeared to be unique, while the other phages fell into five groups, including twelve, five, four, and three members respectively.

Whole genome sequencing

Sequencing of all 30 phages was performed using the Illumina technology, and the reads were assembled producing a single contig. In some phages, upon alignment of reads against the assembled sequences, and in agreement with a previous observation for Ab01, high peaks of reads could be observed at two positions, delimitating a Direct Terminal Repeat (DTR) at the genome ends ([S2 Fig](#)). Assembled genomes were aligned with all available *P. aeruginosa* phage genomes, in order to identify the closest one. For all phages except Ab31 and the Ab18 group (Ab18, Ab19, Ab20, Ab21), 85–95% alignment was possible along the whole sequence of a known genome. The genome size of the new phages, and the genus they belong to, are indicated on [Table 2](#). Some genome sequences appeared to be almost identical suggesting that the same phage was isolated several times independently. The 30 recovered phages correspond to 22 distinct phages. Interestingly, the GC content of most phages was largely inferior to that of the bacterial chromosome (66,6% for PAO1), except for phages of the YuA and DMS3 genera, and for Ab05. The putative ORFs were identified for each phage and annotation was performed by comparison with closely related genes. The proteins were categorized into different functional groups: morphogenesis and packaging, DNA replication, modification and recombination, regulatory functions, nucleotide synthesis. In total 21 among the 22 independent phages could be assigned to nine known genera, which are described below, and one phage, Ab31, was a combination between a virulent and temperate phage. The detailed analysis of Ab31 which showed limited homology at the DNA level with several Lambda-like phages, has been reported independently [36].

PAK_P1-like viruses. The twelve phages from this virulent phage genus, including the previously reported phage Ab01 [26], were isolated at four different locations in Abidjan mostly in 2010 but also once in 2011. They were distributed into five different subgroups within which the genome sequences differed at only few nucleotides. They presented more than 90% similarity with the genomes of PAK_P1 from France [45], PAP1 from China [25] and JG004 from Germany [24]. For all twelve phages of this genus, DTRs of 1153 bp (Ab01, Ab07) or 1165bp (Ab02, Ab08, Ab10, Ab13, Ab14, Ab15, Ab16, Ab23, Ab24 and Ab25) were found, similarly to those observed on closely related PAK_P1-like genomes. The DTR size difference was due to insertion/deletions of a few base pairs. All genomes possessed 14 tRNA

Table 2. Characteristics of the new phages.

Phage ID	Genome size (bp)	Family	Genus	Average similarity	Other similar phages ^a	DTR (bp)	tRNA	GC (%)
Ab01	92777				Ab07	1153	14	49,3
Ab02	93848					1165	14	49,4
Ab08	93503	Myovirus	PAK_P1	90%	Ab14, Ab16, Ab13	1165	14	49,2
Ab10	93053				Ab25	1165	14	49,3
Ab15	93308				Ab23, Ab24	1165	14	49,3
Ab03	86246					771	3	54,7
Ab04	86668					771	3	54,6
Ab06	84759	Myovirus	KPP10	90%		756	3	54,6
Ab11	85783					771	3	54,5
Ab17	83598					771	3	54,6
Ab27	66299					none	0	55,7
Ab29	66326	Myovirus	PB1	97%		none	0	55,6
Ab28	66181					none	0	54,9
Ab09	72028	Podovirus	N4	93%		641	0	54,9
Ab05	43639	Podovirus	ϕKMV	98%		431	0	62,3
Ab22	45808	Podovirus	LUZ24	91%		184	3	52,4
Ab18	56537					none	0	63,5
Ab19	58139	Siphovirus	YUA	70%	Ab21	none	0	63,3
Ab20	57745					none	0	63,5
Ab26	43055	Siphovirus	PA73	87%		none	0	53,4
Ab30	37238	Siphovirus	DMS3	95%		none	0	64,1
Ab31	45550	Podovirus	New			none	0	57,1

^a according to [30]

^b showing only a few SNPs

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genes and coded for their own DNA polymerase, and for the control of nucleotide metabolism. Fig 1 shows the organization of ORFs on phage Ab02, and S1 Table lists the ORFs with a putative function. One genome of each subgroup was selected for multiple alignments revealing patches of high heterogeneity, but also several regions (3–9 kb) with only a few single nucleotide polymorphisms (SNPs) (S3 Fig). Patches of sequences showing a high level of divergence and low dN/dS values were observed in the first 17 kb of the genome, reflecting events of horizontal genetic transfer (HGT) [46]. By contrast some regions were devoid of traces of HGT and differed by a few SNPs and high dN/dS values. Presence/absence of sequences was noted. Ab08 possessed a gene encoding a putative endonuclease with an H-N-H motif (Ab08 ORF38, between ORF 39 and ORF40 of Ab02). The gene is absent from the other phages from Abidjan, but present in JG004 (PJG4_036) and PAK_P2 (00161c). In Ab02 a 759bp sequence encoding ORF 104 was inserted into the polymerase gene, separating it into two genes, encoding DNA polymerase part I (ORF103) and part II (ORF105). The insert was related to an intron described in the DNA polymerase gene of LUZ24 (PPLUZ24_gp35), and encoding its own endonuclease. Comparison with other phages of the same genus (JG004, PaP1 and PAK_P1) showed a higher level of diversity as reflected in a minimum spanning tree representation (Fig 2). The very high sequence similarity level in the African phages in regions of the genome not affected by HGT is in favour of a recent diversification from a common founder. A remarkable conservation of protein sequences was observed, such as for the major capsid protein which was identical in all the phages, as previously described [25]. A region of 827bp, present in

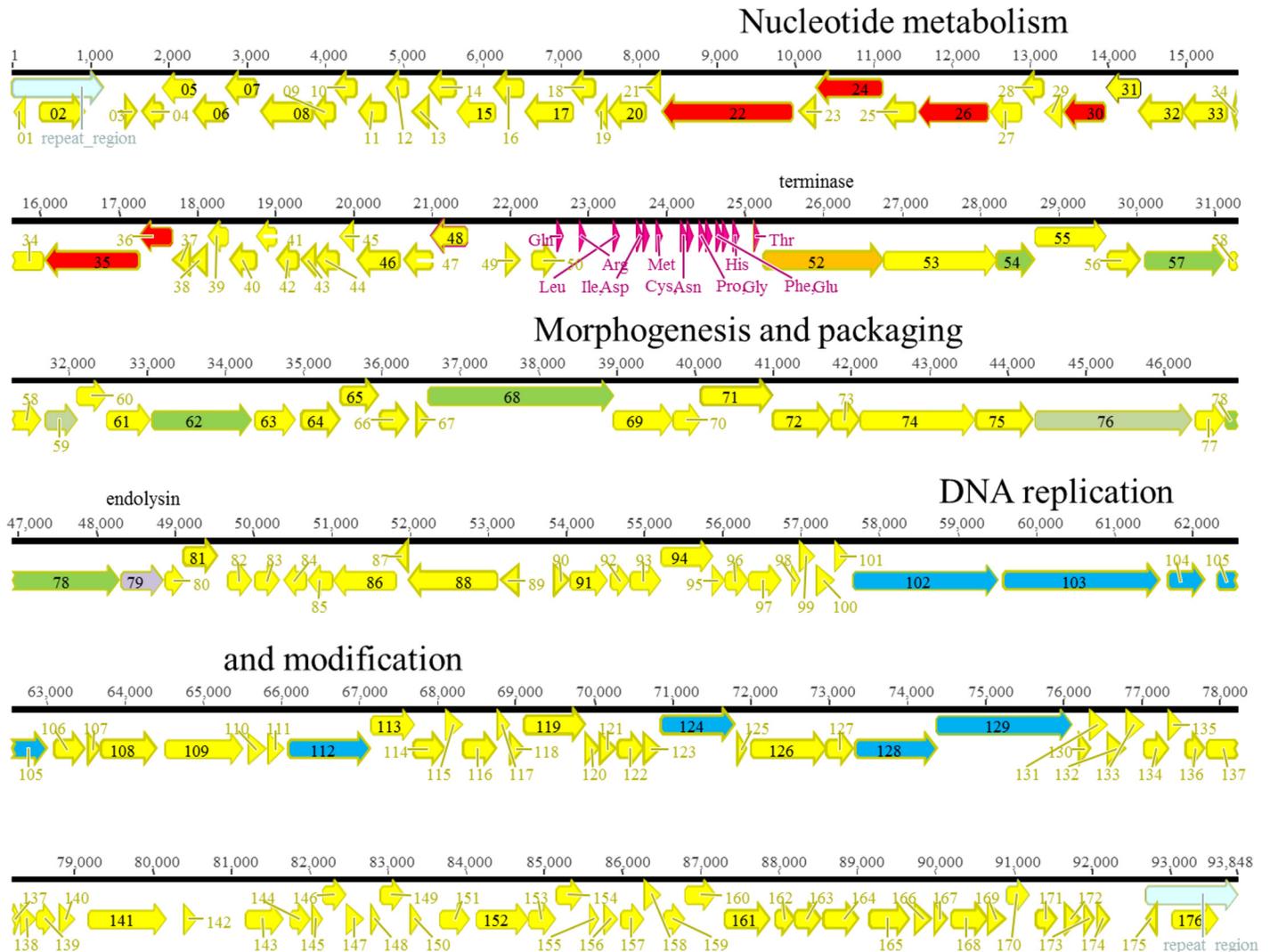


Fig 1. Genomic organization of PAK_P1-like phage Ab02. ORFs are shown as arrows. The different colors correspond to the putative function: yellow, unidentified, red, nucleotide metabolism, orange, terminase, green, morphogenesis and packaging, dark blue, DNA replication, light blue, DTR.

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JG004 only (position 34,438 to 35,264), encompassed the gene for an endonuclease (PJG4_070), inserted between the genes homologous to Ab02 ORF62 and ORF63.

A 21 bp polymorphism in the phages of this group corresponded to the length of a short duplication. It encoded a seven amino acid peptide (VGAPWYS), part of the hypothetical ORF75 of phage Ab02, containing a putative leucine zipper-like domain. Phages Ab02, Ab15, Ab24 and Ab25 had one copy whereas the other phages had two copies, and this polymorphism was confirmed by PCR amplification (data not shown).

KPP10-like viruses. Five phages (Ab03, Ab04, Ab06, Ab11, Ab17) isolated in 2010 and 2011 at four different locations in Abidjan, belonged to the KPP10-like virulent phage genus. They were all different, and shared 88–96% sequence identity with the genomes of KPP10 from Japan [27], and PAK_P3 from France [23,45]. At position 23,000, a region with very low read coverage was observed in all phages except Ab17 and corresponded to an AT-rich region. A putative DTR of 771bp (Ab03, Ab04, Ab11, Ab17) showing an internal 15 bp deletion in Ab06, was found, defining the genome ends. The inferred nucleotide one position was different from

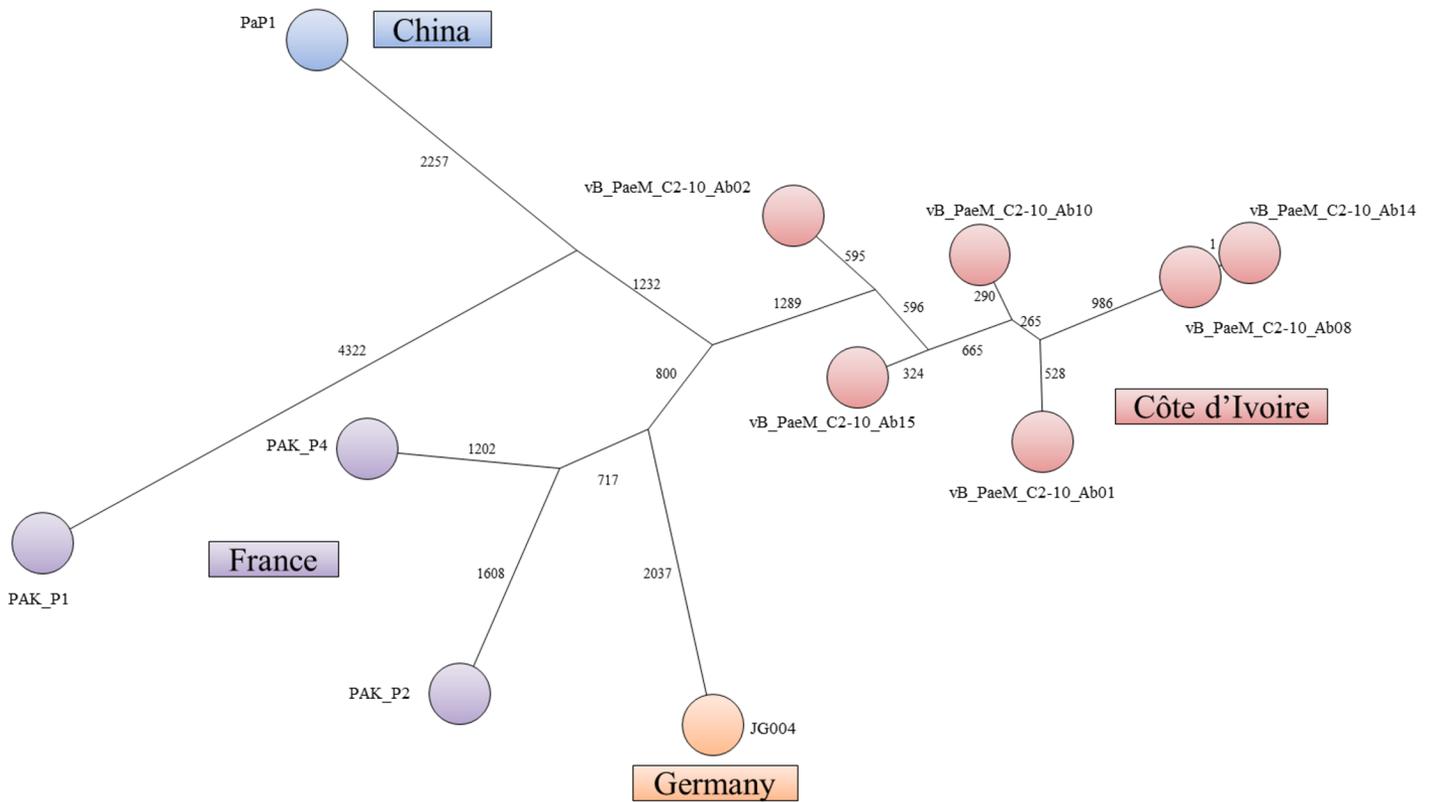


Fig 2. Minimum spanning tree representation of PAK_P1-like phages genomes. The numbers indicated on each branch represent the number of SNPs making this branch. A total of 12125 SNPs were identified and the tree size is 19714 indicating a high level of homoplasia. Homoplasia might result from independent HGT events with unknown phages infecting other *Pseudomonas* species. Colors indicate the phages country of origin.

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the reported first nucleotide of phage KPP10. The genomes of the five phages were annotated (Ab06 is shown on Fig 3) showing that, overall, the ORF organization was very similar to that of the related phages, with a few differences for some very short hypothetical proteins. In keeping with KPP10, these phages had three tRNA genes (tRNA^{Asn}, tRNA^{Tyr} and tRNA^{Gln}). Upon alignment of the five genomes using Geneious, several regions of variability were observed within otherwise largely similar sequences (S4 Fig). In regions most probably encountering frequent HGTs, the percentage of SNPs was 20% as compared to 0.1% on average in the rest of the genome, and dN/dS ratios were low. Similarly to PAK_P1 phages, the first 22 kb, and the last 8kb showed the largest traces of HGT. It is interesting to note that some recombination sites appeared to be inside coding regions leading to the production of putative proteins with high levels of heterogeneity. This was the case for example for ORF14, encoding an hypothetical protein corresponding to ORF120 in KPP10, which was very different in the five phages, and appeared to be a patchwork of short regions from different origins. Similarly to PAK_P1-like phages, the five KPP10-like phages from Abidjan clustered at a large genetic distance to phages isolated in other countries (Fig 4). Several regions of short insertion/deletions were observed between the different phages, sometimes resulting in the fusion of two putative ORFs. Ab17 lacked a 3271 bp region encompassing seven hypothetical ORFs (ORF89 to ORF96 in Ab06), perfectly conserved in the other phages. As a result, a putative RNA ligase (ORF91 in Ab17) was formed by the fusion of the beginning of ORF89 and ORF97 (also a putative RNA ligase in the other phages) in Ab06. This region corresponded to nucleotides 22,874 to 26,077 in KPP10, and also encompassed seven putative genes of unknown function (ORF34 to

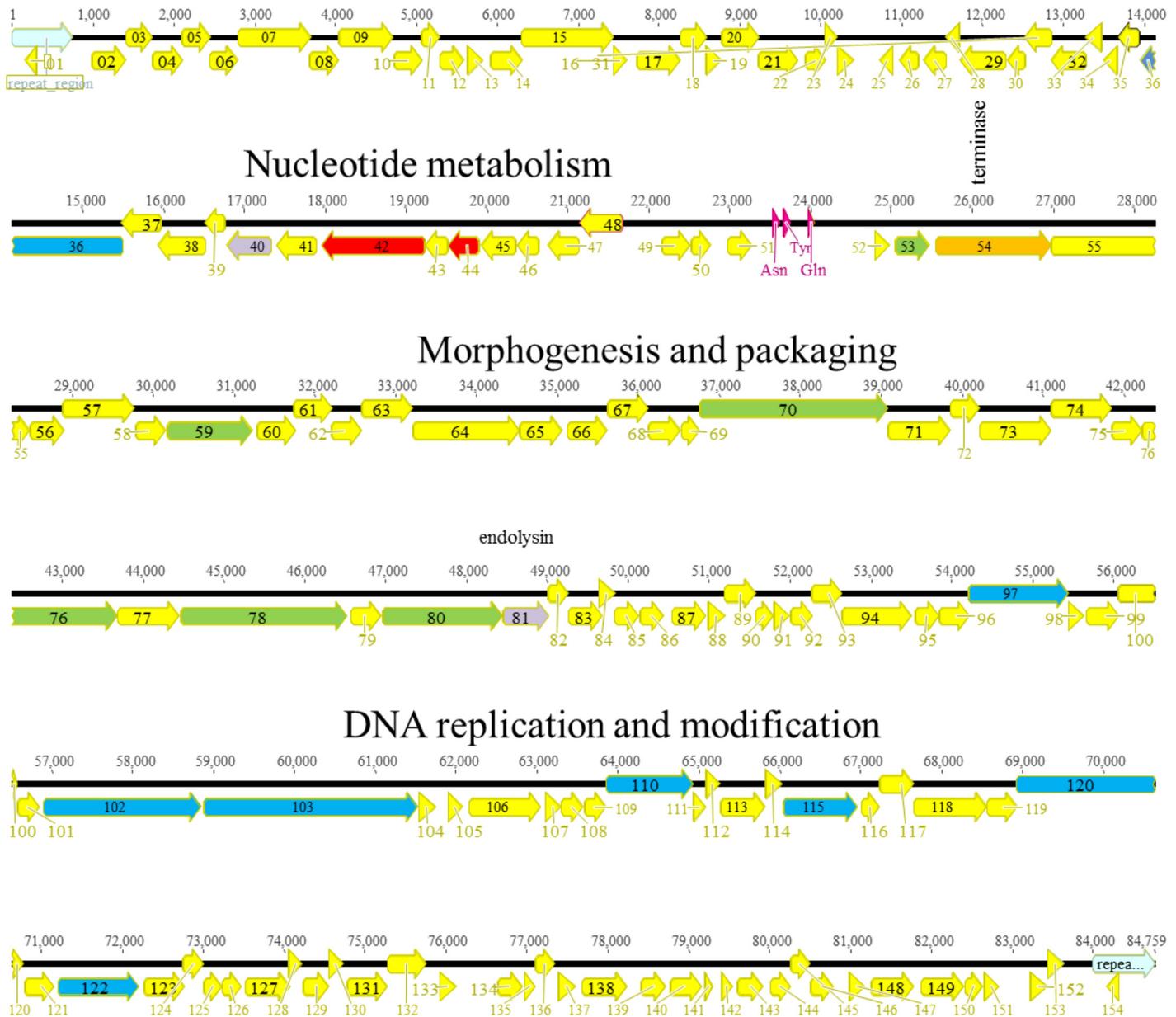


Fig 3. Genomic organization of KPP10-like phage Ab06. The different ORFs are colored according to their putative function: yellow, unknown; red, nucleotide metabolism; orange, terminase; green, morphogenesis; blue, DNA replication.

doi:10.1371/journal.pone.0130548.g003

ORF40). Another region of 528 bp was absent in Ab03 and Ab06, and corresponded to DNA with no homology in Genbank/EMBL at the nucleotide level.

PB1-like viruses. Three phages (Ab27, Ab28 and Ab29) presented on average a 97% similarity level with several PB1-like virulent phages such as JG024 from Germany (66,275bp) [47], KPP12 from Japan (64,144bp) [48], NH-4 from Ireland (66,116 bp) [49], and SN from Russia (66,390bp) [50]. The presence of a category of reads with a single fixed termination tentatively marked the position of the phages genome ends, but there was no indication of a DTR. Based on this information, the first nucleotide could be positioned about 7,500bp upstream that reported for phage PB1. Alignment of the three phage genomes showed that Ab27 and Ab29

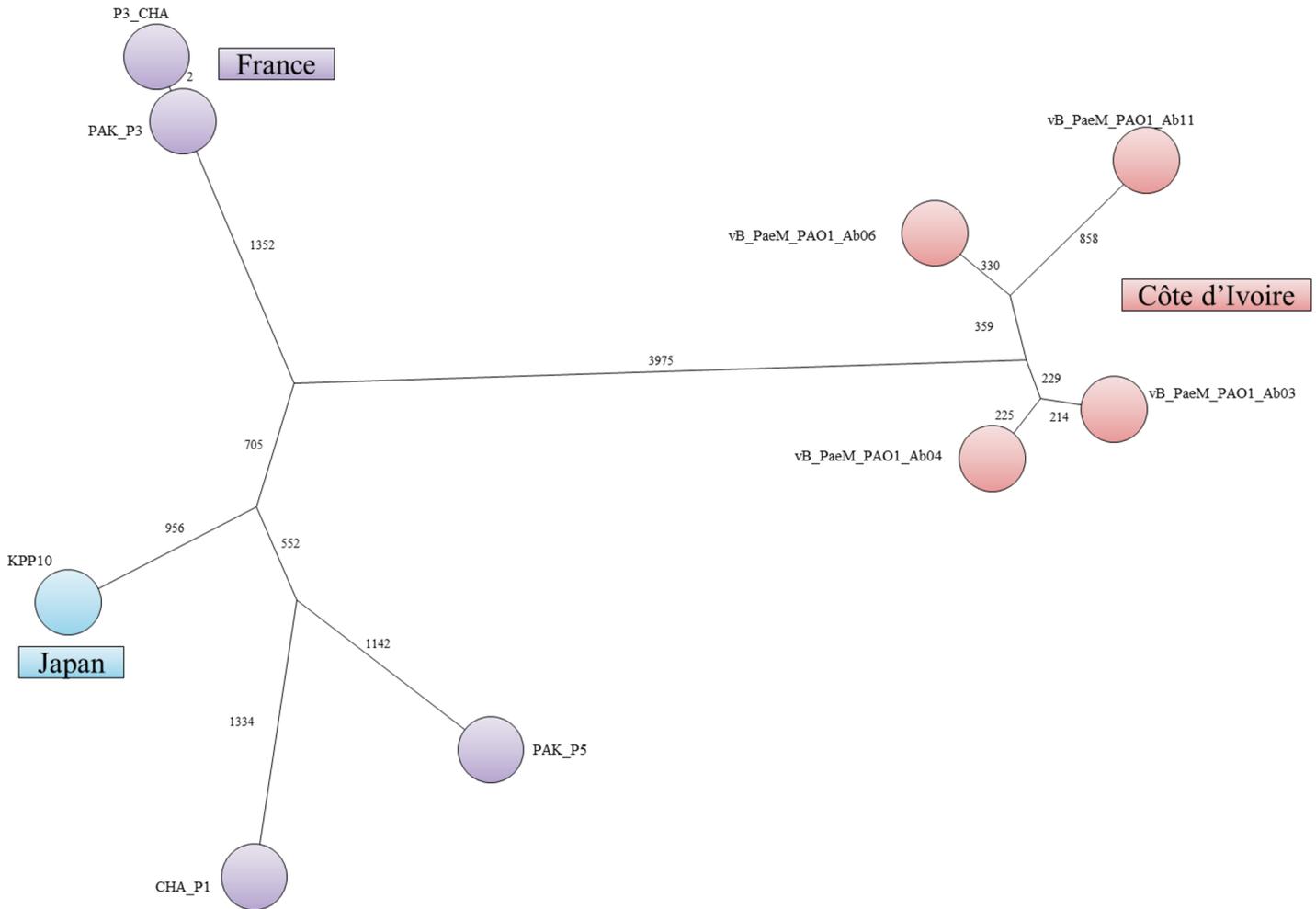


Fig 4. Minimum spanning tree representation of KPP10-like phages genome. The numbers indicated on each branch are the number of SNPs constituting this branch. A total of 9097 SNPs were identified and the tree size was 12233 indicating a significant level of homoplasia. Colors indicate the phages country of origin.

doi:10.1371/journal.pone.0130548.g004

were quite similar, except for the first 4,400bp and the last 11,400bp where traces of recombination events could be seen (S5 Fig), characterized by a high density of nucleotide variations. Ab28 was very different from the other two at the nucleotide level, but the ORFs were remarkably well conserved in the three phages, and other PB1-like phages. The genome organization of phage Ab27 is shown on Fig 5. There was no RNA polymerase, but there seemed to be a complete DNA replication machinery as observed in other PB1-like phages. Between Ab27 ORF60 and ORF61 (position 36,360), reads contained either 9 or 10 G suggesting a possibility of phase variation at this site. Interestingly a 30 bp sequence (GATGCCCCGGCGAACC GGG GCGGGGTGGTT) at position 8,087–8,187 of the phage genome was present as a spacer in the Clustered Regularly Interspaced Region (CRISPR) of several *P. aeruginosa* genomes. This structure is part of an adaptive immune system believed to play a role in *P. aeruginosa* resistance to bacteriophages and plasmids [51], but is usually not associated with resistance to lytic phages. Several studies have shown that in *P. aeruginosa*, CRISPRs carry mostly sequences of temperate phages [26,51]. Our observation suggests that the CRISPR-Cas system may play a role in regulation of PB1-like phages infection.

