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

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## Abstract

Coevolution between bacteriophages 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 favored 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 in three of them no chromosomal mutation could be found. Upon further growth of these pseudolysogens, some variants with new chromosomal mutations were recovered presumably due to continuous evolutionary pressure.


## 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 are key actors in diversification of *P. aeruginosa* by selecting for resistant mutants, and in turn adapting to new bacterial genotypes in a coevolution

arm race (Brockhurst *et al.*, 2005; Dennehy, 2012). A large variety of *P. aeruginosa* bacteriophages has been isolated, some showing a wide host range. However, several studies have illustrated that from 6 to 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 (Φ-KMV) (Chibeu *et al.*, 2009) and siphoviruses, or in lipopolysaccharide (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 on 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 (Brockhurst *et al.*, 2005; Hahn, 1997). Scanlan *et al.* 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 (Scanlan *et al.*, 2015).

It is generally accepted that the outcome of virulent bacteriophage 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; Ripp & Miller, 1998). Pseudolysogeny was first described as an unstable interaction which is not productive and eventually resolves into true lysogeny or virulent growth

(Baess, 1971). Los et al. (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 (LIN) (Bode, 1967) caused by the T4rI gene product (Bode, 1967). Later, pseudolysogeny was defined as a stage in the bacteriophage 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; Ripp & Miller, 1998). The role played by pseudolysogeny in the emergence of bacterial mutants has not been demonstrated. Early work by Demerec and Fano described mutants of *E. coli* obtained on agar medium following infection by seven different phages (T1 to T7) (Demerec & Fano, 1945). The authors noted that phages were present for a long time after they re-isolated 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 bacteriophages 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, thereafter 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 different 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* Transposon Mutant Library" (grant #NIH P30DK089507), UW Genome Sciences, USA.

**Isolation of phage-resistant bacteria**. Bacteria were inoculated at a 600 nm absorbance ($A_{600}$) of 0.01 into glass vials with aeration, containing 5 ml of Luria broth (LB) medium, and grown (37°C, shaking at 180 rotations per minute (rpm)) to an $A_{600}$ of 0.2. Infections were performed at a multiplicity of infection (MOI) of 0.1. Infections on solid medium used a 10 µl inoculum of the bacterial culture (2 x $10^6$ colony forming units (CFU)) mixed with 10 µl of a suspension, containing either a single phage genera, a cocktail of two, or a cocktail of all four phages ($10^5$ plaque forming units (PFU) for each phage). Ten µl of SMG (saline magnesium gelatin) phage buffer (SMG comprises NaCl at 5.8 g $l^{-1}$, $MgSO_4$ at 2 g $l^{-1}$, 1 M Tris-HCl, and gelatin at 0.1 g $l^{-1}$ [pH 8.0]) were used in negative controls. The mixture was kept for 15 min at room temperature (RT), before being poured on a fresh LB agar plate (1.5% wt/vol agar) with 4 ml of soft agar (0.7% wt/vol agar) and incubated at 37°C for 3 days. Because 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 log phase ($A_{600}$ of 0.6) at an MOI of 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 an $A_{600}$ of 0.1. Bacterial cultures in the late log phase ($A_{600}$ of about 1, equivalent to $10^9$ bacteria per ml, determined by titrating the bacteria), were 10-fold serially diluted. One hundred µl of each dilution were mixed with 10 µl (about $10^6$ PFU) of a single

phage suspension or a mixture of two or four phages as described above. The samples were kept for 15 min at RT and then poured on fresh LB 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. The divisor was the number of plated bacteria.

**Phage susceptibility assay**. Aliquots (500 µl) from the liquid culture of variants ($A_{600}$ of 0.8 to 1.2) were mixed with 6 ml of 0.7% wt/vol LB agar and poured onto a square LB 1.5% wt/vol agar plate. Five dilutions ($10^{10}$, $10^{9}$, $10^{8}$, $10^{7}$, $10^{6}$ PFU $ml^{-1}$) 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 EOP (efficiency of plating) using $PAO1_{Or}$ as a control.

**Virucide assay**. The protocol described by de Siqueira et al. (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 volumes of virucide, followed by centrifugation, washing with phosphate-buffered-saline (PBS) and incubation at 37°C for 30 min with 50 µg $ml^{-1}$ DNaseI. Then total bacterial DNA was purified.