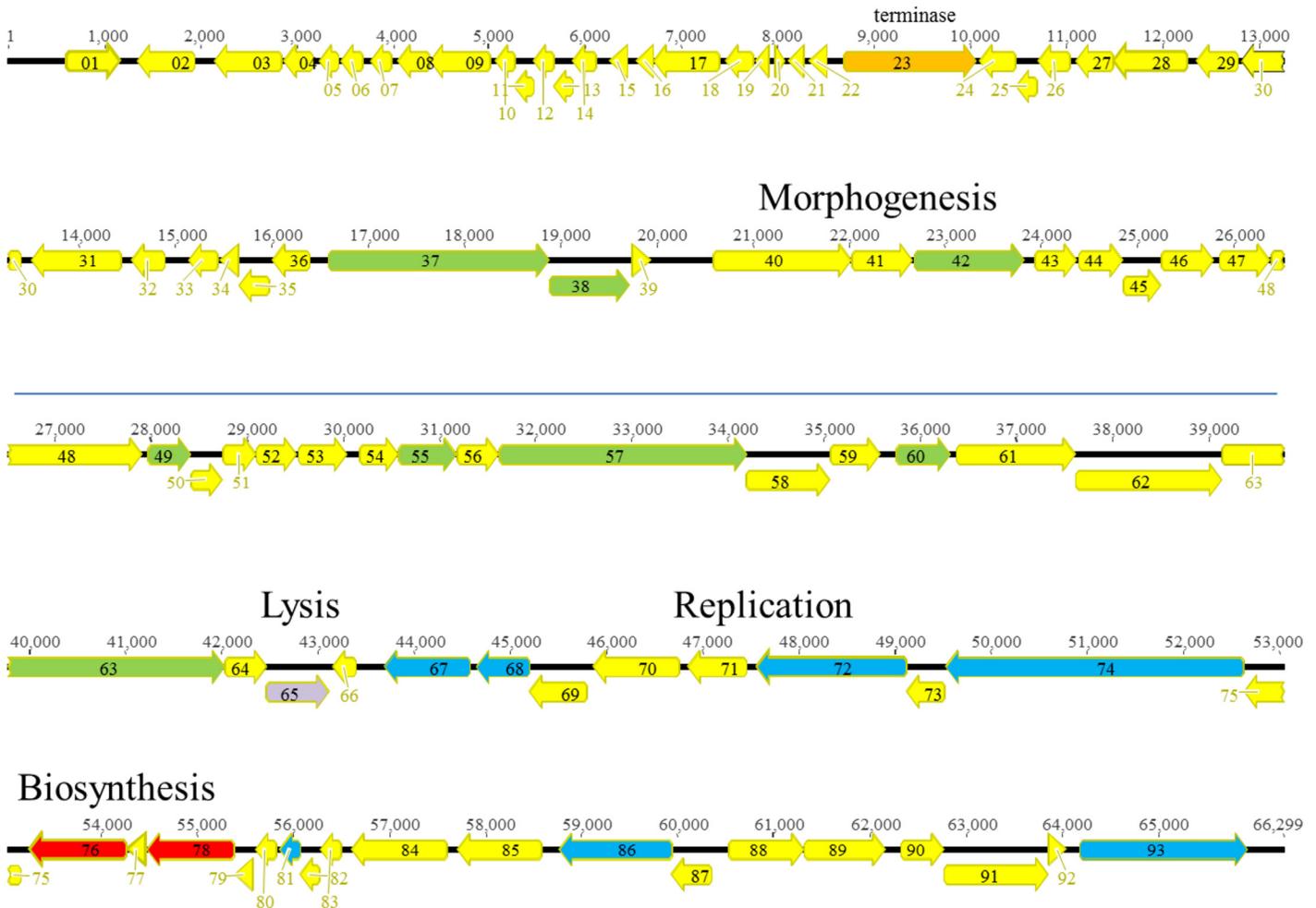


Fig 5. Genomic organization of PB1-like phage Ab27. The different ORFs are colored according to their putative function: yellow, unknown; red nucleotide metabolism; orange, terminase; green, morphogenesis; blue, DNA replication; purple, lysis.

doi:10.1371/journal.pone.0130548.g005

N4-like virus. The genome of Ab09 was 72,028 bp long. It showed a mean 93% similarity with lytic phage LIT1, a N4-like virus isolated in Belgium, (72,544 bp) [52], although some regions had less than 80% similarity with this phage. By contrast the mean similarity at the nucleotide level with phage LUZ7, another N4-like virus from Belgium, was only 65%. A 641bp DTR was found corresponding to the 655bp DTR of LIT1. The genome encoded 83 hypothetical proteins, among which a giant protein of 3398 amino acids (ORF66), the characteristic virion-encapsulated RNA polymerases of N4-like viruses (Fig 6) [52] which allows transcription of early genes in these phages. Ab09, like other N4-like phages encoded a second type of RNA polymerase (ORF18 and ORF19), a heterodimeric T7-like RNAP. Similarly to ORF 56 in phage LUZ7, Ab09 ORF48 aligned with ORF52 and ORF53 of LIT1, both putative tail proteins separated by 195 nucleotides [52]. No tRNA genes were identified. Similarly to other lytic phages with large genomes, a group of small hypothetical ORFs was found at one end of the genome.

φKMV-like viruses. Ab05 (43,639bp) showed, on average, 98% sequence similarity with lytic phages LUZ19 from Belgium (43,548bp) [53], and φKMV from Russia (42,519bp) [54]. A 431bp DTR was observed, similar to that of LUZ19 (472 bp DTR). Overall the organization of

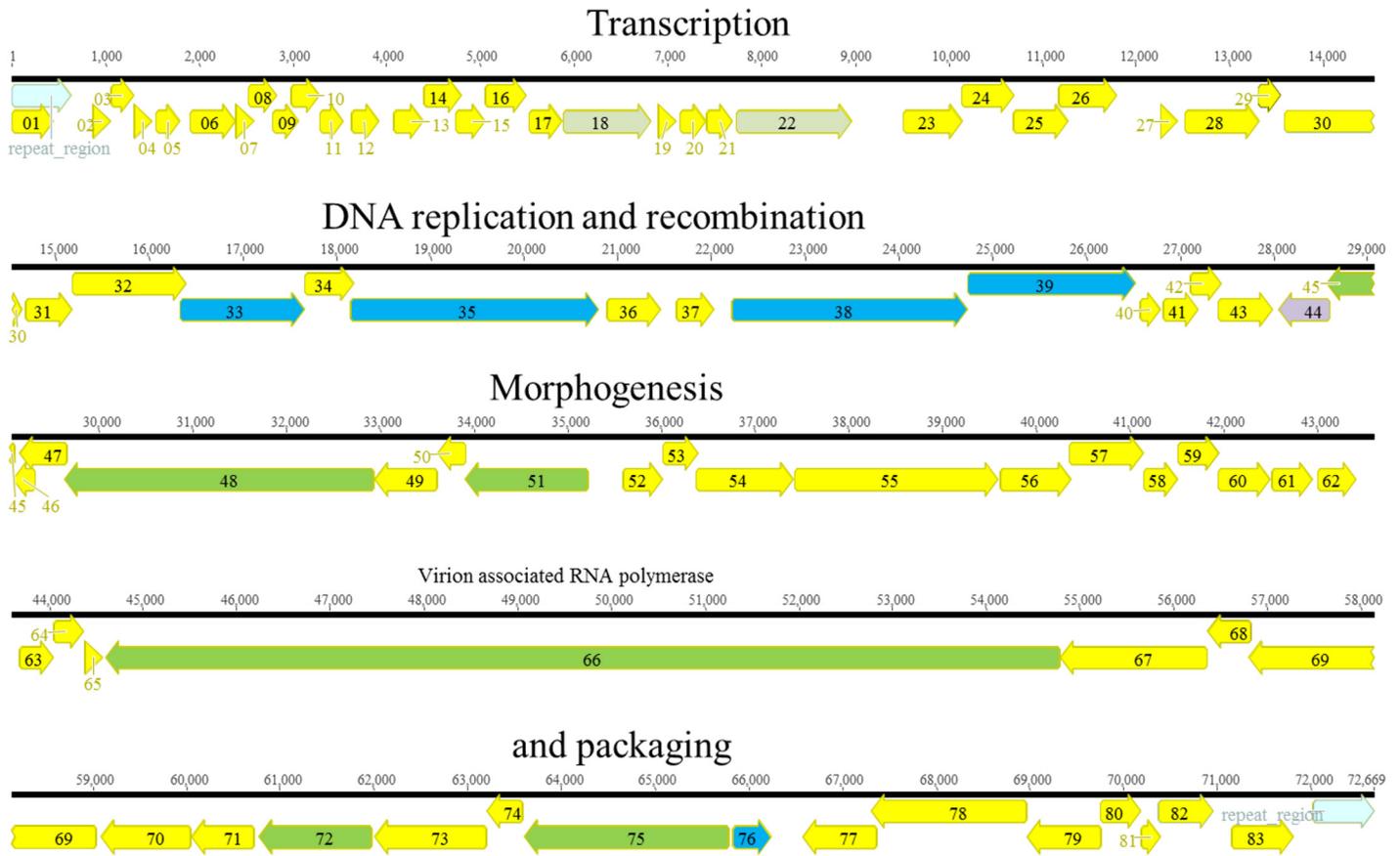


Fig 6. Genomic organization of N4-like phage Ab09. The different ORFs are colored according to their putative function: yellow, unknown; grey, transcription; green, morphogenesis; blue, DNA replication; purple, lysis.

doi:10.1371/journal.pone.0130548.g006

putative genes was that of ϕ KMV-like phages [10,55] (Fig 7). Contrarily to the large-genome viruses described above, Ab05 and the other ϕ KMV-like genomes were more compact and essentially expressed genes for morphogenesis and replication, in addition to a group of small hypothetical ORFs. An RNA polymerase was found (ORF31), as typically observed in phages of this genus. The first genomic region ending after the gene for RNA polymerase, and encompassing genes for host conversion and DNA replication (early region), was the region showing the most diversity [55]. A lysis cassette formed of a pinholin (ORF49), endolysin (ORF50), and spanins (ORFs 51–52) was similar to the one described in ϕ KMV-like phages [56]. Interestingly no putative ORFs were found in the first 1900 nucleotides, a region holding three to five strong promoters in other ϕ KMV-like phages. The canonical nucleotide sequence 5'-CGACX XXXXCCTACTCCGG-3', localized at putative sites for single-strand DNA interruptions [57], was found three times in the Ab05 genome (arrows on Fig 7). Ab05 showed, in addition, the variant sequence 5'-GGGCXXXXXCCTACTCCGG-3'. At these positions an excess of sequencing reads could be observed. Compared to other ϕ KMV-like phage genomes, four deletions of putative genes, as well as many regions with low level of similarity at the nucleotide level were observed, reflecting recombination events. The two smaller deletions corresponded in phage LKD6 to a region containing a putative promoter and to an intergenic sequence [55]. Deletion 3 encompassed the short ORF17.1 of phiKF [58]. Deletion 4 encompassed gp20 in LUZ19, a short ORF present in all the sequenced ϕ KMV-like phages.

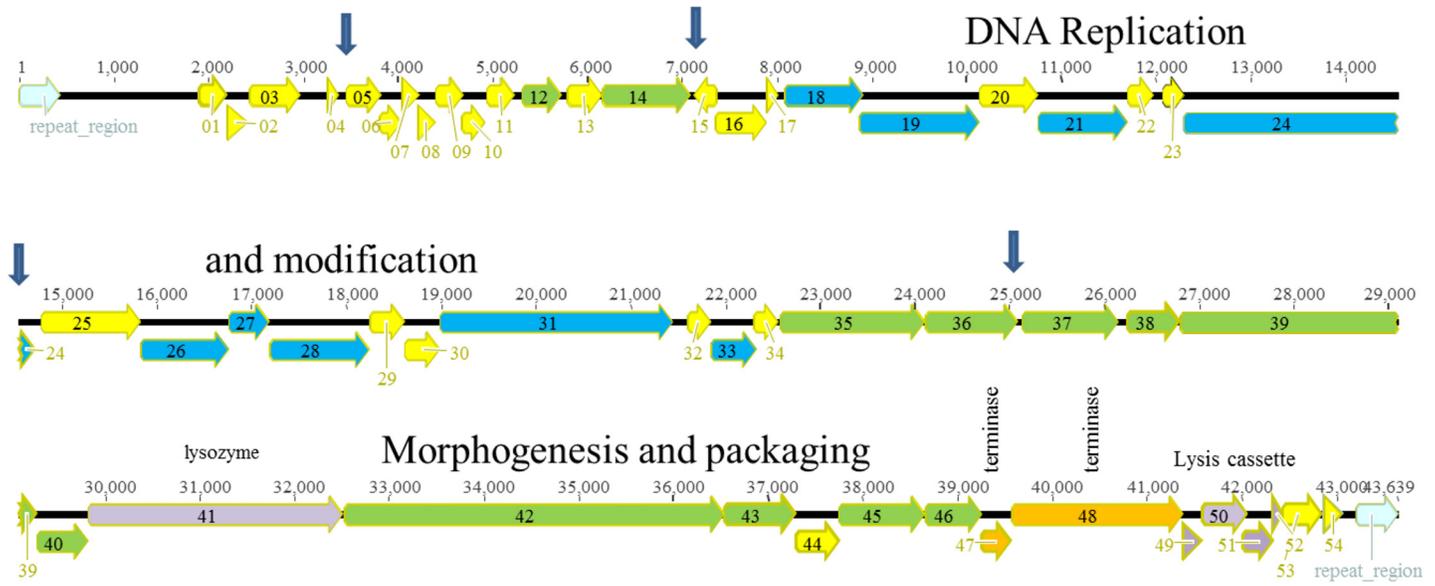


Fig 7. Genomic organization of ϕ KMV-like phage Ab05. The different ORFs are colored according to their putative function: yellow, unknown; orange, terminase; green, morphogenesis; blue, DNA replication; purple, lysis. Vertical arrows indicate the position of single-strand DNA interruptions.

doi:10.1371/journal.pone.0130548.g007

LUZ24-like virus. The Ab22 genome was 45,808 bp long and showed 86–96% similarity with lytic phage LUZ24 from Belgium (45,625 bp; AM910650 [59]) and lysogenic phage PaP3 from China (45,503bp NC_004466 [60]). The genome was also close to that of phage 1-14_{Or01} that we previously isolated in France [26]. Ab22 possessed a 184 bp DTR as observed for 1-14_{Or01} (182bp) [26]. Annotation predicted the existence of 71 putative ORFs and three tRNAs (tRNA^{Pro}, tRNA^{Tyr} and tRNA^{Asn}) (Fig 8). By comparison, LUZ24 showed 74 ORFs and two tRNAs, and PaP3 71 ORFs and 4 tRNAs. Several regions of insertion or deletion with respect

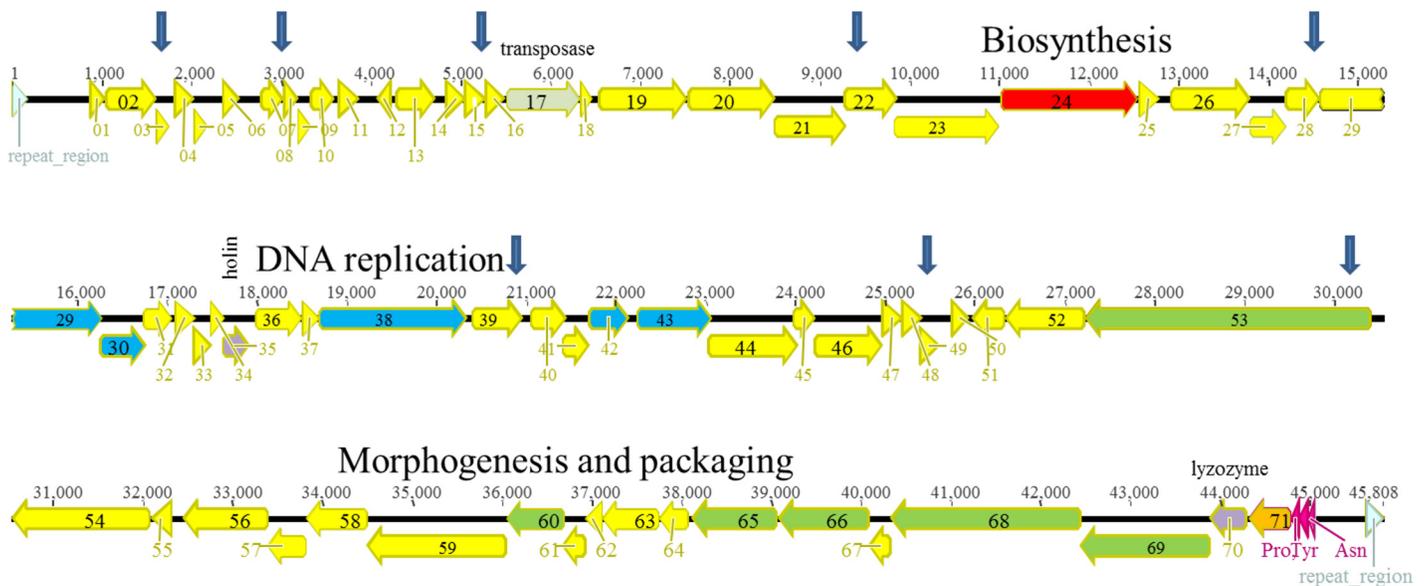


Fig 8. Genomic organization of LUZ24-like phage Ab22. The different ORFs are colored according to their putative function: yellow, unknown; red, biosynthesis; green, morphogenesis; blue, DNA replication; purple, lysis. Vertical arrows indicate the position of single-strand DNA interruptions.

doi:10.1371/journal.pone.0130548.g008

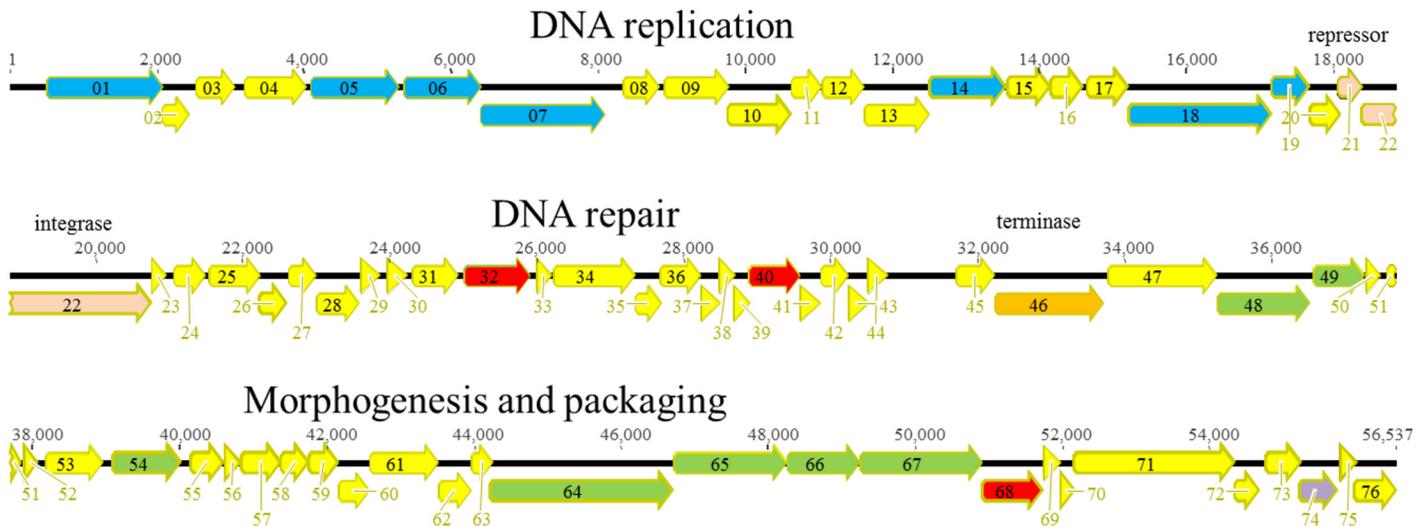


Fig 9. Genomic organization of YuA-like phage Ab18. The different ORFs are colored according to their putative function: yellow, unknown; orange, terminase; red, DNA repair; green, morphogenesis; blue, DNA replication; purple, lysis, pink, prophage insertion.

doi:10.1371/journal.pone.0130548.g009

to LUZ24 were observed. The longest (1206 bp) was present in Ab22 (ORF17) and absent in LUZ24, and all other closely related *P. aeruginosa* phages. It showed 100% identity with the transposase fusion protein of phage TL from Russia (YP_009007804), suggesting that this gene possibly contributes to the insertion of the phage genome in the bacterial DNA. The second largest region of difference was a 665 bp fragment, absent in Ab22, encoding the gp35 endonuclease (self-splicing intron) in LUZ24, separating the polymerase part II and III (ORF34 and ORF36). These two genes were fused into a single ORF in Ab22 (ORF38). The first 1000 nucleotides did not encode any putative protein and probably contained promoters, although the consensus sequence described by Ceyskens et al. [59] could not be found at this position. At six positions (arrows on Fig 8), an excess number of reads corresponded to the sequence 5'-GTA CTATGAC-3', or to the variant 5'-GTACTGTGAC-3' marking the single-strand DNA interruptions observed on the viral genome. We and others previously reported the existence of such sites with phages of LUZ24-family [26,61].

YuA-like viruses. Ab18, Ab19, Ab20 and Ab21, isolated in 2010 and 2011 at two locations and showing related restriction profiles, appeared to be YuA-like phages. Genome sequencing showed that there were in fact only three different phages, Ab19 and Ab21 being identical. The sequencing reads aligned as a circular genome, with no abnormal peaks of reads and consequently the first nucleotide was assigned by comparison with the closest genome, YuA from Russia (58,663 bp) [62]. The genome of the three phages showed at best 70% similarity with that of YuA and MP1412 from South Korea (61,167 bp) [63]. At the protein level, additional similarities could be observed particularly in structural proteins. Moreover, the DNA polymerase (ORF18) and the terminase gene (ORF46) also showed an elevated degree of homology with those of YuA. Fig 9 shows the organisation of the 76 Ab18 ORFs, all oriented in the same direction. Overall the organisation was that of YuA with some remarkable differences, particularly at the level of small ORFs of unknown function. ORF6 and ORF7 of Ab18 and Ab19 encoded the small and large subunits of a ribonuclease reductase of class Ia which corresponded to a single gene in YuA. A putative repressor (ORF21) and an integrase (ORF22) showing 55% identity to that of YuA were present, suggesting that the phage could possibly lysogenize its host. Other parts of the genome had no homology with any phage in the public

databases, either at nucleotide or protein level. Ab18, Ab19 and Ab20 genomes showed an average 95% similarity with each other, and displayed several regions of insertion/deletion. The genomes were aligned showing that overall the SNPs were evenly distributed, except for one large region of low percentage of similarity encompassing genes for the tail fiber proteins (S6 Fig). This is in contrast with the high level of divergence observed with the closest phage YuA, and provides a direct estimate of the relative role of mutation by descent versus recombination in this homogenous group of phages. YuA-like phage genomes were reported to be resistant to many restriction enzymes including *EcoRI*, although *EcoRI* restriction sites exist in their genome [7,62]. We observed a similar resistance in the four phages from Abidjan suggesting the existence of DNA modifications.

PA73-like virus. Ab26 genome was 43,055 bp long and encoded 52 putative proteins (Fig 10). It showed 87% homology with vB_Pae-Kakheti25 from Georgia (42,844 bp; NC 007806) considered a lytic phage [64] and with PA73 from the Lindberg set (42,999 bp; DQ163913) [65]. The phage ends and the orientation of the genome map were aligned to that of related phages, and alignment showed a high overall conservation of gene organization between these phages except for three regions. The 296 bp at the beginning of the genome, encoding a protein found in a lysis cassette in phage vB_Pae-Kakheti25, were very different in the three phages. Interestingly, Ab26 possessed well conserved holin, endolysin, Rz and Rz1 spanin genes (ORF01 to ORF04) forming a lysis cassette similar to that described in ϕ KMV-like phages [56]. One region of 1,600 bp encoding proteins found in mature virions (ORF21-ORF22), showed about 70% similarity with vB_Pae-Kakheti25 but 95% with PA73. Finally, from position 36,000, after the gene encoding a putative primase/helicase (ORF35), to the end of the genome a series a short hypothetical ORFs was observed showing a high degree of divergence as compared to the two closest phages. No integrase or other protein that could be involved in lysogeny were clearly identified. However ORF31, a *recA*-like recombinase, was shown to belong to the sak4 family of proteins, almost exclusively associated with temperate phages [66].

D3112/B3-like virus. Ab30 had a 37,238 bp genome and showed between 91 to 99% similarity with transposable phage DMS3 from the USA (36,415 bp) [67], and phages MP38

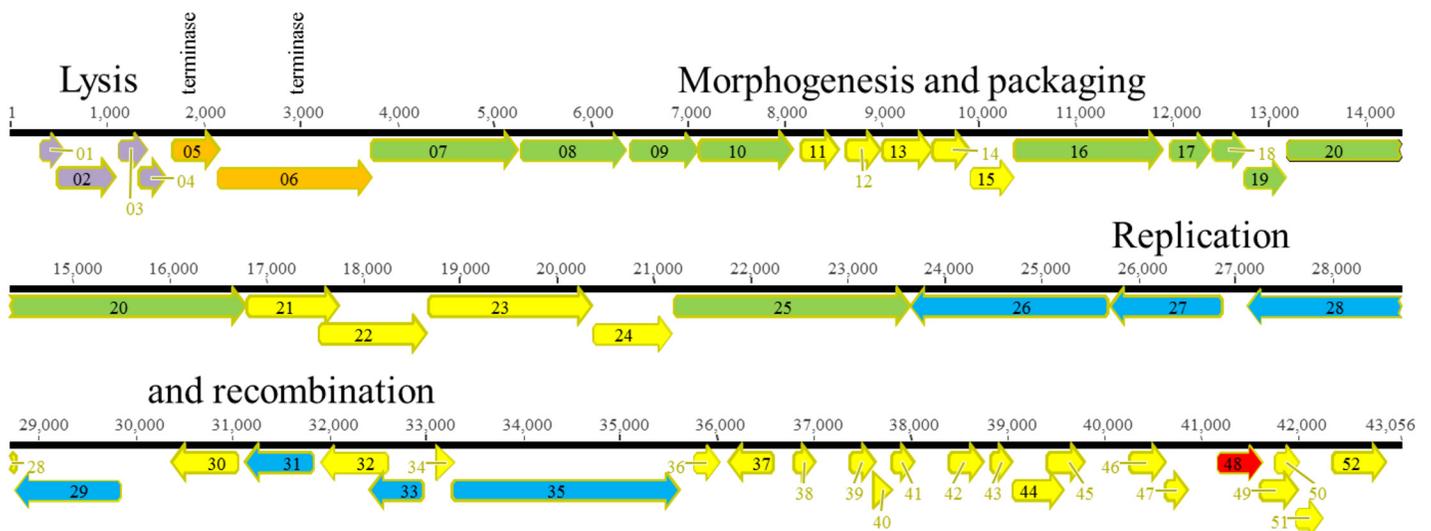


Fig 10. Genomic organization of PA73-like phage Ab26. The different ORFs are colored according to their putative function: yellow, unknown; orange, terminase; red, biosynthesis; green, morphogenesis; blue, DNA replication; purple, lysis.

doi:10.1371/journal.pone.0130548.g010

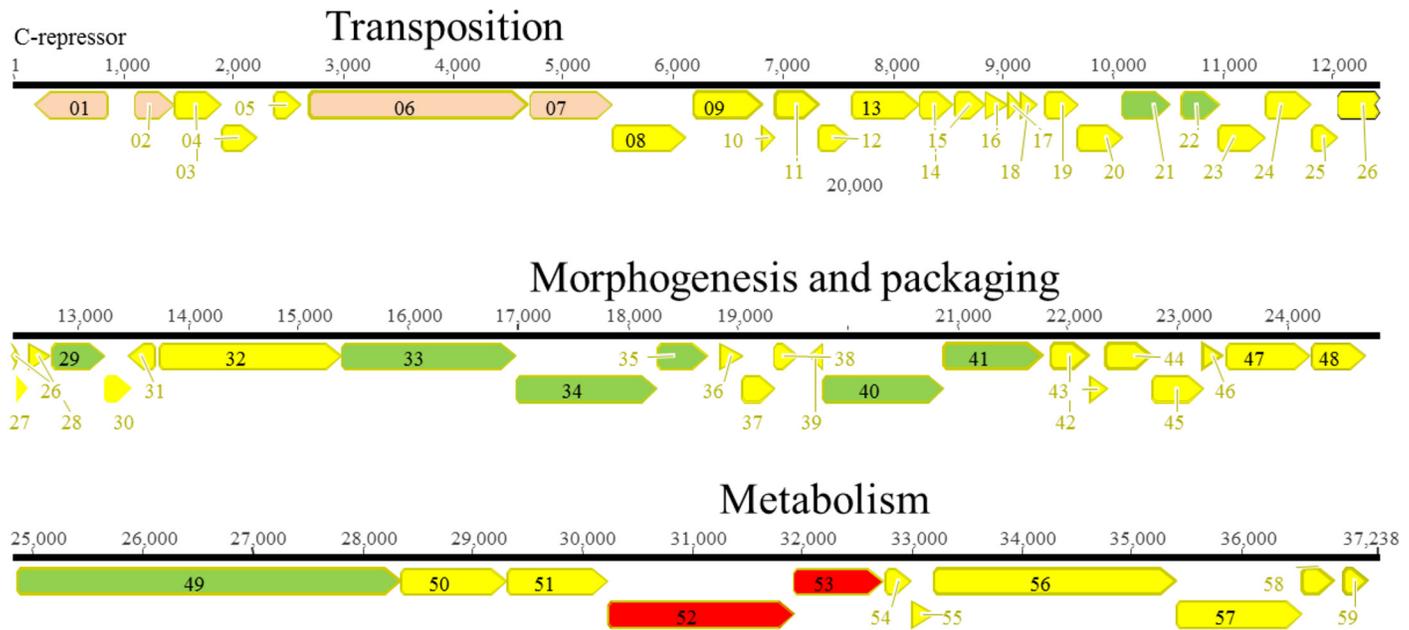


Fig 11. Genomic organization of D3112/B3-like phage Ab30. The different ORFs are colored according to their putative function: yellow, unknown; red, metabolism; green, morphogenesis; pink, transposition.

doi:10.1371/journal.pone.0130548.g011

(36,885bp; EU272037) and D3112 from Russia (37,611bp) [68]. These phages are related to the *Escherichia coli* phage Mu which replicates by transposition [69] and possesses chromosomal c repressor gene (ORF01) and of transposases A (ORF06) and B (ORF07) genes in the early genome region suggested a similar mechanism of lytic-lysogenic switch (Fig 11). Similarly to other phages of this genus, Ab30 possessed an extensive mosaic structure but the gene organization was well preserved. Alignment with phage DMS3 revealed regions of high diversity such as ORF01, and additional genes encoding short hypothetical proteins. Previously, it was shown that inhibition of biofilm formation as well as swarming motility of DMS3-lysogenic bacteria was mediated by the CRISPRs-Cas system [67]. Atypical genes able to inactivate bacterial CRISPR-Cas system were identified within Mu-like phage genomes [70,71]. By comparison with published sequences of Mu-like phages, the group of hypothetical genes encoding ORF36 to ORF39 may represent an anti-CRISPR region.

Virion structure

EM examination was performed for at least one phage of each genus (Fig 12). As expected from the genome sequence, phages belonging to the PAK-P1, KPP10 and PB1 genera were myoviruses (A1 morphotype) with long contractile tails. Five PAK-P1-like phages were analysed and all showed the same morphology, with a 130 nm tail and a 67–70 nm head. Ab11, a KPP10-like phage, had a 70 nm head and a 120nm long tail, while virion particles of Ab29, a PB1-like phage, possessed a 74 nm head and a 140 nm long tail. Among podoviruses with short tails (C1 morphotype), Ab05 and Ab22, belonging to the ϕ KMV-like and LUZ24-like genera respectively, displayed 60nm icosahedral heads, while Ab09, a N4-like phage, had a 70 nm icosahedral head. Ab18, Ab26 and Ab30 were siphoviruses with a B1 morphotype. Ab18 showed an elongated 85×60nm head and a 130 nm tail similar to that of phage YuA. Ab26 had a head of 60 nm and tail of 170 nm, Ab30 a head of 55 nm and tail of 180 nm.

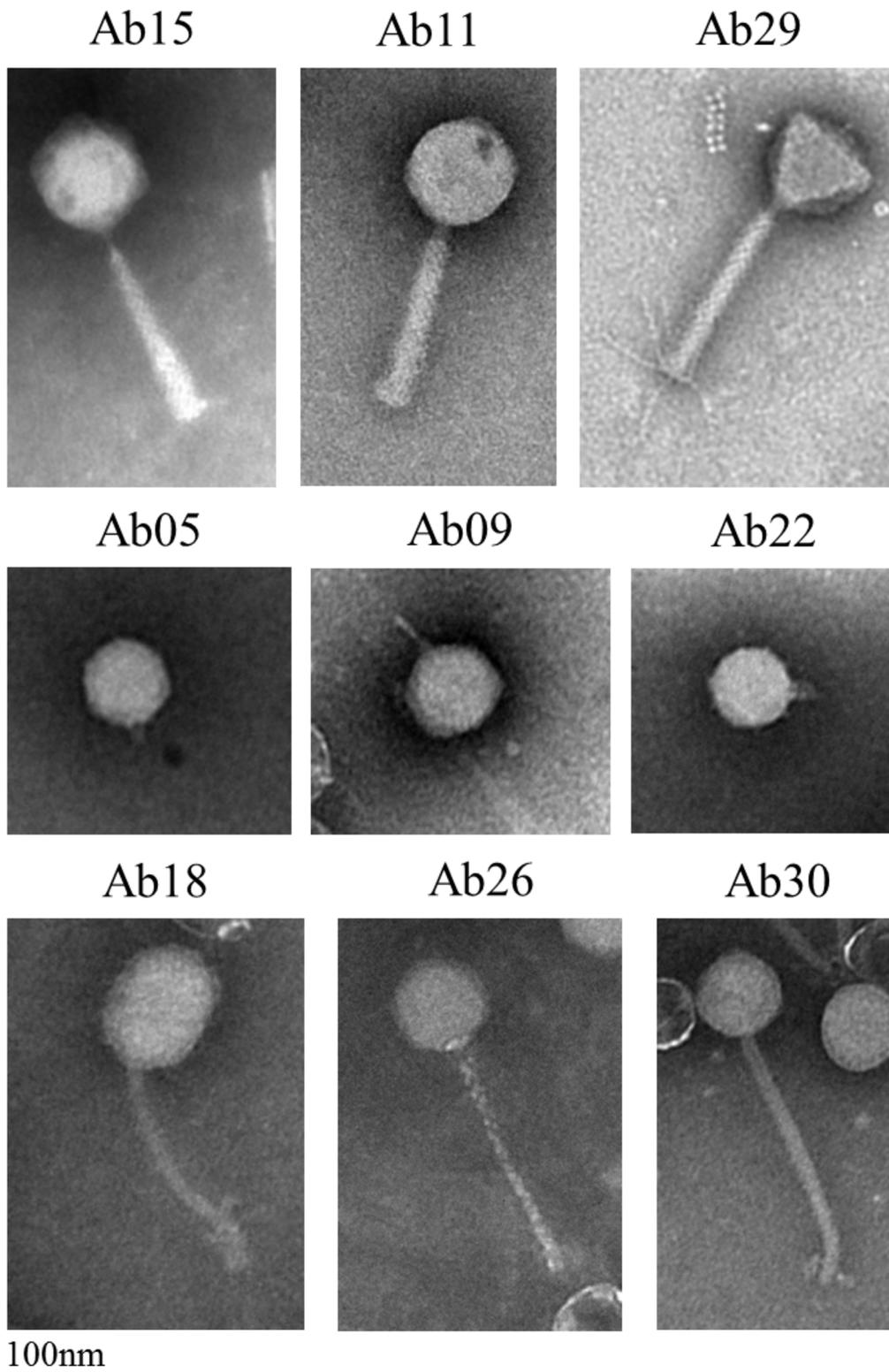


Fig 12. Electron microscopy examination of nine phages representative of the different genera observed in the present phage collection. Scale bar represents 100 nm.

doi:10.1371/journal.pone.0130548.g012

Table 3. Host-range of representative Abidjan and Pyo-Phage-derived phages.

Genus	PAK-P1	KPP10	PB1		N4		φ-KMV		LUZ24		YUA	PA73	D3112
	Ab08	Ab06	Ab27	p1-14 _{pyo}	Ab09	p8-13 _{pyo}	Ab05	1-15 _{pyo}	Ab22	p2-10 _{pyo}	Ab18	Ab26	Ab30
PAO1	-	c+	c+		c+	c+	c+	c+	t		c+	-	t
PA14	t	-	t	c+	-		c+	-	-	t	-	-	c+
Tr60	-	-	-	c+	-		t	t	t	c+	-	-	-
C50	-	-	-	-	-	-	-	-	-	-	c	-	t
SCH	c+	-	c+		c+		-	c+	-		c	c+	t
C1-1	-	t	c	t	c+	c+	t	c+	-		t	-	-
C1-2	c	t	-	t	c+	t	-	-	t	t	c	-	-
C1-14	c+	t	c+	c+	c+	c+	t	c+	t	t	t	-	-
C2-10	c+	-	t	-	c+	-	-	c	c+	c+	-	c	-
C3-16*	-	-	c+	-	t	c+	-	t	t	-	-	-	t
C3-20	-	-	-	-	-	t	-	-	c	c+	-	-	-
C5-2	-	-	t	-	c+	-	-	c+	-	-	t	t	t
C5-12	t	-	c	t	-	-	-	-	c	t	c	-	-
C5-13	-	-	-	-	-	-	-	-	-	-	-	-	-
C7-6	c+	-	-	-	-	-	t	t	-	-	t	-	-
C7-12	-	-	c	-	-	-	-	-	-	-	-	-	-
C7-25°	-	-	c	-	-	-	-	-	-	-	-	-	-
C8-5	c+	-	t	-	-	t	-	t	t	t	t	t	t
C8-7*	c	-	t	-	-	-	-	-	t	-	-	-	t
C8-14	-	-	-	-	-	-	-	-	-	-	-	-	-
C8-15	-	-	-	-	-	-	-	-	-	-	-	-	-
C8-20*	-	-	-	-	-	-	-	-	-	-	-	-	-
C9-5	c	-	c+	-	-	-	-	c	t	-	c	t	-
C9-6	c	-	c	-	-	-	t	-	-	-	-	-	-
C9-11	c+	t	t	c+	c+	c+	-	t	t	-	c	t	t
C9-17	c+	t	t	t	t	t	-	t	t	t	c	t	t

* slow growing strain

°continuous release of prophage

c: clear plaque; c+: maximum growth; t: turbid plaques

doi:10.1371/journal.pone.0130548.t003

Host-range spectrum

Phage host range was determined on the collection of *P. aeruginosa* strains used to isolate the phages of this study, and on additional strains selected for their resistance to Pyo-Phage [26]. The complete result is shown in S2 Table, and Table 3 shows the virulence spectrum of one phage for each genus and that of four phages isolated from Pyo-Phage (p1-14_{pyo}, p8-13_{pyo}, p1-15_{pyo} and p2-10_{pyo}). The members of a genus generally showed similar virulence towards the selected panel of bacterial strains with some exceptions. Together the present collection of lytic phages (all the myoviruses and the podoviruses except Ab31) which belong to six genera, could lyse 16 out of 20 tested strains. Four strains, C5-13, C8-14, C8-15 and C8-20 were resistant to all phages. Phages of the PAK_P1 genus showed the highest virulence both in term of spectrum and efficiency of plating. Seven of them showed a high plating efficiency on C7-6, a Pyo-Phage-resistant strain, in which they produced large clear plaques. On the same strain, Ab01, Ab15 and Ab23 showed a ten times lower growth, and Ab24 and Ab25 did not grow. No clear plaques could be observed on C7-6 with any phage belonging to the other genera. Similarly,

most phages of the PAK_P1 genus could lyse two other Pyo-Phage-resistant strains, C8-5 and C8-7. The three PB1-like phages displayed a large host range, infecting Pyo-Phage-resistant strains C7-12, C7-25, C9-5 and C9-6, but with a lower efficiency as compared to growth on PAO1, SCH and C3-16. This resembled the broad host-range of related T4-like viruses. As previously shown [72], KPP10-like phages were very specific toward certain clinical strains, and displayed strong virulent activity on strain PAO1 on which clear plaques were observed. They produced turbid plaques in a limited number of other strains. Several members of lytic PAK_P1, PB1 and KPP10-like phages could be obtained, possibly reflecting, for the first two genera, their wide host range. However it is more surprising for the KPP10-like phages which efficient growth is restricted to PAO1 in our collection of strains. This might suggest that *P. aeruginosa* is not the preferred host for this genus. Ab22, a LUZ-24-like phage, produced clear and turbid plaques in strain C2-10 (Fig 1), and uniform turbid small plaques in strain PAO1. The host-range of ϕ KMV-like phage Ab05 was similar to that of phage p1-15_{pyo}, another ϕ KMV-like phage, but with different efficiency, Ab05 being clearly less virulent. In particular p1-15_{pyo} produced large plaques with a halo characteristic of this genus in strain PAO1 [10], whereas Ab05 produced plaques with a small halo, only on PA14. The N4-like phage Ab09 had a rather large virulence spectrum but different from that of PAK_P1-like phages. It formed clear 1-2mm plaques on PAO1 without a halo, as opposed to other *P. aeruginosa* N4-like phages [52].

The siphoviruses of *P. aeruginosa* are majoritarily temperate phages and we expected them to display a rather specific host range. The group of phages related to YuA (Ab18, Ab19–Ab21, Ab20) showed a variety of virulence profiles, but the highest efficiency of plating was seen with PAO1 on which they produced small clear plaques (0.5–1mm). Interestingly only phages of this group infected the widespread European strain C50 (reference strain for clone C) that is also resistant to pyophage. Ab26 grew efficiently only on strain SCH and produced a more limited growth on several other strains. Phage vB_Pae-Kakheti25, belonging to the same genus possesses a very large host range toward clinical strains [64]. Ab30 showed a lytic activity only on PA14.

The ability of phages from the three siphovirus groups to form lysogens was tested by the presence of viral DNA by PCR in resistant colonies recovered from the centre of turbid plaques, and then passaged at least three times. Lysogens for Ab30 could be obtained in strain Tr60, as suggested by the stable presence of phage genome, and by mytomycin C induction of virions. Lysogens for Ab18 could not be obtained in strain PAO1, the most susceptible strain for this phage, as reported for YUA, and despite the presence of an integrase gene in the phage genome. In strain C2-18 in which only moderate growth was observed, the Ab18 DNA was maintained for several generations but disappeared upon further replating, suggesting a pseudolysogenic state. Ab26 genomes could be detected in resistant variants of SCH, the strain which supports its growth, but no stable lysogens could be obtained.

By mixing one member of each genus in the present collection, we were capable of lysing all *P. aeruginosa* isolates except four that appear to be also resistant to ϕ KZ-like viruses [26]. Host range is dependent on interactions between the phage tail fibers and bacterial receptor, but additional mechanisms are involved in bacterial susceptibility to phages. Phage infection failure may be due to exclusion of superinfection as the majority of strains isolated from CF patients possess one or several prophages [26]. Indeed, the presence of prophages in a bacterial chromosome immunizes bacteria against infection by phages of the same nature [73]. During lysogeny, D3-like phages modify the LPS receptors, making bacteria resistant to LPS-dependent phages, including virulent phages [74]. Another mechanism for specificity of virulent phages could be related to inhibition of the general phage defense mechanism built up by the CRISPR-Cas

system. Recent studies showed that the CRISPR-Cas system may be an actor in the bacterial resistance to virulent phages [75].

Conclusions

P. aeruginosa, a frequent opportunistic pathogen for humans, is abundant in waste water, together with a large variety of lytic and temperate phages. The phages have a profound influence on bacterial communities, through regulation of populations by mortality, and through modification of bacterial fitness and physiology by gene transfer and selection of resistant mutants [5,76]. We describe here *P. aeruginosa* phage diversity inside a single environment over a two-year period in a large African city. By comparison with published phage genomes, we show that the African phages, although belonging to already known genera, form a distinct population with important internal similarities. All the collect sites were somewhat connected which could explain that very similar phages were sampled at distant locations. It is thus likely that the phages grow inside a bacterial community that colonize the waste water system, providing here a unique view of their evolution in the environment.

The advance of high throughput sequencing technologies allows investigations of microbial populations on a large scale. Whole genome sequencing was applied to all isolated phages with high accuracy, and assembly produced a single contig. We expect that such an approach will open the way to new studies of phage-bacteria coevolution, either in natural environment or following therapeutic use of bacteriophages.

Supporting Information

S1 Fig. Plaque morphology of phage Ab22 on bacterial isolate C2-10. Both clear and turbid plaques are visible.

(TIF)

S2 Fig. Determination of the DTR. A) Detail of a region showing two peaks of reads and corresponding to the existence of the DTR at both ends of the genome. B) Sequencing reads showing a fixed end.

(PDF)

S3 Fig. Alignment using Geneious of five genomes of PAKP1-like phages, Ab01, Ab02, Ab08, Ab10 and Ab15.

(PDF)

S4 Fig. Alignment using Geneious of five genomes of KPP10-like phages Ab03, Ab04, Ab06, Ab11 and Ab17.

(PDF)

S5 Fig. Alignment using Geneious of 3 genomes of PB1-like phages Ab27, Ab28 and Ab29.

(PDF)

S6 Fig. Alignment using Geneious of 3 genomes of YuA-like phages Ab18, Ab19 and Ab20.

(PDF)

S1 Table. Putative function of ORFs observed in annotated phage genomes.

(XLS)

S2 Table. Host range of 29 Abidjan phages.

(XLS)

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Author Contributions

Conceived and designed the experiments: GV CP GL SN SL. Performed the experiments: CE LL AC AK CP. Analyzed the data: CE CM YB GV CP. Contributed reagents/materials/analysis tools: CE SL AC AK CP. Wrote the paper: CE GV CP.

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ANNEX II

A novel *Pseudomonas aeruginosa* bacteriophage, Ab31, a chimera formed from temperate phage PAJU2 and *P. putida* lytic phage AF: characteristics and mechanism of bacterial resistance



A novel *Pseudomonas aeruginosa* Bacteriophage, Ab31, a Chimera Formed from Temperate Phage PAJU2 and *P. putida* Lytic Phage AF: Characteristics and Mechanism of Bacterial Resistance

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Abstract

A novel temperate bacteriophage of *Pseudomonas aeruginosa*, phage vB_PaeP_Tr60_Ab31 (alias Ab31) is described. Its genome is composed of structural genes related to those of lytic *P. putida* phage AF, and regulatory genes similar to those of temperate phage PAJU2. The virion structure resembles that of phage AF and other lytic *Podoviridae* (*S. enterica* Epsilon 15 and *E. coli* phiv10) with similar tail spikes. Ab31 was able to infect *P. aeruginosa* strain PA14 and two genetically related strains called Tr60 and Tr162, out of 35 diverse strains from cystic fibrosis patients. Analysis of resistant host variants revealed different phenotypes, including induction of pigment and alginate overproduction. Whole genome sequencing of resistant variants highlighted the existence of a large deletion of 234 kbp in two strains, encompassing a cluster of genes required for the production of CupA fimbriae. Stable lysogens formed by Ab31 in strain Tr60, permitted the identification of the insertion site. During colonization of the lung in cystic fibrosis patients, *P. aeruginosa* adapts by modifying its genome. We suggest that bacteriophages such as Ab31 may play an important role in this adaptation by selecting for bacterial characteristics that favor persistence of bacteria in the lung.

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Introduction

Cystic fibrosis (CF) is one of the most common life-threatening, autosomal recessive genetic diseases in Caucasian children. This is due to mutations that occur in a single gene encoding the CF transmembrane regulator. The life expectancy of CF patients is above all related to the development of lung disease: the persistence of abundant mucous secretion in the lungs leads to chronic coughing at a young age, followed by frequent lung infections [1]. The microorganisms that colonize the CF patients' lungs belong to various bacterial genera. For 30% of CF patients, the predominant bacterial species during early life is *Staphylococcus aureus*, whereas in early adolescence, chronic infection with *Pseudomonas aeruginosa* is common: up to 80% of adult CF patients are colonized by this pathogen [2]. Later during colonization of the lungs, non-motile, anaerobic, mucoid variants of *P. aeruginosa* form a biofilm, a structure that confers resistance to several antimicrobial agents [3]. Usually, the microorganisms account for less than 10% of the dry weight of the biofilm, while 90% is composed of bacterially-produced extracellular polymeric substances (EPS) that form a matrix in which the bacterial cells are embedded [4].

The most abundant component of the EPS produced by *P. aeruginosa* is a polyanionic alginate, a copolymer of mannuronic

and glucuronic acids [5]. Typically, *P. aeruginosa* mucoid strains arise in the lungs of CF patients due to mutations in the *mucA* gene or when MucA is degraded by regulated intramembrane proteolysis [6]. Conversion of non-mucoid *P. aeruginosa* strains to mucoid variants can also be the consequence of selective pressure operated by bacteriophages [7,8]. Recent studies have shown that bacteriophages can drive the emergence of numerous variants with enhanced virulence potential [9,10].

The majority of *Pseudomonas* tailed phages belong to the order Caudovirales with three main families. Strictly lytic phages are found among the *Myoviridae* with a long contractile tail, and the *Podoviridae* with a short tail, whereas members of the *Siphoviridae* are temperate phages, implying the possibility to undergo lytic or lysogenic interactions with their host [11]. The most striking feature emerging from phage genome comparative analyses is that they are extensively mosaic, with different segments having distinct evolutionary histories. A simple general explanation is that horizontal genetic exchanges play a dominant role in shaping these genome architectures [12,13]. Gene modules are exchanged using host- or phage-encoded recombination machinery. Although some phages can switch host using different mechanisms, the host preferences represent a significant barrier to genetic exchange. Moreover, phages infecting a common host can also exhibit substantial diversity, creating additional barriers to genetic

exchange [12,13]. Horizontal gene transfer and the pattern of vertical, divergent evolution of phage genomes has led to the definition of different phage genera, and consequently, to a classification based on criteria related to phage genome organization and replication strategy [11]. Despite rapid phage evolution and the short generation time, viral genomes can be stably maintained over ecologically significant time and distance, and this allows their classification. Viral species can be identified and they appear to be globally widespread. Indeed, related members of specific genera with sequence identity up to 99%, can be isolated from different habitats across the globe [14,15]. The part of the phage genome that varies greatly within each genus is confined to genes encoding the metabolic conversion proteins (early region) and the tail spikes, indicating a local adaptation necessary to infect specific hosts in specific environmental conditions.

In the present study we describe a new phage, vB_PaeP_Tr60_Ab31, whose genome is the result of recombination between two phages belonging to two different families. This phage exerts a selective pressure on *P. aeruginosa*, which could be deleterious to chronically infected patients.

Materials and Methods

Ethics Statement

The present project is in compliance with the Helsinki Declaration (Ethical Principles for Medical Research Involving Human Subjects). Strains were collected from sputum as part of the patients' usual care, without any additional sampling. The ethic committee "Comité Consultatif pour la Protection des Personnes dans la Recherche Biomédicale (CCPPRB) Ile-De-France", who was consulted, specifically approved this study, and declared that patient informed consent was not needed.

Bacterial Strains

The two reference *P. aeruginosa* strains UCBPP-PA14 [16] and PAO1 [17] were purchased from the "Collection de l'Institut Pasteur" (CIP, Paris, France), and C50 was a gift of U. Römling (Karolinska Institute, Sweden) [18]. The other strains were isolated from sputum of French CF patients, and were previously genotyped using Variable number of tandem repeats (VNTR) analysis (MLVA) [19,20]. Strains were considered to belong to the same clonal complex when they shared at least 10 VNTR size alleles out of 15. Serological typing was performed using 4 polyvalent and 16 monovalent antisera (Bio-Rad), as described [21]. Briefly, the slide agglutination procedure was performed on 24 h cultures of *P. aeruginosa*: one loop (0.01 ml) of bacterial culture (approximately 3×10^6 CFU) was mixed with one drop (0.01 ml) of each antiserum (firstly the four polyvalent sera, then the four monovalent sera, corresponding to the positive polyvalent serum). The slide was gently shaken with a rotary movement, and the mixture was examined with the naked eye over a dark surface. A positive reaction was defined as the appearance of agglutination in a maximum of 2 min.

Phage Amplification and Purification

Phages were amplified on fresh LB agar plates at a ratio of 1 phage for 1000 bacteria. An overnight culture of bacteria grown in LB medium was concentrated 10 times in saline magnesium (SM) phage buffer (50 mM Tris-HCl pH 7.5, 100 mM NaCl, 8.1 mM MgSO₄, 0.01% gelatin). Phages were added and, after 15 min of incubation at room temperature, the mixture was poured onto a round plate together with 4 ml of soft agar. After complete bacterial lysis (\approx 8 h), 5 ml of SM phage buffer supplemented with a drop of chloroform were added to the recovered soft agar,

containing phage particles. After centrifugation, the supernatant was filtered through a 0.22- μ m pore size membrane, and kept at 4°C.

Small Drop and Double Agar Plate Assay

For the small drop assay, 50 μ l of 10X concentrated *P. aeruginosa* overnight culture were added to 4 ml molten soft agar (0.7%), and poured over an LB agar plate. Then, 10 μ l of serially diluted test lysate were spotted onto the bacterial lawn. For the double agar plaque assay, a mixture of 50 μ l of bacterial suspension and 10 μ l of phages at ten-fold serial dilutions was kept for 15 min at room temperature, and then poured onto a solid agar plate with 4 ml of soft agar. Plates were inverted and incubated overnight at 37°C.