**Adsorption assay**. An overnight bacterial culture was diluted to an $A_{600}$ of 0.1-0.6 and left to equilibrate at 37°C. Approximately $10^{6}$ phages were added to 1 ml of the diluted bacterial culture ($1x10^{8}$ to $6x10^{8}$ bacteria). At a fixed time point, 50 µl of the mixture were transferred to a 1.5 ml conical centrifuge tube containing 940 µl of LB medium and 10 µl of chloroform. The suspension was vortexed for 5 sec and centrifuged in order to pellet the phages adsorbed on the bacterial surface. Then, 10 µl of the 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 of a fresh LB agar plate was used as a reference in all experiments. To test for hemolytic activity, 10 µl of an overnight culture of phage-resistant mutants ($A_{600}$ of 2) were spotted onto Sheep blood (5% wt/vol) agar, and plates were incubated for 24 h at 37°C. For twitching motility assessment, one µl of an overnight bacterial culture ($A_{600}$ of 2) was inoculated between the agar and the plastic surface of LB 1.5% wt/vol agar plates. The diameter of the motility zone around the inoculation site was measured after 24 h incubation at 37°C. Lipopolysaccharides (LPS) were purified using the method of Hitchcock and Brown (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 (Tris 1M, 2% SDS, 4% β-mercaptoethanol and 10% glycerol), prior to 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 *et al.*, 1990).

**Biofilm formation**. 96-wells microtiter plates (Greiner) containing LB were inoculated with an overnight bacterial culture ($A_{595}$ of ≈ 0.1) and incubated at 37 °C for 48 h. Before proceeding with biofilm quantification, the $A_{595}$ was recorded. The wells were washed three times with PBS, 200 µl of 0.1% wt/vol 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 it into 200 µl of absolute ethanol. The $A_{595}$ was then divided by the $A_{595}$ 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 fifty-two colonies had been plated. After 5 min, the membrane was lifted using forceps and treated successively for 2 min with NaOH 0.4 N twice, Tris 1 M pH 7.5 twice, 2X Saline Sodium Citrate (SSC) twice. After this, the membrane was 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 of hybridization buffer (Church & Gilbert, 1984) per membrane. The probe was labeled using the Megaprime$^{TM}$ kit (GE Healthcare Amersham) and hybridization was performed overnight at 65°C in hybridization buffer. Washes were done successively with 2XSSC and 0.1% wt/vol SDS, 0.5XSSC and 0.1% wt/vol SDS, 0.2XSSC and 0.1% wt/vol SDS.

**DNA extraction, PCR and sequencing**. PCR was performed on thermolysates or purified DNA using oligonucleotides listed in Table S1. 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, pH 7.8, EDTA 10 mM, NaCl 10 mM, SDS 0.5% wt/vol), treated with proteinase K at 50 µg 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 VNTRs ms216 and ms217 as previously described (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 lacZα. The PAO1 *mucA* gene was PCR-amplified using oligonucleotides mucA_Clon_F_Bam 5'TGGGATCCCGAGAAGCCTGACACAGC3' and mucA_Clon_R_Hind 5'GAAAGCTTACCGCCATCAGGCTGCCA3', which included restriction sites for *BamH*I and *Hind*III. The amplicons were digested with *BamH*I and *Hind*III, ligated into the vector similarly digested 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. (Choi *et al.*, 2006). Transformants were selected

using 10 µg $ml^{-1}$ Gentamycin, and the presence of the plasmid was verified by PCR amplification using a *mucA* forward oligonucleotide mucA-int_F5'ACGCAGGTAGATCGGCAGAC3' and a plasmid reverse oligonucleotide pUCP24_MCS_R 5'GGCCTCCTTCGCTATTACGCC3'. The colony aspect was observed under the stereomicroscope. The transformants were then tested for their susceptibility to the four bacteriophages.

**Whole genome sequencing.** Ten µg purified bacterial DNA 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 250 bp paired-end reads were produced. One million up to 5 million reads were obtained corresponding to a 40-200 fold average coverage. The mutations were identified by comparison with the genome of the $PAO1_{Or}$ sequence using native GeneiousR9 tools default parameters (Biomatters, New Zealand). 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 a 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 localisation 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 GeneiousR9 native assembler using the Medium-Low Sensitivity/Fast parameter.

**Nucleotide sequence accession number.** The DNA sequence of the PAO1 strain representative $PAO1_{Or}$ has been deposited in the EMBL-EBI database under accession number LN871187, available from the European Nucleotide Archives (ENA) browser at http://www.ebi.ac.uk/ena/data/view/ project PRJEB9838.