Liquid Infection

LB medium (10 ml), supplemented with 10 mM CaCl₂, was inoculated at 2.5% with an overnight culture of the indicator strain, and incubated at 37°C. When an OD₆₀₀ of 0.6 was reached, phage suspensions at different multiplicities of infection (M.O.I.) were added. The OD₆₀₀ was periodically measured, and when a significant reduction of the culture density was recorded, 50 μ l of chloroform were added in order to facilitate bacterial lysis and release of phages. The suspension was centrifuged at 2,500 \times g for 10 min at 4°C to eliminate bacterial debris, and the supernatant was filtered through a 0.22 μ m filter.

Electron Microscopy Examination

Phage preparations were stained with 2% potassium phosphotungstate (pH 7.0), and then visualized using an EM208S transmission electron microscope (FEI, Eindhoven, The Netherlands) operating at 80 kV.

Isolation of Resistant Bacteria

Putative resistant bacteria were isolated by simply picking bacterial colonies growing inside the lysis zone of a small drop assay, and streaking them onto new plates. Putative resistant bacteria were also recovered at the end of the liquid infections, by directly streaking 1 μ l of the phage-bacterial mixture on a solid agar plate. Up to twenty colonies were picked and challenged with phages through the small drop assay. Some of them were susceptible to phage Ab31, and were thereafter called "non-resistant". Thermolysates of both resistant and non-resistant strains were prepared by resuspending a colony in 100 μ l of water, heating at 95°C for 10 min, followed by cooling on ice for 5 min. Centrifugation was performed at 2,500 \times g for 10 min at 4°C to pellet cell debris, and 2 μ l of the supernatant were used for PCR amplification.

Phage DNA Purification

Phage DNA was purified using a rapid method adapted from [22], as described in [14]. Briefly, phages were amplified on fresh LB agar plates for 8 h at 37°C, then 5 ml of SM buffer were added to the plate, followed by overnight incubation at 4°C. The buffer was transferred to a tube, and bacterial debris were pelleted by centrifugation at 2,500 \times g for 10 min at 4°C. A mixture of 0.2 ml 2 M Tris-HCl pH 7.5, 0.4 ml 0.5 M EDTA, 0.2 ml 10% SDS and 10 μ l diethylpyrocarbonate was added to 4 ml of supernatant. Following incubation at 65°C for 30 min, the tube was cooled on ice, and 1 ml of 5 M KOH was added. After 1 h incubation on ice, centrifugation was performed at 25,000 \times g for 20 min at 4°C. DNA contained in the supernatant was precipitated with 2 vol of absolute ethanol, pelleted by centrifugation, washed twice with 70% ethanol, dried and dissolved in 0.4 ml of TE buffer (10 mM

Tris-HCl pH 7.5, 1 mM EDTA). Bacterial DNA was purified using the classical CTAB (cetyl-trimethylammonium bromide)-phenol extraction method as described [19]. Purified DNA was resuspended in TE buffer. The quality and concentration of DNA was measured using a ND-1000 Spectrophotometer (NanoDrop®, Labtech, Palaiseau, France).

Sequencing

Whole genome sequencing was performed by the CNRS sequencing facility in Gif sur Yvette (IMAGIF) using the Illumina platform (Illumina Genome Analyzer IIx). Assembly of short sequence reads was performed using BioNumerics tools (Applied Maths, Sint-Martens-Latem, Belgium) as described [14]. The phage genome was annotated automatically using the BaSyS annotation tools [23]. Bacterial genome contigs were annotated using BioNumerics annotation tools. Detailed methods are available on the website <http://bacteriophages.igmors.u-psud.fr>.

The annotated Ab31 phage sequence has been deposited at EMBL-EBI under accession number HG798806. Total reads of bacterial genome sequences have been deposited at EMBL-EBI under accession number PRJEB5001.

PCR Detection of Phage DNA

Oligonucleotides selected to test for the presence of phage DNA in resistant bacteria and to analyze the deletions in bacterial genomes, are listed in Table 1. PCR was performed using purified DNA and Taq polymerase as recommended by the supplier (VWR, Strasbourg France). PCR products were analyzed on 2% agarose gels in 0.5X TBE buffer.

Results

Ab31 Virulence Spectrum

P. aeruginosa phage vB_PaeP_Tr60_Ab31, subsequently called Ab31, was isolated in Abidjan (Ivory Coast) as part of a study to

determine the phage diversity in waste water of this city [Essoh *et al.* submitted]. Ab31 was originally enriched on *P. aeruginosa* strain PA14, and subsequently amplified in this strain or in Tr60 (both of serotype O10). A total of 36 *P. aeruginosa* strains were tested for their susceptibility to the phage, including strains from the most frequently encountered clonal complexes in CF patients, PA14 and C50 [24], and reference strain PAO1 (Table 2). Six strains were shown to belong to the PA14 clonal complex (Tr60, Tr162, C7-11, C5-17, C9-12 and C8-12), and seven strains (Tr60, Tr162, C1-3, C3-1, C3-11, C4-14 and C9-5) were of serotype O10. The latter strains were selected in case the host O antigen would serve as a receptor for the phage, as described for *Vibrio cholera* phage VP4 [25]. Ab31 was responsible for complete lysis of Tr60 and Tr162, two non-mucoid strains with the same genotype, isolated from two CF patients at a one year interval, in the same hospital [19]. With the other strains no significant signs of lysis were detected.

Phage Characteristics

The morphology of phage Ab31 was determined by transmission electron microscopy (Fig. 1). The phage possesses an icosahedral head with a diameter of approximately 60 nm, and a short non-contractile tail. Moreover, the subterminal tail spikes were similar to those of phage AF of *P. putida* [26].

Infections in solid agar plates and in liquid medium were performed to analyse the Ab31 multiplication characteristics. When a phage suspension was analyzed on indicator strains using the double agar plaque assays, small clear plaques without a halo were observed. Dot assay revealed a clear zone with only a few small resistant colonies. Since it was known that many phages require CaCl₂ to adsorb on the bacterial surface, the infection in liquid medium was performed using LB supplemented or not with 10 mM of CaCl₂. Upon infection at an M.O.I. of 0.01, in the presence of 10 mM CaCl₂, production of PFUs was stimulated 100-fold. In these conditions, the adsorption time was 4 min and the burst size was 30–50 phages per cell.

Infection in liquid LB medium at an M.O.I. of 0.1 never led to a complete clearing of the bacterial culture (Fig. 2), but we observed that not all the bacteria that survived after infection were resistant, when later challenged with Ab31. This resembles the phenomenon of persistence, in which a subset of an isogenic bacterial population occurring within a susceptible population, tolerates antibiotics [27].

Interestingly, we observed that phage infection led to a change in color of the bacterial culture from yellowish-green to green. It is likely that the presence of the phage affects the production of the pyoverdine, a virulence factor of *P. aeruginosa* [28].

Genome Characteristics

The Ab31 genome encompasses 45,550 bp, and the overall GC-content is ≈ 57%, which is lower than that of the *P. aeruginosa* PA14 genome (66.3% G+C), a characteristic shared by other *P. aeruginosa* phages [29]. Comparing the virtual gel obtained by *in silico* restriction endonuclease analysis with the experimental restriction enzyme banding pattern (Fig. 3), it was possible to establish that Ab31 DNA is apparently circular. Indeed, the number of fragments expected from *in silico* digestion of the circular Ab31 DNA with *EcoRI*, *HindIII*, *SmaI*, *SspI*, *ClaI*, *SaII* and *SphI* was 17, 12, 12, 6, 18, 23 and 19 respectively, perfectly matched, in number and in size, with the bands obtained by experimental agarose electrophoresis. Ab31 DNA digestion using *NotI* and *PvuII*, from which 3 and 2 fragments, respectively, were expected using *in silico* digestion analysis, produced many faint bands in addition to the expected ones. This phenomenon, which

Table 1. List of primers used for PCR amplification.

Phage Ab31	
Ab31-Reg1-F	GACTCAGACCACTGAGATGA
Ab31-Reg1-R	ACGTGTTGGCAGTTGTAGAA
Ab31-Term-F	TACAACCGGATATCCGTGT
Ab31-Term-R	TGTCCTCTGATGGACAAA
<i>P. aeruginosa</i>	
PaTr60_Del22kb_F	TCATCCACTGTACGCCCG
PaTr60_Del22kb_R	CCGTTCTGATGCTCGACCACT
PaTr60_Del11kb_F	GACCATGACCTTGTCCGCAT
PaTr60_Del11kb_R	AGGAGGAAATGGGTGCGGAA
Porin1_PaerDel234_F	GAAATAGAGATTGCGCAGGC
Porin1_PaerDel234_R	CACCTTCGACGAGAGACACA
CupA_Paer_F	AGGATCGTCGGCGAGTAGTA
CupA_Paer_R	CTCTATAGCGGCTACTACAC
Porin2_PaerDel234_F	CTCAAGGACATCTACCGACA
Porin2_PaerDel234_R	AAGTCGCCGATCTGGATGAA
PaerDel234_Flank_F	TCCATCGCCTGCATGGCTTC
PaerDel234_Flank_R	CGGCATACTTCAATCAGGC

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Table 2. List of the strains used and susceptibility to Ab31.

Strain	Description	Serotype ^a	Source or reference	Ab31 growth ^b
PA14	Sequenced	10	[16]	C+++
PAO1	Sequenced	5	[17]	0
C50	Clone C	UN	[18]	0
Tr60	PA14-clone	10	[19]	C+++
Tr162	PA14-clone	10	[19]	C+++
C7-11	PA14-clone	15	[14,20]	trace
C5-17	PA14-clone	17	[14,20]	0
C9-12	PA14-clone	17	[14,20]	0
C8-12	PA14-clone	6	[14,20]	trace
C1-1		5	[14,20]	0
C1-2		3	[14,20]	0
C1-3		10	[14,20]	0
C1-11	Mucoid	15	[14,20]	trace
C1-14		1	[14,20]	0
C2-10		4	[14,20]	0
C2-18		12	[14,20]	0
C3-1		10	[14,20]	0
C3-2		13	[14,20]	trace
C3-11		10	[14,20]	0
C3-15		17	[14,20]	0
C3-16		1	[14,20]	0
C3-18		16	[14,20]	0
C3-19		6	[14,20]	0
C4-14		10	[14,20]	0
C5-2		17	[14,20]	0
C5-13		17	[14,20]	0
C7-6		3	[14,20]	0
C8-5		5	[14,20]	0
C8-7		12	[14,20]	0
C8-14		1	[14,20]	0
C8-15	Mucoid	1	[14,20]	0
C8-20		2	[14,20]	0
C9-5		10	[14,20]	0
C9-11		17	[14,20]	0
C9-17		17	[14,20]	0
C10-5		UN	[14,20]	0

^aUN, unknown.

^b5 μ L of Ab31 stock suspension ($\approx 10^8$ PFU/ml) were spotted on *P. aeruginosa* lawns. C+++; complete clearing; trace, a few individual plaques; 0, a turbid spot where the pipette tip touched the agar.

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was observed repeatedly, indicates the existence of a non-specific digestion also called relaxed sequence recognition or star activity [30].

Overall the Ab31 genome did not align with any known phage sequence. However, at the nucleotide level it showed some rare regions of homology with structural genes of phage AF [26] and of a prophage of *P. putida* GB-1 strain [CP000926-1] (up to 70% DNA-DNA similarity), and with genes involved in replication in phage PAJU2 [31] (from 85 to 97%). At the protein level it was possible to observe additional similarities with AF (shown in red on Fig. 4) and PAJU2 (shown in blue in Fig. 4). AF and PAJU2 are

two lambdoid phages whose genomes are respectively 42,689 bp and 46,872 bp long. Attempts to localize the genome ends by PCR analysis were not successful, as expected if the genome adopts a circular configuration. Therefore we were not able to determine the position of the first nucleotide, and we decided to assign it by comparison with the related phage AF.

In the Ab31 genome, 69 putative “open reading frames” (ORFs) were identified. Thirty five ORFs were transcribed on the positive strand (Fig. 4). On the basis of sequence similarity comparisons in the GenBank database, 25 ORFs could be assigned to putative functions. The majority of the other ORFs

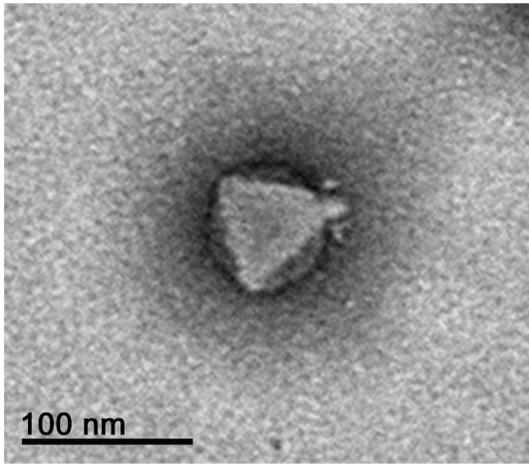


Figure 1. Electron microscopy analysis of phage Ab31. Scale bar represents 100 nm.
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exhibited similarity to uncharacterized bacterial or phage proteins. No tRNA genes were predicted.

Nucleotide position 1 was 60 bp upstream of the ATG codon of a putative protein containing a Helix Turn Helix domain, and sharing similarities with the small terminase subunit encoded by *Escherichia coli* phage phiv10 [32] and *Salmonella enterica* phage epsilon 15 [33]. A putative phage large terminase subunit, encoded immediately downstream of the predicted first coding sequence, shared similarities (maximal identity of 77%, E-value lower than e^{-200}) with a prophage-encoded sequence located within the *S. enterica* serovar Wandsworth str. A4-580 genome. The terminase genes constitute the packaging module typically located at the beginning of the so-called late region of the phage genome. A large part of the late region usually encodes the morphogenesis proteins, whereas genes located in the early region are necessary to initiate the phage multiplication cycle. Following phage adsorption to the bacterial surface, early genes are injected first and, in some phages, they allow complete injection of the phage genome [34]. Phage Ab31 showed high similarity in the late region with the

podovirus AF of *P. putida* and the prophage of *P. putida* strain GB-1: indeed, eight coding sequences reported in Fig. 4 resembled those of phage AF. These included the putative major capsid protein (maximal identity of 75%, E-value of $8e^{-180}$), internal virion protein B (maximal identity of 35%, E-value of $4e^{-6}$), the structural lysozyme (maximal identity of 33%, E-value of $8e^{-32}$) and the tail spike protein (maximal identity of 40%, E-value of $4e^{-37}$). Moreover, three hypothetical proteins located in the same region showed similarities with hypothetical proteins gp4, gp9 and gp12 of phage AF.

The second major block of genes of the Ab31 genome, downstream of the putative tail spike coding region, constitutes the so-called early/middle region. Twenty-three putative and hypothetical proteins encoded by genes located in this region shared similarities with the *P. aeruginosa* siphovirus PAJU2, showing maximal identity percentages that vary from 39% to 100%. This region starts with a putative acetyl-transferase sharing 46% identity with the PAJU2 acetyl-transferase (E-value lower than e^{-200}), and a putative PAJU2-like integrase (identity 99% with PAJU2; E-value lower than e^{-200}). Other related proteins included putative replication proteins O and P, NinB protein, phage antitermination protein Q and endolysin (Fig. 4).

Bacterial Resistance

Different conditions were used for infecting bacteria, in order to favor different resistance mechanisms. Most of the resistant and/or lysogenic bacteria were obtained from typical infection at 37°C in liquid medium of *P. aeruginosa* strains PA14, Tr60 and Tr162, at an M.O.I. of 1, 0.1 or 0.01. Some resistant Tr60 bacteria were also recovered from an infection in liquid medium performed at 42°C at an M.O.I. of 1. In this experiment the incubation was prolonged for 72 h, and the phage suspension was added each 24 h at the same M.O.I., for a total of three infections. Ab31-resistant variants of PA14 and Tr162 were also recovered from infections performed on solid agar plates at 37°C or 30°C, using a small drop plaque assay, and extending the incubation for 72 h in order to allow the growth of resistant bacteria inside the lysis zone. With the different approaches, about 80% (91 out of 114) of recovered bacteria were confirmed to be resistant to the phage. A majority of the resistant bacteria obtained from plates formed mucoid colonies, with entire margins and smooth surfaces, a particular characteristic often

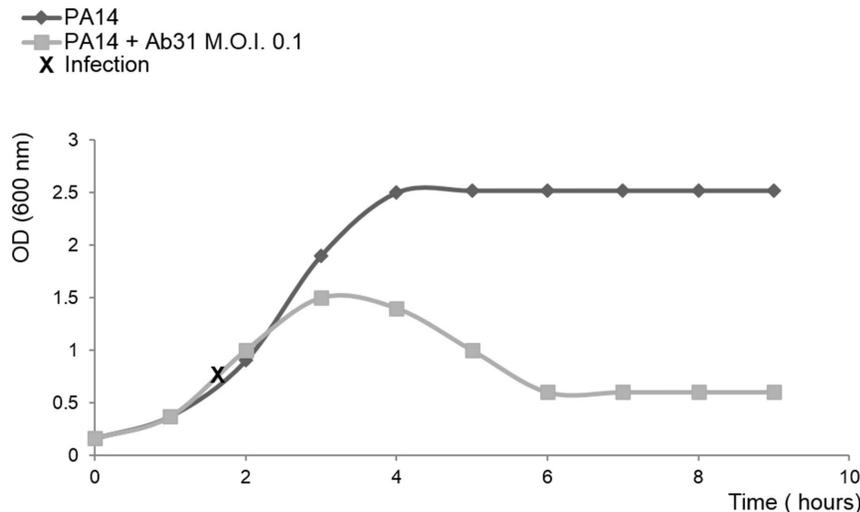


Figure 2. Growth curve of uninfected PA14 (dark grey curve) and of PA14 infected by Ab31 at an M.O.I. of 0.1 (light grey curve).
doi:10.1371/journal.pone.0093777.g002

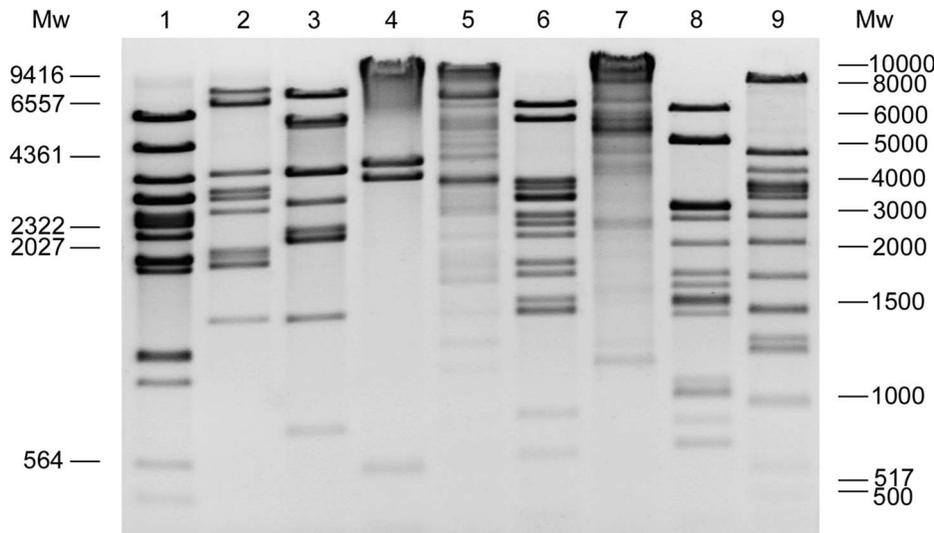


Figure 3. Restriction enzyme analysis of the Ab31 genome. Ab31 phage DNA (2 μ g) digested with *EcoRI* (1), *HindIII* (2), *SmaI* (3), *SspI* (4), *NotI* (5), *ClaI* (6), *PvuII* (7), *Sall* (8) and *SphI* (9) were analyzed by electrophoresis on a 0.8% agarose gel. On the left the λ DNA/*HindIII* and on the right 1 kbp ladder are reported and they were used as molecular weight markers (Mw). doi:10.1371/journal.pone.0093777.g003

observed with *P. aeruginosa* strains isolated from sputa of CF patients [Sousa, 2013 #1739]. In contrast, non-resistant bacterial strains derived from the same experiment showed a non-mucoid phenotype and shared the same morphological characteristics with the wild type uninfected bacterial strain. From infected bacteria incubated on plates at 30°C, some mucoid resistant variants showing a brown pigmentation were also isolated. Ten resistant isolates were serotyped to check whether a switch had occurred, but they were all serotype O10 like the parental strains.

In order to test for the existence of lysogens, the presence of the phage DNA was searched in the Ab31-resistant bacteria after two passages on solid agar medium. Two regions of the phage genome were amplified, designated as Reg1 and Term, and predicted to produce 550 bp and 600 bp long amplicons respectively. The results for some of the resistant isolates and for Ab31 phage DNA used as a control, are shown in Fig. 5. When the DNA of the resistant bacteria was amplified using the Reg1 (Fig. 5A) or Term primers (Fig. 5B), a band of the expected size was detected with every tested isolate except for Tr60-100A. This Ab31-resistant variant was derived from infection in liquid medium at 37°C at an M.O.I. of 0.1. All the resistant variants from solid infection were positive for phage DNA, whereas only 40% of resistant from liquid infection contained phage DNA. The isolates showing a brown pigmentation were not lysogenized.

Bacterial Genome Sequence Analysis

In order to study the basis of the resistance and to identify the possible integration site of the phage genome in the bacterial chromosome, the original Tr60 strain and four Ab31 resistant variants were chosen for whole genome sequencing. Tr60-10A and Tr60-100B were two putative lysogenic bacteria derived from infection in liquid medium at an M.O.I. of 1 and 0.1, respectively. Tr60-100A was a non-lysogenic isolate derived from infection in liquid medium performed at an M.O.I. of 0.1. PA14-P1 was a putative lysogenic bacteria. Tr60-10A and Tr60-100A showed a mucoid phenotype. The bacterial draft genomes were assembled and partially annotated using *P. aeruginosa* PA14 as a reference [16,35].

Upon alignment of the sequenced genomes, two deletions were found in the *P. aeruginosa* Tr60 genome as compared to that of PA14. The first deletion encompassed approximately 22 kbp (coordinates 1919495 to 1941370). It started with 2.5 kbp of DNA of unknown function and ended inside a gene encoding a pirin-like protein (ORF PA14_22080 to ORF PA14_22280), and also contained genes for a resolvase and a recombinase. The second deletion encompassed approximately 11 kbp, and covered exactly the sequence of the Pfl prophage of *P. aeruginosa* PA14 (Genbank: AY324828). Both deletions were identified in Tr60, in all Tr60 variants that were sequenced and in the genome of reference strain PAO1. The existence of the two deletions was confirmed by PCR in Tr60 and its Ab31-resistant variants using primers localised in the flanking regions (Table 1; Fig. 6). The deletions were also observed in Tr162 and in PAO1, as expected. In addition to the two deleted regions, approximately 215 “single nucleotide polymorphisms” (SNPs) were found when the genome sequences of Tr60 and PA14 were compared.

We then looked at the differences between Tr60 and its Ab31-resistant variants. A deletion of about 234 kbp was found in Tr60-10A and Tr60-100A, two mucoid variants obtained from different infections performed with the same Tr60 bacterial culture. Using *P. aeruginosa* PA14 as a reference genome, 175 coding sequences were found to lie within this region (Table 3, genome coordinates approximately 3190870 and 3424480 in PA14). Some of these coded for enzymes involved in amino acid uptake or biosynthesis, for glucose metabolism and for transmembrane proteins. No homologous genes were found in the other regions of the bacterial genome except for the porins. A cluster of five genes involved in the CupA fimbrial organelle assembly was possibly relevant to phage resistance: the chaperone CupA1, the fimbrial subunit CupA2, the usher CupA3, CupA4, an atypical adhesin, and the chaperone CupA5 [36]. Primer pairs were selected within this region (in two porin-encoding genes and in the *cupA4* gene), and in the flanking sequences, in order to confirm the existence of the deletion by PCR amplification. When amplification was performed with primers localised inside the region of deletion, an amplicon was observed for all the samples tested except for Tr60-10A and Tr60-100A (the result for *cupA* is shown on Fig. 7A). In

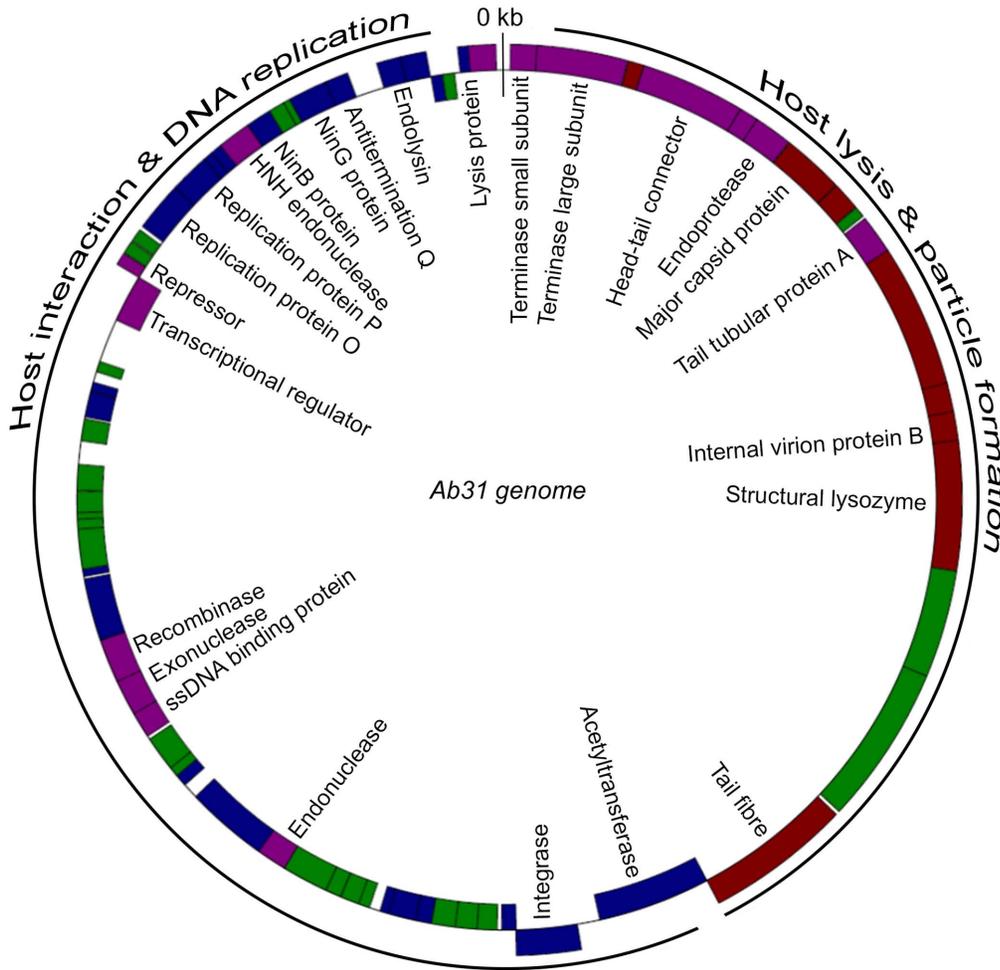


Figure 4. Annotation of the Ab31 genome. The Ab31 hypothetical terminal ends are at the 1 position. The morphogenesis module includes proteins similar to those encoded by phage AF (red), whereas the replication, recombination and lysis modules consist of genes similar to those of phage PAJU2 (blue). Genes encoding hypothetical proteins which have homologies with other phages are shown in purple color. Genes encoding hypothetical proteins of unknown function are shown in green color.
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contrast, amplification with the Flank234 primers, localised on both sides of the deleted region, produced a 600 bp amplicon only for Tr60-10A and Tr60-100A (Fig. 7B). In order to check whether the deletion was pre-existing in a subpopulation of the Tr60 culture before phage infection, 94 bacterial colonies were picked and PCR was performed on thermolysates with the Flank 234 and

porin primers. The results showed that none of the isolates were deleted for the 234 kbp region (data not shown).

Compared to Tr60, Tr60-10A and Tr60-100A showed approximately the same amount of SNPs (respectively 29 and 26). The mutations occurred in the same coding sequences for both resistant bacteria. In particular, mutations were found in a

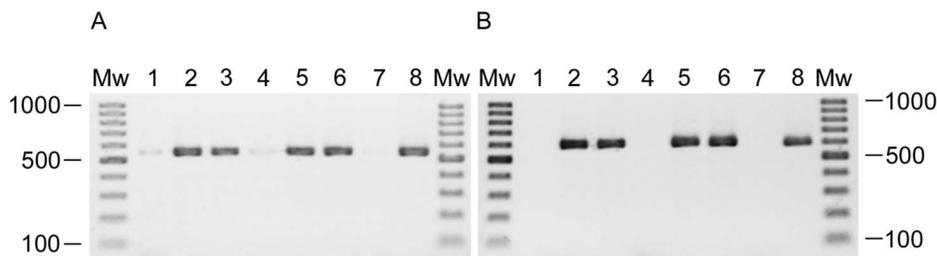


Figure 5. PCR detection of the Ab31 genome in resistant bacteria using Reg1 (A) and Term primers (B). Tr60-E (5) and PA14-P1 (6) are derived from infection on solid agar plates at 37°C and 30°C, respectively. Tr60-10A (2), Tr60-100B (3) and Tr60-100A (4) are derived from an infection assay performed at 37°C in liquid medium at an M.O.I. of 0.1. PA14 (7), Tr60 (1) and Ab31 (8) are used as negative and positive controls, respectively. Samples were run on a 2% agarose gel for 45 min at 135 V. Mw, 100 bp ladder molecular weight markers.
doi:10.1371/journal.pone.0093777.g005

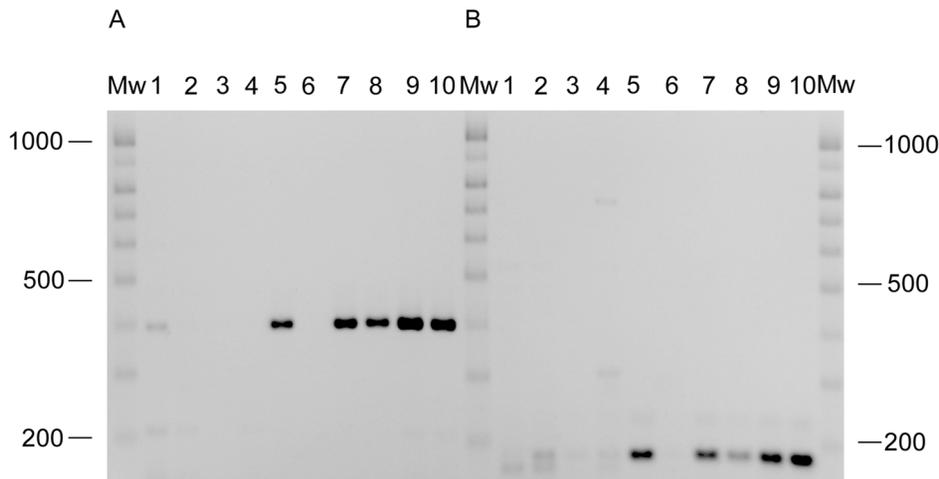


Figure 6. PCR investigation of the 11 kbp (A) and 22 kbp (B) deletions observed in Tr60. C9-12 (1), C5-17 (2), C7-11 (3), C8-12 (4), PAO1 (5), PA14 (6), Tr162 (7), Tr60 (8), Tr60-10A (9), Tr60-100A (10). Samples were run in a 2% agarose gel for 45 min at 135 V. Mw, 100 bp ladder molecular weight markers.

doi:10.1371/journal.pone.0093777.g006

transcriptional regulator, in the NADH dehydrogenase I subunit F, the pyoverdine biosynthesis protein PvcA, phenazine biosynthesis protein PhzD, C32 tRNA thiolase and in an MFS transporter. Specific mutations in a gene for an Usher protein and in a gene for a lipase chaperone, were detected in Tr60-10A and Tr60-100A, respectively. The sequence of the Tr60-100B isolate was compared with that of Tr60, and no SNPs were found.

The PA14-P1 genome showed 129 SNPs as compared to PA14. They affected genes encoding the hemolysin activator, the pyochelin synthase, an RNA methyltransferase, an acetyltransferase, the pyoverdine synthase, and several membrane proteins, including multidrug efflux pumps, type III secretion system proteins and an ABC transporter.

Search for the Phage Integration Site

To identify integrated Ab31 genomes, we first searched for phage reads among the three isolates found to possess phage DNA by PCR, but they were detected only in Tr60-100B. We then looked for phage-bacteria hybrid sequences among the total reads obtained for this isolate. The hybrid reads centered on a 64 bp region found at position 4552973–4553038 in the *P. aeruginosa* PA14 genome (and also in the Tr60 genome), and at position 21471–21534 on the Ab31 genome. These regions correspond in PA14 to the serine tRNA gene (PA14_51230) localized downstream of a glycosyltransferase gene, and, in the Ab31 genome, to a region that covers part of the phage integrase and part of a non-coding sequence upstream of this gene (Fig. 8).

Discussion

Phage Ab31 is a temperate phage genomically related to both the virulent podovirus AF phage of *P. putida* and the temperate siphovirus phage PAJU2 of *P. aeruginosa*. The Ab31 virion structure resembles that of phage AF, *S. enterica* phage Epsilon 15 and *E. coli* phage phiv10, with similar spikes, previously shown to bind and cleave the O-antigen component of the host's cell surface lipopolysaccharide [37]. Some *Pseudomonas* phages can diffuse through alginate present in *Pseudomonas* biofilms [38] owing to a depolymerizing enzyme that is part of the phage particles. One of the most significant examples of such an activity has been reported for phage AF. A halo surrounds the AF plaques at 30°C, due to an

EPS-degrading activity within the tail spikes [26]. We did not see such a halo around Ab31 plaques, whatever the strain used or the temperature. Alignment of the tail spike protein sequences of phage Ab31 and phage AF showed limited homology only at the N-terminus (Fig. 9). Conservation of the N-terminal part is necessary for association of the spikes with the tail structure, whereas the C-terminal part of the spike protein, involved in recognition of and binding to the cell receptor, shows the highest level of variation [39,40,41]. This finding provides evidence that bacteria and bacteriophages have co-evolved in order to overcome the barriers that are imposed by one on the other. Recent observations suggest that bacterial resistance to phages in cystic fibrosis patients evolves with the duration of colonization [42]. The infection by Ab31 causes a slight change in the colour of the bacterial culture from yellowish-green to green, as also observed with phage PAJU2 [43]. Mutation in genes for pyoverdine biosynthesis were identified in some resistant isolates, but further analyses are necessary to determine their significance.

Among the diverse strains tested, Ab31 is specific for PA14 and for Tr60 and Tr162, two strains genetically close to PA14, and isolated from French CF patients in the same hospital. Other strains belonging to the same clonal complex but isolated at other locations were found to be resistant to Ab31, as were strains with the same O serotype. Sequencing of Tr60 revealed the presence of two regions of deletion, one of which corresponds to prophage Pf1, also designated Pf5 in the PA14 genome [44]. It was previously shown that Pf4, a Pf1-like prophage in PAO1, mediated the formation of small-colony variants, and was also a factor contributing to the bacterial virulence, but this was not confirmed for Pf5 in PA14 [45,46]. The 22 kbp region corresponding to the second deletion encodes a resolvase and a recombinase, proteins that usually participate in DNA transfer.

Differences in the bacterial response to phage infection were observed in this study depending on the infection method used. From the infection on solid agar plate, clear lysis zones were observed, an indication that most cells were killed, whereas in liquid culture complete lysis was never obtained. This observation may be related to the formation of pseudodysogens after bacterial infection. The genomes of two sequenced Ab31-resistant variants (Tr60-10A and PA14-P1), shown to contain phage DNA when analysed by PCR, surprisingly did not contain phage reads. A

Table 3. List of coding sequences (CDS) and their position in the 234 kbp deleted region.

CDS	Position	Product
1	87..1418	amino acid permease
2	1482..2921	gamma-aminobutyraldehyde dehydrogenase
3	2967..4220	diaminobutyrate-2-oxoglutarate aminotransferase
4	4267..5229	dehydrogenase
5	- strand (5522..7501)	acetate permease
6	- strand (7558..9828)	acyl-CoA synthetase
7	- strand (9291..10061)	dehydrogenase
8	- strand (10058..11284)	acyl-CoA dehydrogenase
9	- strand (11634..12851)	FadE36, aminoglycoside phosphotransferase
10	12886..14838	propionate catabolism operon regulator
11	- strand (14904..15443)	hypothetical protein
12	- strand (15466..17769)	paraquat-inducible protein B
13	- strand (17762..18619)	paraquat-inducible protein A
14	- strand (19040..20638)	aldehyde dehydrogenase
15	- strand (20752..21747)	hypothetical protein
16	- strand (21775..22950)	hypothetical protein
17	- strand (22981..24408)	MFS transporter
18	- strand (24489..25907)	porin
19	- strand (25912..26925)	4-hydroxythreonine-4-phosphate dehydrogenase
20	- strand (26922..27881)	hypothetical protein
21	- strand (27874..29298)	MFS transporter
22	29301..30479	hypothetical protein
23	30464..32554	hypothetical protein
24	32544..33569	LysR family transcriptional regulator
25	33651..34127	hypothetical protein
26	34342..35232	ABC transporter substrate-binding protein
27	35313..36029	amino acid permease
28	36031..36708	amino acid ABC transporter permease
29	- strand (36724..37608)	hypothetical protein
30	- strand (37734..39329)	signal transduction protein
31	- strand (39418..40800)	dehydrogenase
32	- strand (40640..41758)	hypothetical protein
33	- strand (41808..43745)	TetR family transcriptional regulator
34	- strand (42545..44821)	hydrogen cyanide synthase HcnC
35	- strand (43801..45267)	hydrogen cyanide synthase HcnB
36	- strand (45192..45626)	hydrogen cyanide synthase HcnA
37	45789..47114	adenylate cyclase
38	- strand (47111..47767)	hypothetical protein
39	- strand (48272..49708)	carboxylate-amine ligase
40	48802..51048	hypothetical protein
41	50993..51982	hypothetical protein
42	- strand (51966..52247)	hypothetical protein
43	52256..52957	hypothetical protein
44	- strand (52961..55336)	sensor/response regulator hybrid
45	56396..56686	hypothetical protein
46	- strand (56717..57031)	hypothetical protein
47	- strand (56994..57581)	hypothetical protein
48	- strand (57371..58447)	hypothetical protein
49	- strand (59123..60370)	hypothetical protein
50	- strand (59701..60759)	hypothetical protein

Table 3. Cont.

CDS	Position	Product
51	- strand (60474..61766)	hypothetical protein
52	- strand (61900..62265)	hypothetical protein
53	62570..64174	glycogen synthase
54	64033..65925	glycosyl hydrolase
55	65918..67972	4-alpha-glucanotransferase
56	67752..70745	maltoooligosyl trehalose synthase
57	71060..73210	glycosyl hydrolase
58	76155..77918	cardiolipin synthase 2
59	76625..78910	hypothetical protein
60	- strand (78912..81272)	glycogen branching protein
61	- strand (81107..84409)	trehalose synthase
62	- strand (84420..86534)	hypothetical protein
63	- strand (86558..87439)	KU domain-containing protein
64	- strand (87462..87704)	hypothetical protein
65	- strand (87718..88263)	hypothetical protein
66	- strand (88437..90566)	hydroperoxidase II
67	- strand (90647..90814)	hypothetical protein
68	91301..91711	hypothetical protein
69	- strand (91718..94156)	glycogen phosphorylase
70	- strand (94209..94496)	hypothetical protein
71	94740..94928	hypothetical protein
72	- strand (94948..95991)	short-chain dehydrogenase
73	- strand (95834..96352)	ompetence-damaged protein
74	- strand (96363..96608)	metallothionein
75	- strand (96913..99468)	ATP-dependent DNA ligase
76	- strand (99453..100118)	hypothetical protein
77	99851..100615	hypothetical protein
78	- strand (101068..102432)	transporter
79	- strand (102461..103387)	hypothetical protein
80	- strand (103126..104031)	EAL domain-containing protein
81	- strand (103980..104669)	chaperone CupA5
82	- strand (104683..108297)	fimbrial subunit CupA4
83	- strand (106041..108659)	usher
84	- strand (108643..109476)	chaperone CupA2
85	110882..112108	fimbrial subunit CupA1
86	112535..113170	hypothetical protein
87	- strand (113206..114660)	aldehyde dehydrogenase
88	- strand (114676..116313)	dehydrogenase
89	- strand (116418..117350)	LysR family transcriptional regulator
90	- strand (117387..118523)	hypothetical protein
91	118649..119554	LysR family transcriptional regulator
92	119600..120130	hypothetical protein
93	120147..121259	hypothetical protein
94	- strand (121794..122870)	O6-methylguanine-DNA methyltransferase
95	123062..124045	hypothetical protein
96	- strand (124030..124896)	hypothetical protein
97	- strand (124934..125998)	LysR family transcriptional regulator
98	126034..127413	major facilitator transporter
99	127438..128667	porin

Table 3. Cont.

CDS	Position	Product
100	128700..129443	LamB/YcsF family protein
101	129529..131091	hypothetical protein
102	131159..131635	hypothetical protein
103	- strand (131654..133426)	thiamine pyrophosphate protein
104	133525..133995	hypothetical protein
105	- strand (134394..136025)	short chain dehydrogenase
106	- strand (135137..136177)	esterase
107	- strand (136077..137552)	flavin-binding monooxygenase
108	137699..138733	AraC family transcriptional regulator
109	138861..139727	hypothetical protein
110	- strand (139729..141048)	transmembrane sensor protein
111	- strand (141294..142682)	MFS transporter
112	- strand (142477..144135)	permease
113	- strand (144849..147995)	TonB-dependent receptor
114	- strand (147592..148680)	hypothetical protein
115	- strand (148424..149845)	hypothetical protein
116	- strand (149077..150093)	hydrolase
117	- strand (150527..152425)	asparagine synthetase, glutamine-hydrolysing
118	- strand (152447..153649)	ring-hydroxylating dioxygenase, large terminal
119	- strand (153923..154405)	leucine-responsive regulatory protein
120	154451..155173	kynurenine formamidase, KynB
121	155177..156427	kynureninase
122	155773..157989	amino acid permease
123	158230..160149	hypothetical protein
124	160165..162093	hypothetical protein
125	- strand (162130..163506)	transcriptional regulator
126	163546..163755	hypothetical protein
127	163932..165599	hypothetical protein
128	- strand (165992..168586)	sensory box protein
129	- strand (168758..171127)	elongation factor G
130	171142..173784	TonB dependent receptor
131	173892..175688	carbamoyl transferase
132	175732..176895	MFS transporter
133	176840..177565	hydrolase
134	176941..178200	hypothetical protein
135	178277..180169	copper resistance protein A
136	179851..181221	copper resistance protein B
137	- strand (181241..182575)	hypothetical protein
138	- strand (182667..183848)	pyridoxal-phosphate dependent protein
139	- strand (183994..185604)	ABC transporter ATP-binding protein
140	- strand (185606..186622)	ABC transporter permease
141	- strand (186624..187697)	peptide ABC transporter permease
142	- strand (187699..189507)	ABC transporter substrate-binding protein
143	- strand (189511..192537)	TonB-dependent receptor
144	- strand (192730..193722)	LysR family transcriptional regulator
145	193773..195158	major facilitator transporter
146	- strand (195165..196064)	DNA-binding transcriptional regulator CynR
147	- strand (197377..198330)	Fe ²⁺ -dicitrate sensor, membrane protein
148	- strand (198327..199004)	RNA polymerase sigma factor

Table 3. Cont.

CDS	Position	Product
149	199037..201157	hypothetical protein
150	203113..204468	hypothetical protein
151	204410..204787	hypothetical protein
152	204854..206836	hypothetical protein
153	- strand (208017..209408)	serine/threonine transporter SstT
154	- strand (209734..211104)	amino acid permease
155	- strand (211261..212637)	glutamine synthetase
156	213047..213901	hypothetical protein
157	213157..214200	hypothetical protein
158	- strand (214325..215800)	hypothetical protein
159	- strand (215925..216731)	hypothetical protein
160	217181..218839	thiamine pyrophosphate protein
161	- strand (218847..220016)	hypothetical protein
162	- strand (219521..220477)	hypothetical protein
163	- strand (220564..222048)	transcriptional regulator
164	222029..222676	hypothetical protein
165	- strand (222751..223581)	hypothetical protein
166	- strand (223074..223547)	transcriptional regulator
167	223638..224072	hypothetical protein
168	223754..225226	bile acid/Na ⁺ symporter family transporter
169	225251..227203	ring-cleaving dioxygenase
170	- strand (225281..226636)	glutathione reductase
171	- strand (227385..228275)	UTP-glucose-1-phosphate uridylyltransferase
172	- strand (228272..229873)	nucleotide sugar dehydrogenase
173	- strand (230015..230575)	transcriptional regulator
174	230812..232002	periplasmic multidrug efflux lipoprotein
175	232018..233901	multidrug efflux protein

doi:10.1371/journal.pone.0093777.t003

possible explanation could be that the phage genome did not integrate in the bacterial chromosome but was retained in a small proportion of cells after infection. Indeed, there is evidence that some of the putative lysogenic bacteria lose the phage genome after several replatings. In contrast, Tr60-100B is a true lysogenic variant. The phage integrated within the bacterial genome through a site-specific recombination process using the shared 64 bp sequence, as shown for PAJU2 [43].

In this study, mucoid variants of *P. aeruginosa* Tr60 and PA14 were obtained after infection of the host in liquid medium. These variants were stably resistant to the phage, although they were not lysogenic. Miller *et al.* showed that temperate phages with elongated heads and flexible tails (similar to PAJU2 virions), induced from CF-associated *P. aeruginosa* strains, were capable of converting non-mucoid strains to the mucoid phenotype [8]. We may hypothesize that the presence of the phage induced a stress that caused mutations in genes involved in alginate production. An alternative explanation could be that the phage selected a subpopulation of mucoid bacteria, with the mucoid layer inhibiting early stages of infection. MucA is a negative regulator of alginate production through sequestration of AlgU, the primary sigma factor responsible for the expression of the alginate biosynthetic operon from the algD promoter [47]. Alternatively, conversion to mucoidy can occur when MucA is degraded by

regulated intramembrane proteolysis operated by AlgW [6]. The activation of AlgW, and the consecutive proteolysis of MucA, is thought to be in response to extracellular stress, as well as the accumulation of misfolded envelope proteins. Interestingly, genome sequencing of two non-pigmented mucoid Ab31-resistant variants (Tr60-10A and Tr60-100A) revealed that no modifications of proteins involved in the alginate biosynthesis occurred. Among the Ab31 resistant variants we also obtained brown-colored, mucoid variants of PA14. The pigment which likely corresponds to pyomelanin, accumulated when the plates were kept at room temperature. It was shown that pyomelanin production is due to loss of the homogentisate gene, *HmgA* and this favors persistence in the lung of CF patients [48].

Notably, two mucoid variants of Tr60 carry a large deletion of 234 kbp corresponding to, among others, genes coding for proteins necessary to assemble a fimbrial organelle. This gene cluster which encodes components of the chaperone-usher pathway and a fimbrial unit, participates in biofilm formation [36,49]. Conceivably fimbriae could be involved in phage adsorption but further investigation is required to confirm or refute this hypothesis. Other deleted genes that might act as phage receptors, are those for two porins, and one being a member of the LamB/YcsF family protein. Previous studies have shown that an outer membrane porin encoded by the *ompLC* gene in *Edwardsiella*

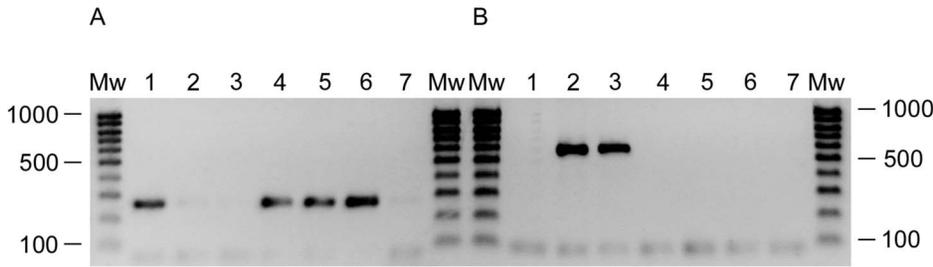


Figure 7. PCR investigation of the 234 kb deletion on Ab31 resistant bacteria using CupA (A) and Flank234 primers (B). Tr60 (1), Tr60-10A (2), Tr60-100A (3), Tr60-100B (4), PA14-P1 (5), PA14 (6), Negative control (7). The experimental conditions are those of Fig.5. doi:10.1371/journal.pone.0093777.g007

ictaluri is required for phage sensitivity [50], while LamB is the receptor for *Escherichia coli* bacteriophage λ. LamB was shown to be sufficient to confer λ phage sensitivity upon transformation of the *lamB* gene into bacteria of different species [51,52].

Since the two resistant isolates, in addition to the 234 kbp deletion, show a few nucleotide differences (≈ 40 SNPs) with Tr60, two hypotheses could be formulated to explain the origin of the deletion. It is possible that a variant subpopulation with the 234 kbp deletion preexisted in the Tr60 stock suspension, and that the phage infection led to its selection. We could not detect such variants by PCR analysis on 94 isolated colonies or on total DNA extracted from a Tr60 culture. Another possible explanation is that phage infection promoted rearrangement of the host genome. This hypothesis is supported by the finding that at both ends of the 234 kbp region present in the original Tr60 strain, there are sequences of 10 bp in length (ctcgcatga and ctcggcgtatga) that differ by a single nucleotide insertion. Notably a similar sequence (c-cggcgtatga) was detected in the phage Ab31 genome at the end of the gene encoding an acetyl-transferase, upstream of the phage integrase. The 10 bp sequence “ctcggcgtatga” constitutes the junction of the deleted region on the resistant bacterial genome. Moreover, the sequence upstream the 234 kbp region encodes several proteins involved in transposition, including a bacterial transposase. This suggests that the origin of the deletion in Tr60

was most probably a recombination and/or transposition event in which the phage was also involved. Large genomic deletions have been observed during early stage adaptation of *P. aeruginosa* in CF patients, but none were as large as 234 kbp, which represents about 3.6% of the genome [24,53]. Rau and colleagues described a deletion of 148 kbp, encompassing the *cupA* cluster [53]. It is not known whether the presence of phages could play a role in the induction of such deletions. Ab31-resistant strain PA14-P1 showed no deletion corresponding to those which characterize Tr60-10A and Tr60-100A. A number of mutations in different genes were observed, but at this time it is impossible to know which one is responsible for phage resistance.

Looking at the Ab31 genome sequence it is possible to distinguish two main modules. The first, showing homologies with the AF phage genome, covers the so-called late region and contains sequences encoding the structural proteins of the phage, such as those for capsid, tail-to-head connector, tail and tail spikes. The second Ab31 genomic region encodes proteins involved in recombination and replication of the phage genome, and constitutes the so-called early/middle region. This contains several genes that show similarities with those of PAJU2 explaining why, although phage Ab31 shows a morphology typical of the virulent AF podovirus, it behaves as a temperate phage capable of lysogenizing *P. aeruginosa* strains. Indeed, the Ab31 insertion site in

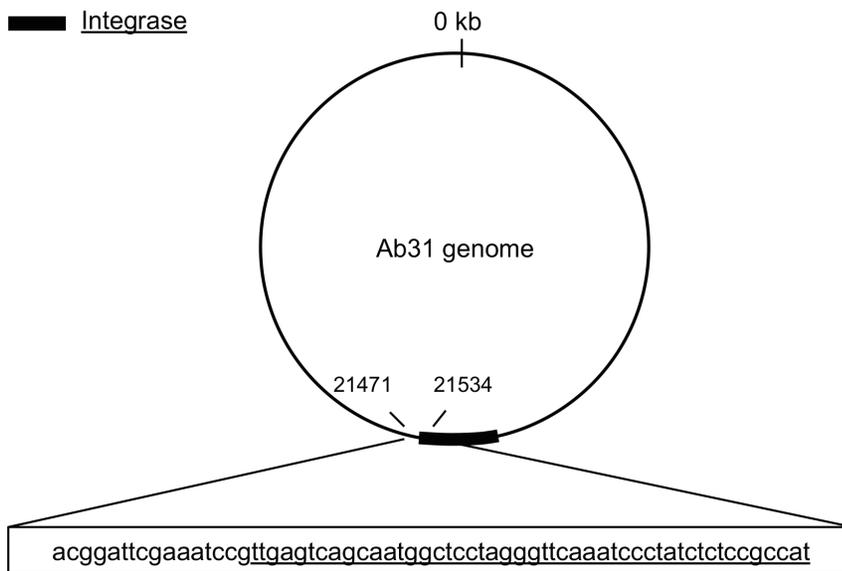


Figure 8. Schematic representation of the Ab31 insertion region. A 64 bp sequence is shared by the phage and the *P. aeruginosa* Tr60 genome. The portion of the shared region that overlaps with the phage integrase encoding gene is underlined. doi:10.1371/journal.pone.0093777.g008

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Ab31_Spike      LTVSTTDSVIEYEGNGVTTAFVPPFKFPANG--DLVVTQVYNDVSTTLLSGANYSVVGAG
AF_Spike        MTVSSSASTASFPNGVTTVFPLPFRFFSNSDVEVSLLNVASGVVTPQSIGTNYTSLGAG
:***::: * . .: *****.*:*:*:* :*. .: : : * ..* *. *:*:*:*: **

Ab31_Spike      -----AQAGGAVIVNIPTPAGATLSISRSLAAVQETDLRNQGRYFAETHESVFDYLTMLI
AF_Spike        DPEVDGNANSTLTMLVAPPVGTTLIVERVMDAVQQTDIINQARFFPEIHENVFDRLVMLI
.:*...:: : :..*.*.*. :.* : ***:*:*: *.*:*.* **.*.* *..**

Ab31_Spike      QQGFSGLSRALRRPIGKDYFDAENRRISNVSDPVLSQDAVTRKWSEEYFSDLISDIQGPV
AF_Spike        QQNASVIGRALLRPIGKDYFDALGYRIANLHDPIGSQDAATMAYVQQAIAALLEIGSGSA
**.* :.* **.* **.* **.* . **:*: **:* **.*.* : :. :. :.*. .*..

Ab31_Spike      NNAQNVLYVD-GDGISTTVQKGI IKQFTSVSDVRGKAGDKDGDQAVLLGYYQDSPGVGGG
AF_Spike        NNAANVLYVPNGPNPTPLTVQNRLRQAI FLDDYKTTVVVDGVTSNQVDLENALLACTTKGA
*** **.* * . . . . :.* :.* : . . * . : * * : . * .

Ab31_Spike      NLYWDSLSVEPDNGGSVFAVTGVPNGRWKRDITHGVWAEWFGARNDGTDAAGTTAAVWAA
AF_Spike        DLFWP-----DPVPRVSTSPPLPT
:*:* . . . :. :

Ab31_Spike      IIALRHDPETIVQYIGGPTVTAYASGRNLFNGVVFALQPDSFDITQDLGLTIVGQFRGK
AF_Spike        LHTVRHVGPGAIKADG---LTFYVDPKYAQQNVIIYMAPGGTGDGLSPSRPLNGFNGLTAA
: :.* :. : * :* *.. : * :. : . : * . : * .

Ab31_Spike      NQAMKAATTLCKGVSSGFFFRHYGNNGARNLTFKMDVYENSDFGTGDIVESLTSPLGTL
AF_Spike        LKNYAPLKSRTVEGSAGTYFEDVQFPNYLASSHDNYLAWNFPAPAGLRAEPGTYPAILS
: . . : *:* :*.. : :* :. :. : * .* * *..

Ab31_Spike      ER--TRLGCGYGLLGTTRVQTARSCIRPTFDEFRLCEDVVFDAQNGFWSGDARTVPGLSF
AF_Spike        GIGNTALTGFHTGIGNRMTVNNLCVANFYDNSLTNVRQVRRGI AVGAYST-LFLVNCASQ
* * : :.*:* . . * : * : * * * : * * *

Ab31_Spike      GGWGTTLMNCTFYDIANVMIIHHAANRGRSIVNVIGCAFNPINISPARCFDITNIEGINVT
AF_Spike        GNGLSNLAALPSGLTIITGGRHKGSRYGIDNTAGRC SMTATTTTYAVVEDALEYG-----
* . :.* . : . :* ..* . . * :. . . : * * :

Ab31_Spike      GNQFTPGTGSQPTQEWIRLLGSGTAMDGNTFSGPSSKLGITGGVDPSSIRFSNNRVSCQG
AF_Spike        ---LYEKHDASTVSDYTEWRNNGKHPAAVLYGAAIFAYKSNASVDTRGNKFYANNICWN-
: . . . . :. . . . : . . . . : . . ** . : * * . . :

Ab31_Spike      GLVVSGGIFTGSGNEYSIADHGVDVSPVAITTLDIGPDI FKSGVSGNSYRISADSSLLGG
AF_Spike        ---ARGGFVADNPDI PDVYGTGVDANTRRFLCRGGGGDDLQTYRAN--RPAEISTRYTGG
. ** :. . . : . . ** . . . : . * * :. : . . . : * *

Ab31_Spike      RINYIFEQDNSNSKFSNVSSRVSIDNVDRRISTIG---SLPSTASPYFSGRTYNVTVAGT
AF_Spike        STTSTTDVLLNQIAEIREGYLANNDQSLRIRLIGRAQVADALITPQLRVNGSGVITLGT
. : * . . . :. : . ** ** : : * : . . * . **

Ab31_Spike      FTLPT----PIPGIRLRVMKSTATALTVSTTSGSNILVGASSARTSAVATAAEIGPAIEF
AF_Spike        YRITAGKYGEIRLVVRPNIARTAFSVEFSC TDAVQNLGAAVQIITTTTDLTVATLQVEV
: :. : * : : ** :. : * * . : * . * . :. : : : * *

Ab31_Spike      VAFSSTSWMAQVLSGSWSFS-
AF_Spike        AGRADAGGTASFLTCMVDLLG
.. :. : *..* : . :
    
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Figure 9. Alignment of the spike protein sequence of phages Ab31 and AF.
doi:10.1371/journal.pone.0093777.g009

P. aeruginosa is the same as in PAJU2. Phages AF and PAJU2 infect *P. putida* and *P. aeruginosa*, respectively. These bacterial species are closely related, and phage genome exchanges probably occurred during infection of a lysogenic host by the virulent phage. As a result of their mosaic structure, some temperate phage genomes can migrate between unrelated bacteria [54]. Although genetically distant, phages AF and PAJU2 share a lambdaoid genome organization which could favor genetic replacement (modular exchanges of gene blocks) [55,56]. Similar events seem to occur between D3112-like phages morphologically identical to phage lambda, and the transposable coliphage Mu belonging to the *Myoviridae* family [57]. Several types of recombination events are thought to build phage genomes. There are examples of conserved sequences at gene boundaries that could serve to target homologous recombination at these positions, via transposition or site-specific recombination [13]. However a major contributor to phage genome building is illegitimate recombination, or recombination between short conserved sequences (a few bases), coupled with functional selection of genes [13,58].

Conclusion

Our observations show that phage Ab31 is the result of a rare recombination event between genomes of two unrelated bacterio-

phages, normally infecting different bacterial species. It is capable of forming lysogens but its genome can also apparently persists unintegrated for a long time in the bacterial cells, with accompanying repression of virulence functions thus allowing the bacteria to escape lysis. In addition, we show that the phage exerts strong pressure on the bacteria by selecting for variants with new phenotypes, possibly improving their adaptation to chronic lung infection.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: CE HVT CP. Performed the experiments: LL CE YB. Analyzed the data: LL CE YB CP. Contributed reagents/materials/analysis tools: CE HVT. Wrote the paper: LL YB CP.

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ANNEX III

Pseudolysogeny and sequential mutations build multiresistance to virulent bacteriophages in *Pseudomonas aeruginosa*

Pseudolysogeny and sequential mutations build multiresistance to virulent bacteriophages in *Pseudomonas aeruginosa*

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Coevolution between bacteriophages (phages) and their prey is the result of mutualistic interactions. Here, we show that pseudolysogeny is a frequent outcome of infection by virulent phages of *Pseudomonas aeruginosa* and that selection of resistant bacterial mutants is favoured by continuous production of phages. We investigated the frequency and characteristics of *P. aeruginosa* strain PAO1 variants resisting infection by different combinations of virulent phages belonging to four genera. The frequency of resistant bacteria was 10^{-5} for single phage infection and 10^{-6} for infections with combinations of two or four phages. The genome of 27 variants was sequenced and the comparison with the genome of the parental PAO1 strain allowed the identification of point mutations or small indels. Four additional variants were characterized by a candidate gene approach. In total, 27 independent mutations were observed affecting 14 genes and a regulatory region. The mutations affected genes involved in biosynthesis of type IV pilus, alginate, LPS and O-antigen. Half of the variants possessed changes in homopolymer tracts responsible for frameshift mutations and these phase variation mutants were shown to be unstable. Eleven double mutants were detected. The presence of free phage DNA was observed in association with exclusion of superinfection in half of the variants and no chromosomal mutation could be found in three of them. Upon further growth of these pseudolysogens, some variants with new chromosomal mutations were recovered, presumably due to continuous evolutionary pressure.

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INTRODUCTION

Pseudomonas aeruginosa is a bacterium frequently found in the environment and often associated with human infections in clinical settings. This species displays an important genome plasticity due in large part to horizontal gene transfer of genomic islands and mobile elements, but also to *de novo* mutations (Spencer *et al.*, 2003). Bacteriophages (phages) are key actors in diversification of *P. aeruginosa* by selecting for resistant mutants and, in turn, adapting to new bacterial genotypes in a coevolution arms race (Brockhurst *et al.*, 2005; Dennehy, 2012). A large variety of *P. aeruginosa* phages have been isolated, some showing a wide host range. However, several studies have illustrated that 6–10 % of genetically different clinical *P. aeruginosa* strains were not

lysed by currently known phages (Essoh *et al.*, 2013). Spontaneous mutations responsible for phage resistance are frequently related to alterations in the phage receptor (Hyman & Abedon, 2010; Labrie *et al.*, 2010). In *P. aeruginosa*, adsorption mutants are principally affected in type IV pili, often used as receptors by podoviruses (Chibeu *et al.*, 2009) and siphoviruses, or in LPS, a major virulence factor (King *et al.*, 2009; Lam *et al.*, 2011) involved in the binding of myoviruses. We and others showed that large chromosomal deletions could be selected in bacteria resistant to single or multiple phages. Such deletions encompassed genes involved in fimbriae, outer membrane proteins or LPS components (Latino *et al.*, 2014; Le *et al.*, 2014; Tanji *et al.*, 2008). Resistance to phages due to modified type IV pili has consequences for bacterial motility by affecting twitching – a form of solid surface translocation (Chiang & Burrows, 2003). Type IV pili allow the adherence of *P. aeruginosa* cells to the host epithelium and also play a role in biofilm formation (Bucior *et al.*, 2012; Klausen *et al.*, 2003; O'Toole & Kolter, 1998). The mechanism of resistance associated with the loss of pili may drive an increase in *P. aeruginosa* diversity and strongly reduce infectivity

The GenBank/EMBL/DDBJ accession number for the DNA sequence of the PAO1 strain representative PAO1_{Or} is LN871187. All sequence reads have been deposited in the European Nucleotide Archive (ENA) project number PRJEB9838.

Three supplementary tables and seven supplementary figures are available with the online Supplementary Material.

(Brockhurst *et al.*, 2005; Hahn, 1997). Scanlan *et al.* (2015) showed that coevolution of phages and bacteria leads to the emergence of a complex population of cells with mutations that sometimes increase bacterial fitness but also constrain evolution.

It is generally accepted that the outcome of virulent phage infection is a lytic cycle leading to bacterial death, whereas temperate phages can either perform a lytic cycle or lysogenize their host. Pseudolysogeny is a third state, most frequently described for temperate phages as an intermediate between the lytic cycle and lysogeny, allowing the bacteria to survive infection (Ripp & Miller, 1997, 1998). Pseudolysogeny was first described as an unstable interaction that is not productive and eventually resolves into true lysogeny or virulent growth (Baess, 1971). Los *et al.* (2003) demonstrated that *Escherichia coli* phage T4 can form pseudolysogens in starved, slowly growing cells. They showed that superinfection of the host by another T-even phage was responsible for lysis inhibition (Bode, 1967) caused by the T4rI gene product. Later, pseudolysogeny was defined as a stage in the phage development, without multiplication of the genome, allowing subsequent restart and resumption of the virus cycle (Los & Wegrzyn, 2012). In *P. aeruginosa*, pseudolysogeny was documented in slowly growing cells with two phages responsible for generalized transduction: F116 (a temperate phage) and UT1 (a virulent phage) (Ripp & Miller, 1997, 1998). The role played by pseudolysogeny in the emergence of bacterial mutants has not been demonstrated. Early work by Demerec & Fano (1945) described mutants of *E. coli* obtained on agar medium following infection by seven different phages (T1–T7). The authors noted that phages were present for a long time after they reisolated resistant colonies and finally obtained mutants showing different patterns of cross-resistance. A high frequency of what were likely double mutants was observed, but the authors were not able at that time to identify the genetic changes that conferred the heritable cross-resistance.

We wished to go further in the analysis of phage-driven *P. aeruginosa* evolution and investigated the mechanisms by which *P. aeruginosa* survives infection by one or a mixture of virulent phages belonging to different genera. We characterized mutations selected by phages and showed that maintenance of phage DNA in pseudolysogens over many colony purification steps was a major factor in allowing selection of additional mutations.

METHODS

Bacterial strains and phages. A single colony of *P. aeruginosa* PAO1, a reference strain originating from a patient (Stover *et al.*, 2000) and propagated in the laboratory for several years, was cultivated for storage at -80°C , and for genome extraction and sequencing. This representative, hereafter called PAO1_{Or} (where Or stands for Orsay), was used to isolate phage-resistant mutants. Two podoviruses, vB_PaeP_PAO1_Ab05 (Ab05) and vB_PaeP_C2-10_Ab09 (Ab09), and two myoviruses, vB_PaeM_PAO1_Ab17 (Ab17) and vB_PaeM_PAO1_Ab27 (Ab27), representing four differ-

ent genera were used in this study, alone, or combining a podovirus with a myovirus or in a cocktail of all four phages. These phages, isolated in Abidjan (Côte d'Ivoire), have been described in detail in Essoh *et al.* (2015). PAO1 LPS and type IV pilus transposon mutants were obtained from the *P. aeruginosa* Mutant Library (<http://www.gs.washington.edu/labs/manoil/libraryindex.htm>).

Isolation of phage-resistant bacteria. Bacteria were inoculated at OD₆₀₀ 0.01 into glass vials containing 5 ml Luria broth (LB) medium, and grown with aeration (37°C with shaking at 180 r.p.m.) to OD₆₀₀ 0.2. Infections were performed at m.o.i. 0.1. Infections on solid medium used a 10 μl inoculum of the bacterial culture (2×10^6 c.f.u.) mixed with 10 μl suspension containing either a single phage genus, a cocktail of two phages or a cocktail of all four phages (10^5 p.f.u. for each phage). An aliquot of 10 μl SMG (saline magnesium gelatin) phage buffer (5.8 g NaCl l⁻¹, 2 g MgSO₄ l⁻¹, 1 M Tris/HCl and 0.1 g gelatin l⁻¹, pH 8.0) was used in negative controls. The mixture was kept for 15 min at room temperature, before being poured on a fresh LB agar plate [1.5 % (w/v) agar] with 4 ml soft agar [0.7 % (w/v) agar] and incubated at 37°C for 3 days. As no stable resistant variants were obtained with the solid assay for phage Ab27, alone or associated with Ab05, liquid infection was also performed when using Ab27. Bacteria were infected during the exponential phase (OD₆₀₀ 0.6) at m.o.i. 0.001 each 24 h for a total of three infections. Thereafter the surviving bacteria were plated onto LB agar plates.

Calculation of the frequency of resistance. An overnight culture of *P. aeruginosa* PAO1_{Or} was used to inoculate fresh medium to OD₆₀₀ 0.1. Bacterial cultures in the late-exponential phase (OD₆₀₀ ~ 1 , equivalent to 10^9 bacteria ml⁻¹, determined by titrating the bacteria) were 10-fold serially diluted. Aliquots of 100 μl of each dilution were mixed with 10 μl ($\sim 10^6$ p.f.u.) of a single phage suspension or a mixture of two or four phages as described above. The samples were kept for 15 min at room temperature and then poured on fresh LB agar plates using 4 ml soft agar. Plates were inverted and incubated at 37°C for 24 h. The frequency of resistance was calculated considering that all the colonies growing on the plates after 24 h of incubation were resistant to phages used for the infection. The divisor was the number of plated bacteria.

Phage susceptibility assay. Aliquots (500 μl) from the liquid culture of variants (OD₆₀₀ 0.8–1.2) were mixed with 6 ml 0.7 % (w/v) LB agar and poured onto a square LB 1.5 % (w/v) agar plate. Five dilutions (10^{10} , 10^9 , 10^8 , 10^7 and 10^6 p.f.u. ml⁻¹) from a progenitor stock of each phage were spotted (10 μl) onto the soft agar layer, incubated at 37°C overnight and inspected for plaque formation. The resistance of the mutants against the phage was expressed as the efficiency of plating using PAO1_{Or} as a control.

Virucide assay. The protocol described by de Siqueira *et al.* (2006) was used to prepare a virucide solution from Chinese black tea leaves. The phage-containing bacteria were treated for 10 min with 3 vol. virucide, followed by centrifugation, washing with PBS and incubation at 37°C for 30 min with 50 μg DNase I ml⁻¹. Total bacterial DNA was then purified.

Adsorption assay. An overnight bacterial culture was diluted to OD₆₀₀ 0.1–0.6 and left to equilibrate at 37°C . Approximately 10^6 phages were added to 1 ml diluted bacterial culture ($1-6 \times 10^8$ bacteria). At a fixed time point, 50 μl mixture was transferred to a 1.5 ml conical centrifuge tube containing 940 μl LB medium and 10 μl chloroform. The suspension was vortexed for 5 s and centrifuged in order to pellet the phages adsorbed on the bacterial surface. Then, 10 μl unadsorbed phage suspension was titrated. Phage adsorption was expressed as the percentage of the initial amount of phage employed for the infection that did not adsorb to the bacterial surface after 16 min (time necessary for adsorption of the four phages).

Phenotypic assays. A planktonic culture of strain *P. aeruginosa* PAO1_{Or} prepared from a single colony off a fresh LB agar plate was used as a reference in all experiments. To test for haemolytic activity, 10 µl overnight culture of phage-resistant mutants (OD₆₀₀ 2) was spotted onto sheep blood [5 % (w/v)] agar and plates were incubated for 24 h at 37 °C. For twitching motility assessment, 1 µl overnight bacterial culture (OD₆₀₀ 2) was inoculated between the agar and the plastic surface of LB 1.5 % (w/v) agar plates. The diameter of the motility zone around the inoculation site was measured after 24 h incubation at 37 °C. LPS was purified using the method of Hitchcock & Brown (1983). In order to normalize the samples for the subsequent gel analysis, a similar amount of lyophilized bacteria was disrupted in lysis buffer (1 M Tris, 2 % SDS, 4 % β-mercaptoethanol and 10 % glycerol) prior to LPS extraction. The LPSs were resolved by electrophoresis on a 15 % SDS-polyacrylamide gel and the band pattern was visualized using the silver staining method (Fomsgaard *et al.*, 1990).

Biofilm formation. Microtitre plates (96-well; Greiner) containing LB were inoculated with an overnight bacterial culture (OD₅₉₅ ~0.1) and incubated at 37 °C for 48 h. Before proceeding with biofilm quantification, the OD₅₉₅ was recorded. The wells were washed three times with PBS, 200 µl 0.1 % (w/v) crystal violet was added and the plate was kept for 30 min at room temperature. The unattached crystal violet was washed three times with PBS and then the remaining biomass was quantified by resuspending it into 200 µl absolute ethanol. The OD₅₉₅ was then divided by the OD₅₉₅ value measured for planktonic bacteria in each well to account for the difference in growth rates of the mutants.

Colony lift and hybridization. A circular Nylon N+ membrane (Nytran) was applied on the agar plate on which 52 colonies had been plated. After 5 min, the membrane was lifted using forceps and treated successively for 2 min with 0.4 N NaOH twice, 1 M Tris, pH 7.5 twice and 2 × saline sodium citrate (SSC) twice. The membrane was then dried on Whatman filter paper and kept at 20 °C until use.

Pre-hybridization was performed at 65 °C for 4 h with 2 ml hybridization buffer (Church & Gilbert, 1984) per membrane. The probe was labelled using a Megaprime kit (GE Healthcare Amersham) and hybridization was performed overnight at 65 °C in hybridization buffer. Washes were done successively with 2 × SSC and 0.1 % (w/v) SDS, 0.5 × SSC and 0.1 % (w/v) SDS, and 0.2 × SSC and 0.1 % (w/v) SDS.

DNA extraction, PCR and sequencing. PCR was performed on thermolysates or purified DNA using oligonucleotides listed in Table S1 (available in the online Supplementary Material). Thermolysates were produced by diluting 10 µl overnight culture in 200 µl water and heating at 95 °C for 5 min. For DNA purification, bacteria were lysed in lysis buffer [10 mM Tris, pH 7.8, 10 mM EDTA, 10 mM NaCl and 0.5 % (w/v) SDS], treated with 50 µg proteinase K ml⁻¹ for 2 h at 50 °C, followed by one phenol and one chloroform extraction, and ethanol precipitation. The isolates were verified for contamination from other *P. aeruginosa* strains, commonly used in our laboratory, using PCR with oligonucleotides directed against variable number tandem repeats ms216 and ms217 as described previously (Vu-Thien *et al.*, 2007). The isolates were also screened for the presence of phage DNA by PCR performed on thermolysates using the specific phage oligonucleotides listed in Table S1.

Gene cloning and expression. PCR amplicons were cloned into the pUCP24 plasmid, a generous gift of Dr Schweizer (West *et al.*, 1994). This is a shuttle vector which replicates in *E. coli* and in *P. aeruginosa*, and contains a multiple cloning site downstream from *lacZx*. The PAO1 *muca* gene was PCR-amplified using oligonucleotides

*muca*_Clon_F_Bam (5'-TGGGATCCCCGAGAAGCCTGACACAGC-3') and *muca*_Clon_R_Hind (5'-GAAAGCTTACCGCCATCAGGCTGCCA-3'), which included restriction sites for *Bam*HI and *Hind*III. The amplicons were digested with *Bam*HI/*Hind*III, ligated into the similarly digested vector and transformed into *E. coli*, in which replication of pUCP24 is optimal (West *et al.*, 1994). A selected recombinant was then used to transform *P. aeruginosa* strains by electroporation using the fast protocol described by Choi *et al.* (2006). Transformants were selected using 10 µg gentamicin ml⁻¹, and the presence of the plasmid was verified by PCR amplification using a *muca* forward oligonucleotide *muca*-int_F (5'-ACGCAGGTAGATCGGCAGAC-3') and a plasmid reverse oligonucleotide pUCP24_MCS_R (5'-GGCTCTTCGCTATTACGCC-3'). The colony aspect was observed under the stereomicroscope. The transformants were then tested for their susceptibility to the four phages.

Whole-genome sequencing. Purified bacterial DNA (10 µg) was sent for draft whole-genome Illumina sequencing to the IMAGIF platform (CNRS, Gif-sur-Yvette, France). Libraries were made from sheared fragments of DNA with a mean size of 900 bp and paired-end reads of 250 bp were produced. Between 1 and 5 million reads were obtained corresponding to a 40- to 200-fold mean coverage. The mutations were identified by comparison with the genome of the PAO1_{Or} sequence using native Geneious R9 tools default parameters (Biomatters). The Geneious mapper with the 'Medium-Low Sensitivity/Fast' parameter option was used to map the reads of each variant against the PAO1_{Or} genome. The 'Find Variations/SNPs' analysis was used with the parameter 'Minimum Variant Frequency' set to 0.25. When an SNP or an indel was identified, sequencing reads mapping in the mutated gene plus 1 kb on both sides were recovered, reassembled and the contig was aligned with the PAO1_{Or} genome. This allowed the precise localization of short deletions. Mutations were confirmed by PCR amplification of the affected gene and Sanger sequencing (Beckman-Cogenics).

De novo assembly of phage reads was done with the Geneious R9 native assembler using the 'Medium-Low Sensitivity/Fast' parameter.

RESULTS

Phage-tolerant bacteria show a variety of phenotypes and phage susceptibility patterns

Our goal was to evaluate the frequency and diversity of PAO1_{Or} mutants emerging from infection with phages belonging to different genera, used alone or in cocktails. We hypothesized that each phage may select for specific mutations. Four different virulent phages displaying various host ranges (Essoh *et al.*, 2015) were used, alone or in combinations of two or four. On PAO1_{Or}, Ab05 (φKMV-like phage), Ab09 (N4-like phage) and Ab17 (KPP10-like phage) produced clear plaques, whereas Ab27 (PB1-like phage) produced tiny, turbid plaques. First, we investigated the nature of the primary receptor of the four phages by testing the susceptibility of two PAO1 transposon mutants, affected in type IV pili (*pilA* mutant) or LPS O-antigen (*algC* mutant) synthesis genes. Ab05 was not capable of growing on a type IV pilus mutant, as previously reported for most φKMV-like phages (Ceyssens *et al.*, 2011), whereas growth of Ab09, Ab17 and Ab27 was restricted on the LPS-defective mutant. We then designed an experimental procedure to

allow for phage amplification and isolation of independent resistant mutants. For this purpose, PAO1_{Or} was infected at m.o.i. 0.1 (one phage for 10 bacteria) by single phage or cocktails, before plating the bacteria in soft agar on LB solid medium. The cocktails consisted of a 1 : 1 mixture of Ab09 and Ab17 or Ab05 and Ab27, and a 1 : 1 : 1 : 1 mixture of Ab09, Ab17, Ab05 and Ab27. Complete lysis was obtained in 8 h, with the exception of dispersed insensitive bacteria which, after 72 h, produced colonies with different morphologies (Fig. 1a). We calculated the frequency of surviving cells to be 3.2×10^{-5} for single phage infection, 4×10^{-6} for double infection and 3.8×10^{-6} for multiple infection. Colonies of variable shape, size and appearance were picked from seven independent experiments and were purified by three reisolation steps, in order to ensure that a pure population was obtained. A single colony was recovered after the third reisolation step (P3) and used to inoculate an overnight culture which was then stored at -80°C in glycerol. This stock was later used for genomic DNA purification and to perform further tests (Fig. 1a). The majority of PAO1_{Or} variants recovered after Ab05, Ab09 and Ab17 single or multiple infections were 'tolerant' to at least one phage. Tolerance was defined as the capacity to survive the phage infection, whether this was due to a lack of

receptor or to any other mechanism. In contrast, none of the surviving bacteria recovered from infection with Ab27 alone turned out to be stably tolerant to Ab27. A similar observation was made by Hosseinioust *et al.* (2013a) who failed to isolate bacteria resistant to phage E79, another PB1-like phage.

In total, 32 PAO1_{Or} variants were retained and tentatively distributed into five groups according to their phage susceptibility pattern, evaluated by the efficiency of plating (Tables 1 and S2). The 13 Group 1 variants displayed normal susceptibility only to phage Ab05. The six Group 2 variants showed intermediate susceptibility patterns to the different phages. The five Group 3 variants were resistant only to phage Ab05. The four Group 4 variants displayed full resistance to phages Ab05 and Ab27, and reduced susceptibility to phages Ab09 and Ab17, characterized by the production of small plaques instead of large, clear plaques. Four variants resisting all four phages constituted Group 5. In Group 2, a mucoid phenotype was stably observed for PAO1-02, PAO1-06 and PAO1-13, whereas PAO1-17 continuously produced two types of colonies on solid LB media, some with a smooth appearance as seen for the control PAO1_{Or} strain and others surrounded by an irregular transparent edge (Fig. 1b). This phenotype

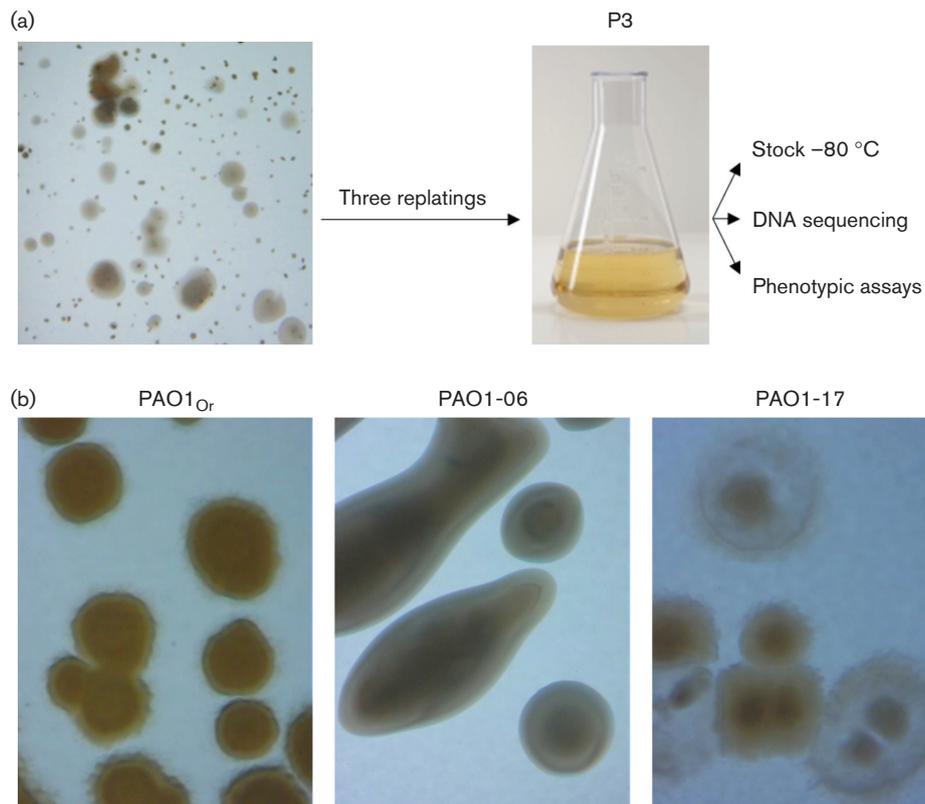


Fig. 1. Isolation of phage-tolerant variants. (a) Colonies surviving phage infection after 72 h on LB agar were replated three times before the P3 culture was prepared and stored at -80°C . (b) Colony morphotype of PAO1_{Or}, PAO1-06 and PAO1-17.

Table 1. Clustering of phage-tolerant variants according to their resistance pattern against the four phages used in the present study

Group	Infection	PAO1 variant	Phage resistance*				Phage DNA†
			Ab05	Ab09	Ab17	Ab27	
1	Ab09, Ab17	01	S	R	R	R	–
	Ab09, Ab17	04	S	R	R	R	–
	Ab09, Ab17	05	S	R	R	R	–
	Ab09	07	S	R	R	R	–
	Ab17	09	S	R	R	R	–
	Ab17	12	S	R	R	R	–
	Ab17	14	s	s	R	R	–
	Ab09	15	s	R	R	R	–
	Ab17	18	s	I	R	R	–
	Ab09, Ab17	19	S	I	R	R	Ab09
	Cocktail‡	21	s	I	R	R	–
	Ab05, Ab27	32	S	R	R	R	Ab27
Ab05, Ab27	34	s	I	R	R	Ab27	
2	Ab09, Ab17	02§	s	s	S	R	–
	Ab09, Ab17	03	S	I	S	I	–
	Ab09, Ab17	06§	S	I	S	S	–
	Ab17	10	s	s	s	I	–
	Ab09	13§	s	s	S	s	–
	Ab09	17	s	R	s	R	Ab09
3	Cocktail‡	25	R	S	S	s	Ab05
	Ab05	26	R	S	S	S	Ab05
	Ab05	27	R	S	S	S	Ab05
	Ab05	28	R	S	S	S	–
	Ab05	29	R	S	S	S	Ab05
4	Cocktail‡	24	R	s	s	R	Ab27
	Ab05	30	R	S	I	I	Ab05
	Ab05, Ab27	36	R	s	s	R	Ab27
	Ab05, Ab27	37	R	s	s	R	Ab05
5	Cocktail‡	20	R	R	R	R	Ab17
	Cocktail‡	22	R	R	R	R	Ab17
	Ab05, Ab27	33	R	R	R	R	Ab27
	Ab05, Ab27	35	R	R	R	R	–

*S, Completely susceptible; R, completely resistant; I, reduced efficiency of plating; s, normal efficiency of plating but small and turbid plaques.

†Detection by PCR.

‡Infection performed with all four phages.

§Mucoid variant.

may be related to an observed decrease in swarming capacity (data not shown). In addition, mucoid colonies appeared after several days of growth. When replated, the PAO1-17 colonies surrounded by a transparent edge again produced both types of colonies, whereas the others stably maintained their phenotype. Growth on sheep blood agar plates showed the existence of new phenotypic characteristics for some variants as compared with the parental PAO1_{Or} strain (Fig. S1). Variants PAO1-02, PAO1-13 and PAO1-17 lacked the haemolytic ability displayed by the WT PAO1_{Or} but, interestingly,

the colonies of PAO1-13 presented zones of reversion to the WT phenotype.

Unexpectedly, PCR amplification showed that phage DNA could still be detected at the P3 reisolation step in 15 of the 32 variants (Table 1). We checked whether the phage DNA was inside the bacteria or adsorbed on the surface by treating two of the variants with a virucide (tea decoction) and DNase I digestion, followed by several washings of the bacteria pellet. Phage DNA was still present in large amounts in the bacteria, as shown by semiquantitative PCR (Fig. S2 shows PAO1-20 and PAO1-32), suggesting that the phage genome was maintained in an episomal state: lysogeny was not likely as these phages are believed to be strictly lytic, based on their genome characteristics and because the amount of phage DNA appears to be in large excess over that of the bacterial DNA (see 'Persistence of phage DNA in pseudolysogens'). The results obtained suggested that some bacterial cells might contain in the order of 100 phage genomes.

An adsorption assay was performed with the 16 variants devoid of phage DNA showing that resistance was linked to absence of phage binding to the bacterial surface (Fig. 2). In order to identify the mutations conferring resistance, and to investigate in more detail the variants containing phage DNA, whole-genome sequencing was performed on DNA extracted from 23 variants selected into the different groups, at the P3 purification step.

A wide range of chromosomal mutations is selected by phages

To identify *de novo* mutations, it was necessary to sequence the genome of the parental PAO1_{Or} strain, prepared from the culture used to derive phage-tolerant variants. The PAO1_{Or} sequencing reads were mapped against the sequence of the reference PAO1 (GenBank accession number NC_002516), allowing the assembly of the full genome and identification of differences (Fig. S3 and Table S3). These differences included a large inversion between rRNA sequences (position 727255–4788575), the presence of a copy of filamentous Pfl prophage in PAO1_{Or} at position 5242103–5254164 and 63 SNPs or short indels events. As expected, some of these differences, including the inversion and the Pfl prophage plus a number of the SNPs and indels, were previously reported by Klockgether *et al.* (2010). Others were specific to the PAO1_{Or} subline.

The sequencing reads from each of the 23 whole-genome sequenced phage-tolerant variants were mapped against the PAO1_{Or} genome, showing a uniform distribution with a mean coverage of 40- to 200-fold and only a few places with low coverage and relatively poor quality sequence, common to all variants. In variants PAO1-30, PAO1-32 and PAO1-34, no chromosomal mutation could be detected. In 14 variants, a single chromosomal mutation was identified. Six variants were double mutants.

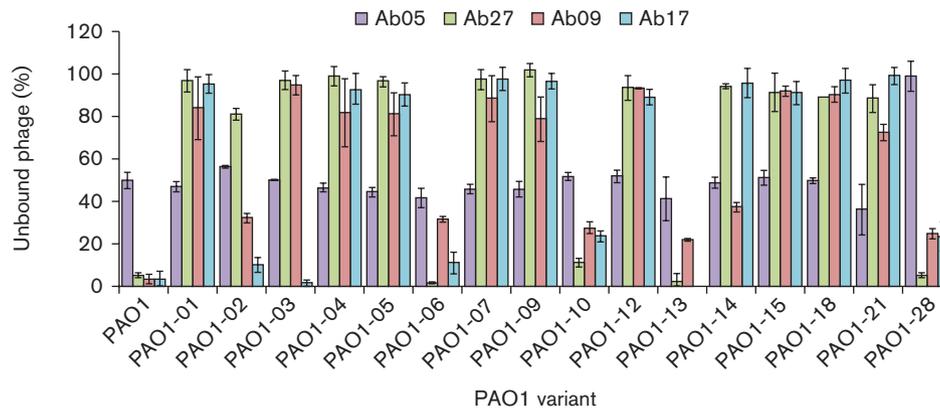


Fig. 2. Phage absorption on 16 PAO1 variants devoid of phage DNA. Phage absorption is presented as the percentage of unabsorbed phage at 16 min after infection. Data represent mean \pm SD of three independent assays.

Coverage at these genetic alterations was always at least 40-fold and up to 250-fold. PAO1-02, with a mucoid phenotype, was analysed by PCR using a candidate gene approach and this allowed us to find a frameshift mutation in the *mucA* gene. Table 2 displays the position and nature of the mutations, as well as the percentage of sequencing reads containing a particular mutation. In several variants, reads corresponding to both the WT and mutated sequence could be found, indicating that the cell population was mixed. This indicated that an unstable mutation had reverted back. Three different kinds of genetic alterations were found: phase variation, deletions of ≥ 10 bp and nucleotide substitutions. Fourteen genes were affected. The *wzy* mutations A(7) \rightarrow A(8) at position 1 976 849 as well as A(7) \rightarrow A(6) at the same position, and G(6) \rightarrow G(5) at position 1 977 338, were found in six, one and two other mutants, respectively. Seven different frameshift mutations due to a single nucleotide insertion or deletion in homopolymer tracts resulted, in six cases, in early termination of protein synthesis. In the last case, PAO1-06, deletion of a T in a stretch of four Ts in the *mucA* gene suppressed normal termination of translation resulting in the production of a longer MucA protein fused with the beginning of MucB. Deletions were found in three type IV pili structural genes: *pilY1*, *pilQ* and *pilJ*. *pilY1* was missing 10 bp in PAO1-37 and 109 bp in PAO1-33, *pilQ* was missing 19 bp in PAO1-26 and 555 bp in PAO1-20, whereas *pilJ* was missing 11 bp in PAO1-35. A 213 bp deletion was detected in the PAO1-22 *algC* gene. The deletions either caused a frameshift and the creation of a premature stop codon or deleted an internal domain. Eight variants showed a single nucleotide substitution. All mutations were confirmed by PCR amplification and Sanger sequencing.

The different mutations potentially affected the biosynthesis of membrane structures that participate in binding of phages to their receptor. Mutations in the gene cluster regulating the production of alginate were selected by

Ab09, and could reduce the efficiency of infection of all the phages. The *wzy*, *wzz2* and *wbpL* genes are members of the heteropolymeric O-specific antigen biosynthesis cluster in PAO1 (Lam *et al.*, 2011). Gene *migA* encodes a rhamnosyltransferase involved in LPS core capping (Poon *et al.*, 2008), whereas *wapH* and *dnpA* are known to be involved in the synthesis of LPS (Hansen *et al.*, 2007; Liebens *et al.*, 2014), and *pgi* encodes a glycosyltransferase (Rocchetta *et al.*, 1999). Mutations in *algC* affect the biosynthesis of alginate, LPS and rhamnolipids, biosurfactants necessary for bacterial swarming motility and biofilm formation (Olvera *et al.*, 1999). Overall, the phage susceptibility pattern of each mutant correlated well with the nature of the mutated genes. Infection with Ab09, Ab17 and Ab27 mainly selected mutations in genes regulating LPS and O-antigen biosynthesis, whilst Ab05 selected mutations in genes involved in type IV pilus synthesis. The number and variety of observed mutations were very high, confirming that the procedure used to isolate the variants allowed for selection of independent events.

Observed mutations are responsible for modifying the phage receptor

To confirm that the observed mutations were responsible for affecting the phage receptor, we investigated the phenotype of the three classes of mutants affected in type IV pilus, LPS and alginate biosynthesis. The motility of the variants was evaluated by performing a twitching assay on semisolid agar. Compared with the PAO1_{Or} control, the diameter of the twitching zone was significantly reduced in all variants, but the strongest effect was observed with those bearing a mutation in *pil* genes and/or resisting Ab05 infection (Fig. 3). PAO1-32 and PAO1-34 were also affected in twitching although no *pil* mutations could be observed, but this was likely related to a continuous cell death due to phage production. Indeed upon culture in LB broth the cells lysed totally

Table 2. Mutations identified in phage-tolerant variants according to their comparison with the reference PAO1_{Or}

Group	PAO1 variant	Phage resistance*	Position on PAO1 _{Or}	Mutation	Locus tag	Protein alteration	Percentage of reads representing		
							Mutation	Phages	Bacteria
1	01	SRRR	1976849	A(7)→A(8)†	wzy	74/438 aa‡	100	0	98
	04	SRRR	1977338	G(6)→G(5)	wzy	224/438 aa	100	0	99
			4736958	G→A	migA	Arg→His	100		
	07	SRRR	1977338	G(6)→G(5)	wzy	224/438 aa	98	0	98
	14	ssRR	1976849	A(7)→A(8)	wzy	74/438 aa	100	0	98
	15	sRRR	1986619	G(9)→G(8)	wbpL	88/339 aa	100	0	99
	18	sIRR	5634829	C→A	wapH	Arg→Leu	99	0	99
	19	SIRR	5632885	G→C	dtpA	67/472 aa	100	24 [Ab09]§	74
	21	sIRR	1976849	A(7)→A(8)	wzy	74/438 aa	100	0	99
	32	SRRR	–	–	–	–	0	85 [Ab27]	13
2	34	sIRR	–	–	–	–	0	73 [Ab27]	25
	02	ssSR	4683540	G(3)→G(2)	mucA	146/194 aa	–	–	–
	03	SISI	4487654	C(5)→C(6)	wzz2	228/443 aa	100	0	98
	06	SISS	4683359	T(4)→T(3)	mucA	253/194 aa	100	0	99
	10	ssSI	5327357	A→C	pgi	Thr→Pro	99	0	99
	13	ssSs	4683943	T→C	mucA ... algU¶	–	100	0	99
	17	sRsR	1977343	C→A	wzy	220/438 aa	47	2 [Ab09]	97
	26	RSSS	5689432	19 bp	pilQ	180/714 aa	81	2 [Ab05]	97
	24	RssR	1976849	A(7)→A(8)	wzy	74/438 aa	40	24 [Ab27]	72
	30	RSII	5095901	G→C	pilR	Arg→Pro	98	0	57
3	37	RssR	5103099	10 bp	pilYI	816/1161 aa	67	3 [Ab05]	96
	20	RRRR	1977570	A→G	wzy	Asp→Gly	100		
	22	RRRR	5688665	555 bp	pilQ	529/714 aa	100	35 [Ab17]	63
	33	RRRR	5095650	C(2)→C(1)	pilR	334/445 aa	97	11 [Ab17]	88
			6005075	213 bp	algC	797/868 aa	63		
			1976849	A(7)→A(8)	wzy	74/438 aa	49	2 [Ab27]	98
			1976848	A(7)→A(6)	wzy	54/438 aa	49		
			5102164	109 bp	pilYI	501/1161 aa	100		
			451455	11 bp	pilJ	751/682 aa	100	0	98
			1976849	A(7)→A(8)	wzy	74/438 aa	100		

*Resistance pattern is reported in order against phages Ab05, Ab09, Ab17 and Ab27. S, completely susceptible; R, completely resistant; I, reduced efficiency of plating; s, normal efficiency of plating but small and turbid plaques.

†Number of repeated nucleotides indicated in parentheses.

‡Length of the mutated protein/WT.

§Phage found by sequencing indicated in square brackets.

¶Mutation found by PCR and Sanger sequencing of the *mucA* gene.

¶¶Intergenic region indicated by ellipsis (...).

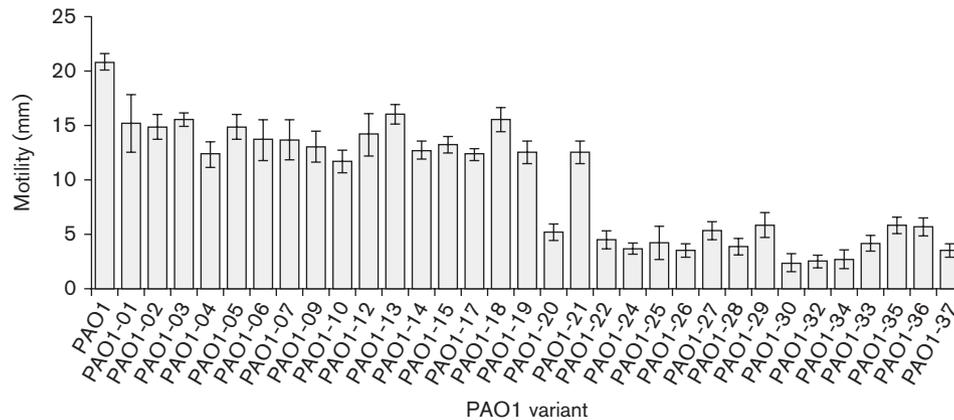


Fig. 3. Twitching motility of original (P3) phage-tolerant variants. Bacterial motility is presented as the diameter (mm) of the growth zone at the bottom of the agar plate. Data represent mean \pm SD of three independent assays.

after reaching OD₆₀₀ 0.8. Inhibition of twitching was accompanied by a decrease in biofilm formation, except for PAO1-20 and PAO1-06 (Fig. 4). This may be due to the existence of a mixed population of bacteria in these variants. The LPS extracted from PAO1-04 (*wzy* and *migA* double mutant) and PAO1-07 (*wzy* mutant) were analysed by PAGE. Fig. 5 displays the banding profiles compared with that of PAO1_{Or}, showing absence of the A and B bands, as well as modifications in the proportion of core and core +1 bands. PAO1-04 possessed only the core +1 oligosaccharide form. In contrast, variant PAO1-07 possessed both bands in equal amounts, whereas PAO1_{Or} had a small proportion of core +1. Absence of core oligosaccharide in PAO1-04 was likely a consequence of the mutation in *migA*. Finally, to confirm that the observed *muca* mutations were responsible for the mucoid phenotype, we tested whether the mutants could be

complemented by the WT gene. A full *muca* amplicon was cloned into an expression vector, which was then introduced into PAO1-02, PAO1-06 and PAO1-13. In the three cases, the transformants no longer showed a mucoid appearance, whereas the vector alone did not reverse the mucoid phenotype. In addition, the *muca* transformants recovered normal susceptibility to all phages.

Persistence of phage DNA in pseudolysogens

In 11 variants, phage DNA represented 2–85 % of sequencing reads. The very high proportion of phage DNA in some samples could only be explained by the presence of free phages, inside bacteria, and/or attached to cells. To confirm that phage DNA was present inside bacteria, we performed further genome sequencing of PAO1-17 and PAO1-20 at the P3 purification step, after treatment

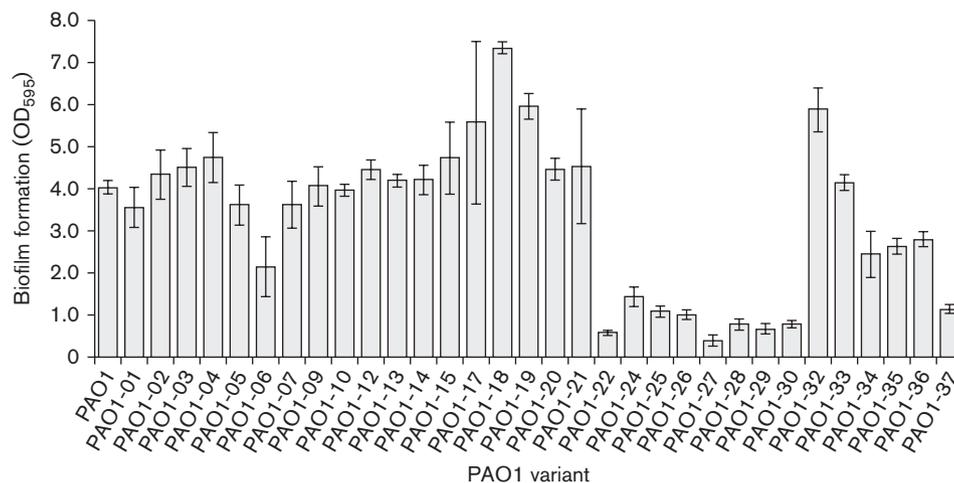


Fig. 4. Biofilm formation assay of original (P3) phage-tolerant variants. The amount of bacteria bound to the wells was evaluated by measuring the OD₅₉₅ as described in Methods. Data represent mean \pm SD of three independent assays.

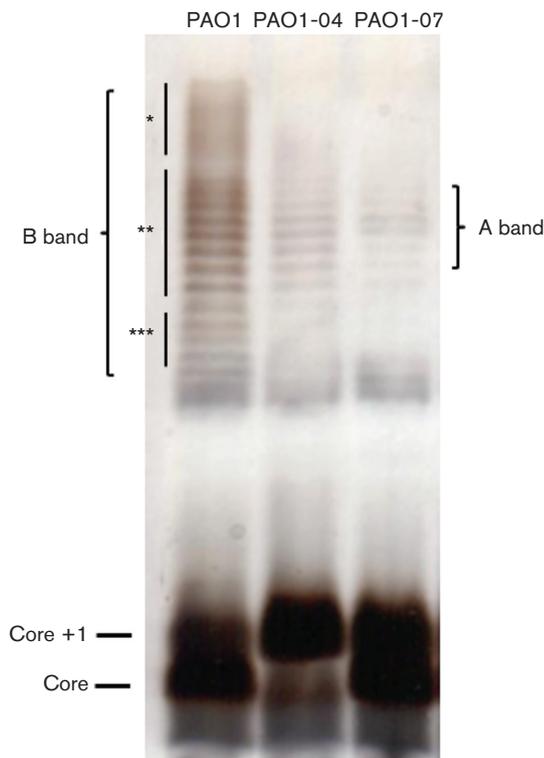


Fig. 5. SDS-PAGE of LPS samples. Positions of the core, A band and B band of the O-antigen were identified according to the work of Islam *et al.* (2013). *Very long B chains, **long B chains and ***short B chains.

of the bacterial pellet with DNase I and the virucide, followed by three washing steps. The results showed that 1.6 % of reads still corresponded to Ab09 in PAO1-17 and 52 % to Ab17 in PAO1-20, similar to what was observed in the first sequencing analysis (Table 2). A search for hybrid reads between phage and bacteria genomes did not produce any significant result, indicating that the phage DNA was not inserted into the bacterial chromosome, and therefore we refer to these bacteria as pseudolysogens. Interestingly, the three variants in which no chromosomal mutation could be found, i.e. PAO1-30, PAO1-32 and PAO1-34, possessed large amounts of phage DNA: PAO1-30, immune to Ab05, Ab17 and Ab27, contained high levels of Ab05 DNA; PAO1-32 and PAO1-34, immune to Ab09, Ab17 and Ab27, contained Ab27 DNA. Intermediate resistance profiles observed in some variants were correlated with the existence of a mixed population of WT and mutated bacteria, and with the presence of phage DNA.

In the sequenced samples in which a high proportion of sequence reads derived from phage DNA were present, it was possible to assemble the full phage genome sequence. This led to the identification of several single nucleotide differences in tail fibre genes, as compared with the parental genotype. In three pseudolysogens obtained

independently, an Ab05 tail fibre gene displayed two SNPs. By PCR and sequencing, we could also observe these SNPs in a fraction of the phages used to select for resistant bacteria (Fig. S4). Similarly, a single SNP was observed in an Ab17 tail fibre gene from variants PAO1-20 and PAO1-22, and in the ribosome-binding site of an Ab27 tail fibre gene from variants PAO1-24, PAO1-32 and PAO1-34. This might reflect the selection of phage variants by strain PAO1_{Or}, possibly affecting the capacity of the bacteria to resist phages. However, we could not see any differences in binding to the host or plating efficiency with these phage genotypes, as compared with the parental genotype.

Stability of the pseudolysogen state

Viable phages were released by pseudolysogens, sometimes at high titres, during overnight culture in LB medium. This suggested that a portion of the bacterial population could achieve a productive viral cycle. To evaluate the dynamics inside pseudolysogen colonies, we measured the percentage of bacteria containing phage DNA and producing viable phages, and evaluated for how long phage DNA was maintained. For this purpose, 1 µl of some bacterial strains from the frozen P3 stock was spread on LB agar (P3₀). In total, 52 colonies were picked and deposited successively on an LB agar plate, then on an LB agar plate covered with a lawn of soft agar containing PAO1_{Or} (see PAO1-30 replatings as an example in Fig. 6). After incubation at 37 °C for 24 h, a lysis zone could be seen around some colonies on the lawn of PAO1_{Or}. One such phage-producing colony from the LB agar plate was streaked onto a new LB plate and the procedure was repeated. The fraction of pseudolysogenic cells contained in a single colony varied from 4 up to 100 %. In PAO1-30, the pseudolysogenic state was observed up to 10 replatings (Table 3). Hybridization with phage DNA probes in a colony lift experiment confirmed that bacteria not releasing phages were devoid of phage DNA, thus excluding the presence of colonies able to maintain phage DNA without releasing functional phage particles (data not shown).

The presence of phage DNA and phage particles in important amounts up to 10 colony replatings, and of bacteria devoid of phages, is in agreement with a model of simultaneous and independent lysis of some infected cells, random production of cured progeny from pseudolysogens and further amplification of phages by infection of these phage-free bacteria. Interestingly, PAO1-30, which kept phage-producing cells for the longest time, showed peaks of phage abundance, reflecting a classical equilibrium between phage production and bacteria predation (Table 3).

Continuous evolution of bacteria from pseudolysogens

A mixture of bacterial WT and mutant reads was clearly observed in PAO1-17, PAO1-22, PAO1-24, PAO1-26,

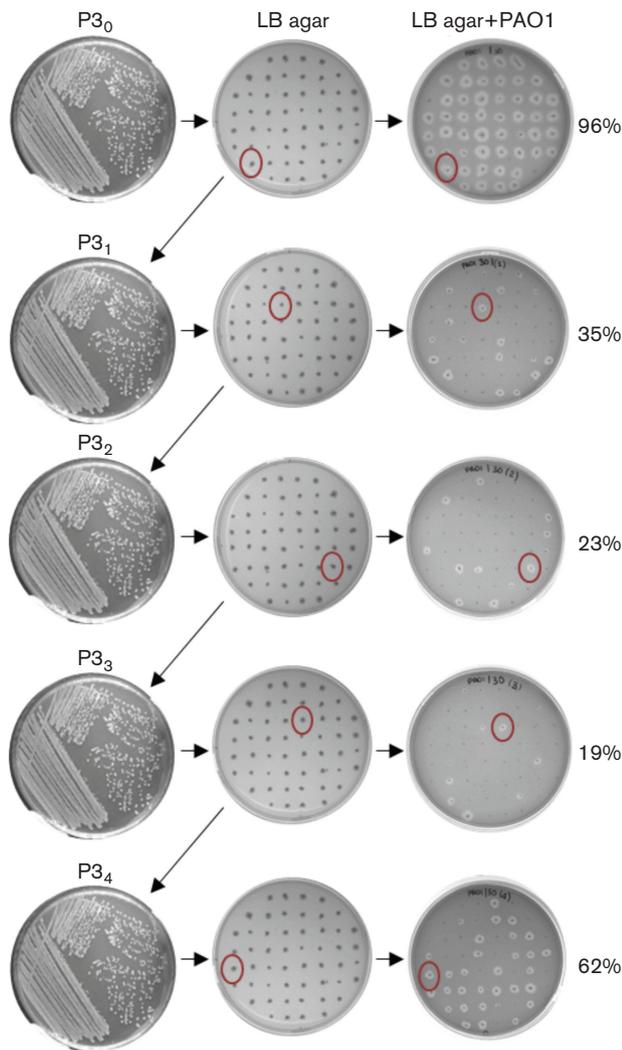


Fig. 6. Evaluation of pseudolysogeny persistence in PAO1-30. In total, 52 colonies recovered from the P3 stock (left column) were simultaneously plated (small horizontal arrows) using a sterilized pipette tip on LB agar (centre column) and on PAO1_{Or} embedded in soft agar overlay (right column). The clear zone around the bacterial colonies in the right column is due to phage lysis of the indicator bacteria. The percentage of colonies producing phages (pseudolysogens) is indicated. Red circles indicate the colony that was chosen and replated at each reisolation step (in this case from P3₀ to P3₄) because of its ability to release phage particles and lyse PAO1_{Or}.

PAO1-33 and PAO1-37, after three purification steps, always accompanied by phage DNA (Table 2). This, added to the high frequency of double mutants, suggested that immunity provided by the phage in a pseudolysogenic state allowed survival and subsequent emergence of mutations. To investigate this hypothesis, we tested whether new mutations would appear in response to the pressure imposed by phages. We went back to the -80°C stocks of seven variants (P3), isolated new colonies,

replated them and tested for the presence of phage DNA by PCR, until a colony devoid of phage DNA was obtained (Table 4). Susceptibility to the four phages was evaluated in these cured colonies, and the mutations previously identified by whole-genome sequencing were searched by PCR and Sanger sequencing. Different situations existed when phage DNA was no longer present. The Group 3 PAO1-26 variant *pilQ* microdeletion was found in about two-thirds of the colonies reisolated after -80°C storage and it was associated with resistance to Ab05. Similarly, upon reisolation of PAO1-37, $\sim 50\%$ of colonies were stable double *wzy/pilY1* mutants, devoid of Ab05 and displaying resistance to Ab05 and Ab27. In other variants, the phage susceptibility profile changed when additional colony reisolation steps were performed and new mutations could be found upon sequencing candidate genes (Table 4). In the mucoid variant PAO1-17_1 devoid of phage Ab09, a new *mucA* frameshift mutation (a deletion of a single C in a stretch of five Cs present in the WT strain) was identified in about half of the sequenced PCR products, resulting in superimposition of two sequencing profiles (Fig. S5a). PAO1-20_1 and PAO1-22_1 acquired additional mutations in *wzy*, providing resistance to LPS-dependent phages. PAO1-24_1, devoid of Ab27 DNA, was shown to resist all four phages, whereas the PAO1-24 progenitor was susceptible to both Ab09 and Ab17 (Table 1). The original *pilR* mutation in PAO1-24 (Table 2) was confirmed through PCR and DNA sequencing. Surprisingly, sequencing of a *wzy* PCR amplicon showed that the original insertion of an additional A in a stretch of seven A residues in the WT *wzy* gene was replaced by a deletion of one A, resulting in a frameshift and early stop. Similar to the *mucA* mutation in PAO1-17_1, the sequencing profile showed the superimposition of a WT and mutated profile (Fig. S5b). PAO1-25_1 and PAO1-36_1, devoid of Ab05, were sequenced, and mutations were found in *pilR*, and in *wzy* and *pilC*, respectively. All the new mutations were confirmed by Sanger sequencing of the PCR amplification products.

Colony reisolation was also performed for the three pseudolysogens for which no chromosomal mutation could be observed, i.e. PAO1-30 (Ab05 infection), PAO1-32 and PAO1-34 (Ab05 and Ab27 co-infection) (Table 1). PAO1-30_1 devoid of Ab05 still resisted Ab05. Three genes involved in type IV pilus assembly were analysed by PCR in a candidate gene approach and a new *pilQ* mutation was identified showing a substitution of a T by a G causing a threonine to proline mutation (Table 4). In contrast, PAO1-32_1 and PAO1-34_1, devoid of Ab27 DNA, recovered full susceptibility to all phages, and Ab27-resistant mutants were not obtained. This confirmed that Ab27 conferred the observed superinfection exclusion in the P3 variant and that it was not selecting mutants on both solid and liquid media.

In summary, it appeared that pseudolysogenic colonies continuously evolved due to the production of new functional phage particles that selected for new phage-resistant variants. Eventually, all variants possessed mutations in one

Table 3. Percentage of phage-producing colonies during replatings of some PAO1_{Or} variants containing phage DNA

PAO1 _{Or} variant	Replating										
	P3 ₀	P3 ₁	P3 ₂	P3 ₃	P3 ₄	P3 ₅	P3 ₆	P3 ₇	P3 ₈	P3 ₉	P3 ₁₀
20	6	0	–	–	–	–	–	–	–	–	–
22	44	0	–	–	–	–	–	–	–	–	–
24	100	0	–	–	–	–	–	–	–	–	–
26	54	0	–	–	–	–	–	–	–	–	–
36	100	0	–	–	–	–	–	–	–	–	–
37	46	0	–	–	–	–	–	–	–	–	–
25	4	8	0	–	–	–	–	–	–	–	–
34	83	6	33	0	–	–	–	–	–	–	–
17	98	56	92	0	–	–	–	–	–	–	–
32	38	31	44	69	38	0	–	–	–	–	–
30	96	35	23	19	62	25	100	100	88	2	0

Table 4. Phage tolerance pattern and mutations in secondary isolated variants

PAO1 _{Or} variant	Resistance pattern*	Sequencing method	Position on PAO1 _{Or}	Mutation	Mutation event	Locus tag	Protein alteration
17_1	IRRR	PCR candidate gene approach	4683508	C(5)→C(4)†	Frameshift	<i>mucA</i>	146/194 aa‡
20_1	RRRR	Illumina	1977343	C→A	Transversion	<i>wzy</i>	220/438 aa
			1976837	C→T	Transition	<i>wzy</i>	Ser→Phe
22_1	RRRR	Illumina	5688664	555 bp	Deletion	<i>pilQ</i>	529/714 aa
			1976848	A(7)→A(8)	Frameshift	<i>wzy</i>	74/438 aa
24_1	RRRR	PCR candidate gene approach	5095649	C(2)→C(1)	Frameshift	<i>pilR</i>	334/445 aa
			1976848	A(7)→A(6)	Frameshift	<i>wzy</i>	54/438 aa
25_1	RSSS	Illumina	5095900	G→C	Transversion	<i>pilR</i>	Arg→Pro
30_1	RSSS	PCR candidate gene approach	5096064	A→C	Transversion	<i>pilR</i>	Thr→Pro
36_1	RRRR	Illumina	5688968	T→G	Transversion	<i>pilQ</i>	Thr→Pro
			1976848	A(7)→A(6)	Frameshift	<i>wzy</i>	54/438 aa
			5071804	G→A	Transition	<i>pilC</i>	Arg→His

*Resistance pattern is reported in order against phages Ab05, Ab09, Ab17 and Ab27. S, Completely susceptible; R, completely resistant; I, reduced efficiency of plating.

†Number of repeated nucleotides indicated in parentheses.

‡Length of the mutated protein/WT.

of the pilus type IV assembly genes, and, as expected, the ability of phages to adsorb on their surface (Fig. S6) and the twitching motility of these variants were defective when compared with the control PAO1_{Or} (Fig. S7).

DISCUSSION

Pseudolysogeny is a major factor in the selection of mutants

In our experimental model, pseudolysogeny appears to be a frequent outcome of infection by the four virulent phages, providing immunity to the bacteria and allowing emergence of mutations in genes involved in receptor synthesis.

In the present investigation, we might even underestimate the frequency of pseudolysogeny as we started the analyses after three replatings for purification purposes. The frequency of single mutants was of the order of 10^{-5} plated bacteria but, surprisingly, we observed that double mutants could be recovered at a frequency of 10^{-6} , which is far higher than expected if these were present at the onset of infection. We show that pseudolysogenic colonies constitute a reservoir for phages that exert a permanent pressure on bacteria, leading to selection of secondary mutations. Many controlled studies have demonstrated the role of starvation and slow growth in the establishment of pseudolysogeny. In contrast, pseudolysogeny in rich medium is not understood (Los & Wegrzyn, 2012; Ripp & Miller,

1998). Being in the inner part of a colony might mimic starvation and slow growth conditions, whereas cells in direct contact with the agar medium would be in a rich medium context.

We observe that pseudolysogeny is established in a situation where the large majority of bacteria have been lysed and high amounts of phages are present, thus resembling the lysis inhibition control observed in T4. We propose a model in which a pseudolysogenic cell, which may contain >100 phage genome copies according to the phage burst size, forms, after several rounds of division, a colony containing bacteria cured of the phage and bacteria in which the phage lytic cycle is resumed, producing new phages (Fig. 7). The cured bacteria become prey for further amplification and production of new pseudolysogens in which phage growth is stalled. This interaction between phages and bacteria is reminiscent of the carrier state life cycle observed in different systems (Siringan *et al.*, 2014). However, in the present study the phage/host equilibrium is not stable. The appearance of pseudolysogenic cells could occur when the amount of phages and resistant mutants is higher

than the total amount of WT susceptible bacteria, allowing phages to be protected against extinction. The relative efficiency of reactivation of the phage cycle and production of cured bacteria determines the duration of the pseudolysogeny stage. It will be interesting to perform *in situ* analyses to check whether the colony is a homogeneous population of cells or if there are sectors in which phage activation is favoured and to follow fluctuations of free phage concentrations within a single colony.

A lack of immunity to superinfection mediated by immunity genes in temperate phages is supposed to differentiate true lysogeny from pseudolysogeny (Wommack & Colwell, 2000). The present pseudolysogens demonstrate inhibition of superinfection by the same phage and, more interestingly, by phages of different genera, which bind to different receptors. Immunity genes have been found in *E. coli* T4 (*imm*) (Lu & Henning, 1989) and P1 (*sim*) (Maillou & Dreiseikelmann, 1990) phages. The genes appear to impair successful injection of phage DNA into the cell. This mechanism could account for inhibition of phage infection by phages using different receptors, but there is

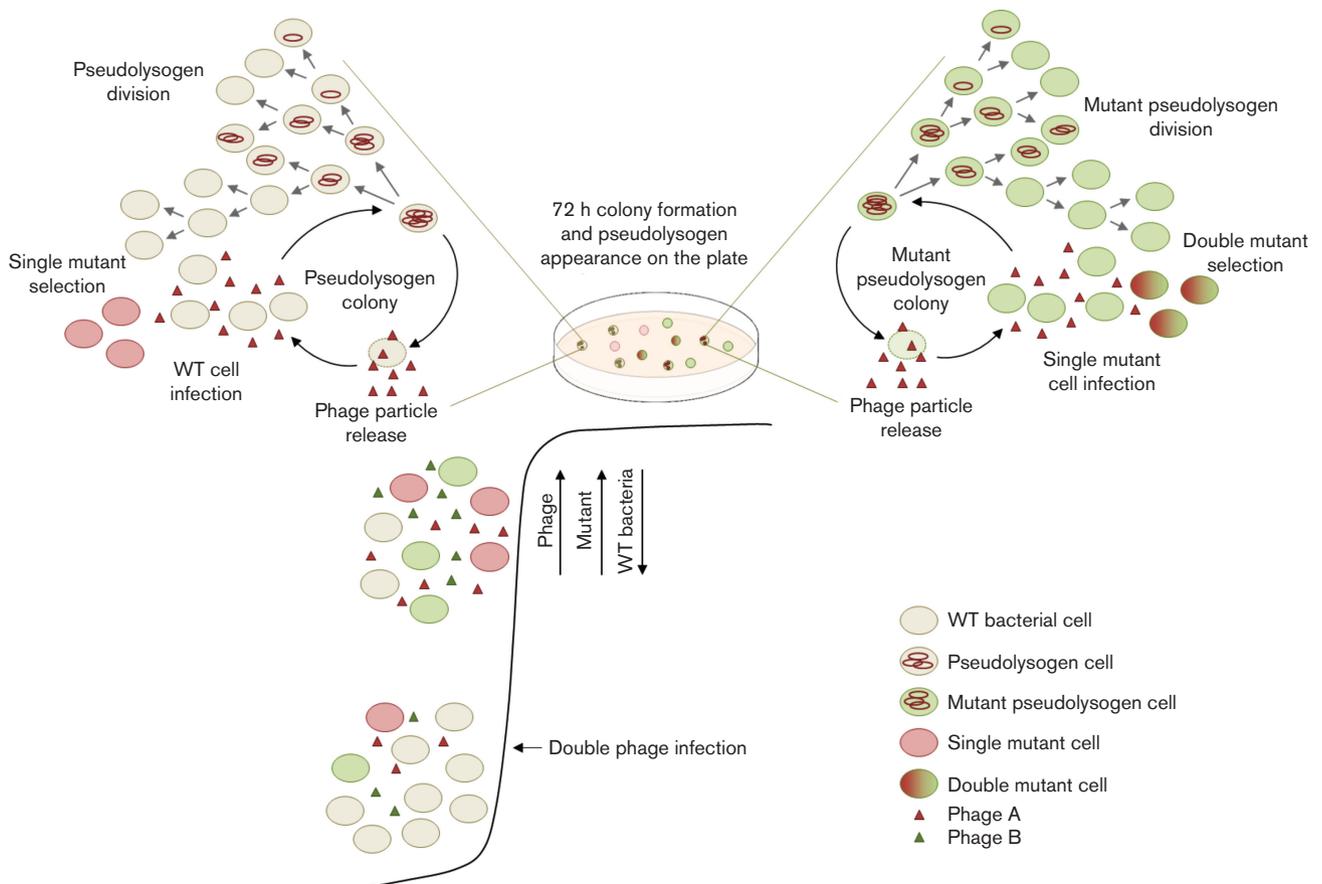


Fig. 7. Model of pseudolysogeny evolution. Continuous production of cured bacteria and release of phages from reactivated lytic cycle in pseudolysogenic cells lead to the emergence of mutations. Single mutants (left) or double mutants (right) selected by phage (phage A) result from the activation of a lytic cycle in a WT pseudolysogen or in a pseudolysogen already containing a mutation, previously selected by another phage using a different receptor for infection (phage B), respectively.

no evidence of such genes in our phages at the current time. Further experiments are needed to understand at which stage phage multiplication is inhibited.

Red Queen dynamics/arms race coevolution

Studies performed in chemostats have addressed the coevolution dynamics of phage and bacteria in controlled growth conditions (Betts *et al.*, 2014; Buckling & Rainey, 2002). Two possible outcomes were described in some assays where prey and predators are left to evolve for a long time. In the arms race, the fittest genotype survives and this limits the diversity, whereas in the Red Queen dynamics, frequency-dependent selection leads to constant production of new mutants, thus maintaining diversity (Dennehy, 2012). In our assay, which takes place in a micro community, after several rounds of coevolution, the population of free phages fluctuates, to the extent that they may seem to almost disappear within the colony. A large diversity of resistant mutants is selected and eventually the colony will be phage-free. Reversion to the WT phenotype is observed for alginate and LPS mutants so that new preys will emerge. We observed, with three phages, the presence of new phage genotypes in pseudolysogens, all three showing one or two SNPs in a tail fibre gene. The mutations were present in a subpopulation of phages used to derive the resistant mutants, and may have been selected during coevolution of phages and bacteria. No particular behaviour of these phages as compared with the parental phages could be demonstrated, such as plaque morphology and growth characteristics. However it is possible that these phages are capable of inducing a pseudolysogenic stage at a higher frequency as compared with the ancestral phage. Our results confirm that success in infection is not sufficient for phage survival, as phages are dependent upon the survival of their host population (Chaturongakul & Ounjai, 2014) and therefore phage/host relationships can be seen not as merely parasitic but as mutualistic (Williams, 2013).

Cross-resistance and reversibility of mutants

We showed that mutations selected by phages were often frameshift mutations known as phase variation (Henderson *et al.*, 1999). Frameshift mutations due to variation in poly(A), poly(G) or poly(T) stretches have been described in several bacterial genes as an adaptation mechanism to different environmental conditions and are reversible when the selective pressure is no longer applied (Segura *et al.*, 2004). Natural mutations of *mucA* observed in strains isolated from cystic fibrosis patients were phase variation mutations (Spencer *et al.*, 2003), or other frameshift mutations (Pulcrano *et al.*, 2012), also resulting in truncated proteins as seen in PAO1-02.

Interestingly, many of the mutations identified in this study occur in the *wzy/wzx*-dependent pathway responsible for the synthesis of LPS O-antigen (Islam & Lam, 2014) and

they are either single nucleotide indels or mutations. LPS is composed of a lipid A membrane anchor, a core oligosaccharide linker and a distal polysaccharide termed O-antigen, in the form of A and B bands (Taylor *et al.*, 2013). Both WT and mutant forms of *wzy* and *mucA* genes were simultaneously found in the presently described mutants, suggesting that the mutation can reverse at a high rate. Constant variations in LPS and alginate biosynthesis pathways may help *P. aeruginosa* face aggression or environmental changes. This might be one explanation for the 'colonial dissociation' frequently observed with *P. aeruginosa*, characterized by colonial differences of a single strain (Zierdt & Schmidt, 1964).

The different assays show that, depending on the phage used, the selected mutants, obtained at a high frequency, display a large variety of phenotypic changes related to membrane permeability and cell motility. Hosseinidoust *et al.* (2013a) described such phenotypes induced by two phages which use type IV pili and LPS as receptors, but they could not identify the mutations. Phenotypic changes can alter bacterial virulence (Lyczak *et al.*, 2000). Indeed, we show that phage Ab09 often selects for mutants with a mucoid phenotype, probably related to an increased capability to produce alginates. In the context of cystic fibrosis infection, mucoidy favours the formation of protected colonies with increased resistance to opsonization, phagocytosis and destruction by antibiotics (Pritt *et al.*, 2007). It has been shown that alterations of a single band or both bands of the O-antigen of *P. aeruginosa* PAO1 can give rise to mutants with increased cytotoxicity mediated by the type III secretion system (Augustin *et al.*, 2007). In addition, changes in O-polysaccharide expression in PAO1 affect the size and protein content of outer membrane vesicles, and the formation of a robust biofilm (Murphy *et al.*, 2014).

A total of 25 components are involved in the type IV pilus biogenesis (Kim *et al.*, 2006). In the present small-scale investigation we observed ten mutations affecting five genes. Half of the mutations are irreversible deletions, which contrast with the high frequency of reversible phase variation mutations in LPS. This suggests that the fitness cost of such mutants would rapidly lead to their elimination and that phages using type IV pili as receptors should be favoured for phage therapy. Several studies have investigated the effect of type IV pilus mutations and phage resistance. Interestingly, phage F6, a dsRNA cystovirus of *Pseudomonas syringae* pathovar *phaseolicola*, selects for several types of mutants that differ in the number of type IV pili expressed per cell, but none of the mutated genes is known to be directly involved in type IV pilus expression (Sistrom *et al.*, 2015).

Phage therapy is considered as a promising approach to fight against antibiotic resistant strains (Abeldon *et al.*, 2011). Either ready-made cocktails or 'sur-measure' phages will be used to treat patients, similar to what is still done in several countries of Eastern Europe. It is

important to investigate the risks linked to the use of phages, particularly in the selection of bacterial mutants that could show deleterious characteristics (Hosseini-doust *et al.*, 2013b) or drive the expression of undesirable bacterial virulence factors (Olszak *et al.*, 2015). In a mouse model of *E. coli* gut infection, it was proposed that virulent phages remained inside bacteria in a pseudolysogenic state, therefore becoming resistant to degradation and allowing persistence of bacteria (Maura & Debarbieux, 2012; Maura *et al.*, 2012). It would be interesting to know whether new variants emerge in such experiments. Alternatively, some phages driving evolution toward loss of virulence could be favoured if they exist (León & Bastías, 2015). Another concern is the potential role of phages in horizontal transfer, which could be favoured by the long-term maintenance of phage genomes inside the bacteria during pseudolysogeny. Additional experiments are needed to further investigate the fate of the phages and bacteria in a pseudolysogen interaction.

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RESUME EN FRANÇAIS

Les bactériophages sont des virus qui injectent leur génome dans une bactérie après fixation à des récepteurs sur la surface de celle-ci, puis effectuent un cycle de multiplication de leur ADN, la synthèse des protéines de structure, l'encapsidation du génome viral et la lyse de la bactérie. Les phages virulents réalisent uniquement des cycles lytiques alors que les phages tempérés peuvent également intégrer leur génome dans celui de la bactérie, donnant ainsi naissance à une bactérie dite lysogène. Les phages peuvent parfois être maintenus dans la bactérie sans effectuer un cycle lytique ni s'intégrer, dans un état encore peu compris, connu sous le nom de pseudolysogénie.

Pseudomonas aeruginosa est une espèce bactérienne présente dans l'environnement et associée à de nombreux hôtes, végétaux et animaux. Elle est responsable de graves infections nosocomiales et on observe de plus en plus souvent des souches multirésistantes aux antibiotiques, ayant une grande capacité à former des biofilms, et en conséquence très difficiles à éradiquer. Il faut donc absolument trouver des approches thérapeutiques nouvelles telle que la phagothérapie. De nombreuses données cliniques obtenues dans les pays de l'est de l'Europe et en Russie attestent de l'efficacité et de l'innocuité de la phagothérapie, mais il reste des incertitudes en particulier concernant la nature et la fréquence des résistances naturelles. Notre projet vise à évaluer le potentiel thérapeutique des phages et à mieux comprendre la dynamique de leur interaction avec leur hôte.

Nous avons étudié les mécanismes de résistance mis en place par la souche de *P. aeruginosa*, PAO1, à quatre bactériophages virulents appartenant à des genres différents: deux *podovirus*, Ab05 (Φ KMV-like) et Ab09 (LIT1-like), et deux *myovirus*, Ab27 (PB1-like) et Ab17 (KPP10-like), tous isolés par notre laboratoire. Des infections simples ou multiples de PAO1 ont été réalisées, et une collection de variants résistants aux phages a été isolée et étudiée. La fréquence des bactéries résistants était de 10^{-5} pour l'infection par un phage seul et 10^{-6} pour les infections par des combinaisons de deux ou quatre phages. Le phénotype et la mobilité des variants résistants étaient fréquemment affectés.

Le génome de 27 variants a été entièrement séquencé par la technologie Illumina, et la comparaison avec le génome de la souche PAO1 a permis l'identification de mutations ponctuelles ou de petites indels. Quatre variants supplémentaires ont été caractérisés par une approche «gène candidat». Des mutations affectant 14 gènes différents et 1 région régulatrice ont été observées. Les gènes mutés codent pour des protéines impliquées dans la biosynthèse des pili de type IV (T4P) et des lipopolysaccharides (LPS), très fréquemment utilisés comme récepteurs par les phages. Des mutations de la synthèse des alginates ont été également observées. La moitié des variants possède des mutations de variation de phase qui se sont révélées être instables. Par contre, les gènes impliqués dans la biosynthèse du T4P montrent des délétions stables.

Nous avons aussi observé que la pseudolysogénie est une conséquence fréquente de l'infection par ces phages virulents et que la sélection de mutants (très souvent des mutants doubles) est favorisée par la production continue de phages par les pseudolysogènes. La présence d'ADN de phage libre a été observée en liaison avec l'exclusion de surinfection.

Pour conclure, si les phages sélectionnent des bactéries résistantes possédant des altérations dans les gènes impliqués dans la biogénèse ou la régulation des déterminants de la virulence, celle-ci sera probablement modifiée, d'une manière bénéfique ou préjudiciable, ce qui reste à étudier.

L'utilisation du cocktail par rapport à l'infection simple, ne réduit pas de manière significative la fréquence de la résistance aux phages et en outre, nous montrons que la pseudolysogénie est un acteur majeur de la sélection de mutations.

Titre : La pseudolysogénie permet la sélection des mutations successives à la base de la résistance multiple de *Pseudomonas aeruginosa* aux bactériophages virulents

Mots clés : Pseudolysogénie, résistance, bactériophages, *Pseudomonas aeruginosa*, coévolution

Résumé : Les bactériophages sont utilisés dans différents pays en complément des antibiotiques, pour traiter des infections bactériennes. Le développement de cette approche qui pourrait aider à combattre des germes multirésistants aux antibiotiques, nécessite une meilleure connaissance des interactions entre phages et bactéries.

Nous avons étudié les mécanismes de résistance que *Pseudomonas aeruginosa*, source majeure d'infections nosocomiales, met en œuvre contre des phages lytiques utilisés pour composer des cocktails pour la phagothérapie. Les bactéries résistantes présentent des mutations des gènes de biosynthèse des récepteurs des phages, ce qui empêche leur fixation sur la surface et l'initiation de l'infection. En outre, nous avons montré que, dans certains variants, la résistance aux phages est due à la persistance du génome viral dans la bactérie, dans un état appelé pseudolysogénie, permettant l'évolution continue de la population bactérienne et la sélection de nouveaux mutants.

Title : Pseudolysogeny and sequential mutations build multiresistance to virulent bacteriophages in *Pseudomonas aeruginosa*

Keywords : pseudolysogeny, resistance, bacteriophages, *Pseudomonas aeruginosa*, co-evolution

Abstract : Bacteriophages are used in different countries in addition to antibiotics to treat bacterial infections. Despite the hopes raised by this approach to fight multidrug-resistant bacteria, it is essential to show that the fate of bacteria can be controlled, in terms of resistance, after the treatment.

This project aimed to study the mechanisms of resistance that *Pseudomonas aeruginosa*, one of the major source of nosocomial infections, employs against lytic phages used to constitute cocktails for phage therapy. The phage-resistant variants that we isolated showed alterations of the phage receptor, preventing phage adsorption on the bacterial surface and the initiation of infection. Moreover, we have shown that, in some variants, phage-resistance was due to the persistence of the viral genome into the bacteria, in a state defined as pseudolysogeny, allowing the continuous evolution of the bacterial population by selecting for new mutants.