**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 combination 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 four phages primary receptor 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 phage (Ceyssens *et al.*, 2011) whereas growth of Ab09, Ab17 and Ab27 was restricted on the LPS defective mutant. Then we designed an experimental procedure to allow for phage amplification and isolation of independent resistant mutants. For this purpose, $PAO1_{Or}$ was infected at an MOI of 0.1 (one phage for ten 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 $3.2x10^{-5}$ for single phage infection, $4x10^{-6}$ for double infection and $3.8x10^{-6}$ for multiple infections. Colonies of variable shape, size and appearance were picked from seven independent experiments and were purified by three re-isolation steps, in order to ensure that a pure population was obtained. A single colony was recovered after the third re-isolation 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 is defined as

246 the capacity to survive the phage infection, whether this was due to a lack of receptor or to any
247 other mechanism. In contrast, none of the surviving bacteria recovered from infection with Ab27
248 alone turned out to be stably tolerant to Ab27. A similar observation was made by Hosseinidoust et
249 al. (Hosseinidoust *et al.*, 2013a) who failed to isolate bacteria resistant to phage E79, another PB1-
250 like phage.

251 In total, thirty-two $PAO1_{Or}$ variants were retained and tentatively distributed into five groups
252 according to their phage susceptibility pattern, evaluated by the efficiency of plating (Table 1 and
253 Table S2). The thirteen Group 1 variants displayed normal susceptibility only to phage Ab05. The
254 six Group 2 variants showed intermediate susceptibility patterns to the different phages. The five
255 Group 3 variants were resistant only to phage Ab05. The four Group 4 variants displayed full
256 resistance to phages Ab05 and Ab27, and reduced susceptibility to phage Ab09 and Ab17,
257 characterized by the production of small plaques instead of large, clear ones. Four variants resisting
258 all four phages constituted Group 5. In Group 2, a mucoid phenotype was stably observed for
259 PAO1-02, PAO1-06 and PAO1-13, whereas PAO1-17 continuously produced two types of colonies
260 on solid LB media, some with a smooth appearance as seen for the control $PAO1_{Or}$ strain, and
261 others surrounded by an irregular transparent edge (Fig. 1b). This phenotype may be related to an
262 observed decrease in swarming capacity (data not shown). In addition, mucoid colonies appeared
263 after several days of growth. When replated, the PAO1-17 colonies surrounded by a transparent
264 edge again produced both types of colonies, whereas the others stably maintained their phenotype.
265 Growth on Sheep blood agar plates showed the existence of new phenotypic characteristics for
266 some variants as compared to the parental $PAO1_{Or}$ strain (Fig. S1). Variants PAO1-02, PAO1-13
267 and PAO1-17 lacked the hemolytic ability displayed by the wild-type $PAO1_{Or}$ but, interestingly, the
268 colonies of PAO1-13 presented zones of reversion to the wild-type phenotype.

269 Unexpectedly, PCR amplification showed that phage DNA could still be detected at the P3 re-
270 isolation step in fifteen 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 DNaseI digestion, followed by several washings of the bacteria pellet. Phage DNA was still present in large amounts in the bacteria, as shown by semi-quantitative PCR reaction (Fig. S2 shows PA01-20 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 dedicated paragraph below). The results obtained suggest that some bacterial cells might contain in the order of 100 phage genomes.

An adsorption assay was performed with the sixteen 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 details 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 (NC_002516) strains, 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 (positions 727 255 to 4 788 575), the presence of a copy of filamentous Pf1 prophage in $PAO1_{Or}$ at position 5 242 103 to 5 254 164, and 63 SNPs or short indels events. As expected, some of these differences, including the inversion and the Pf1 prophage plus a number of the SNPs and indels were previously reported by Klockgether et al. (Klockgether *et al.*, 2010). Others were specific to the $PAO1_{Or}$ sub line.

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-

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 fourteen variants a single chromosomal mutation was identified. Six variants were double mutants. Coverage at these genetic alterations was always at least 40, and up to 250 fold. PAO1-02, with a mucoid phenotype, was analyzed by PCR using a candidate gene approach and this allowed 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 wild-type (WT) and mutated sequence could be found, indicating that the cell population was mixed. This indicates that an unstable mutation has reverted back. Three different kinds of genetic alterations were found, phase variation, deletions of 10 bp or more, and nucleotide substitutions. Fourteen genes were affected. The *wzy* mutations A(7) to A(8) at position 1 976 849 as well as A(7) to A(6) at the same position, and G(6) to G(5) at position 1 977 338, were found in six, one and other two 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 ten 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* or *wbpL* genes are members of the heteropolymeric O-specific antigen (OSA) biosynthesis cluster in PAO1 (Lam *et al.*, 2011). Gene *migA* encodes a rhamnosyltransferase involved in the LPS core capping (Poon *et al.*, 2008), whereas *wapH* and *dnpA* are known to be involved in the synthesis of LPS polysaccharide (Hansen *et al.*, 2007; Liebens *et al.*, 2014), and *pgi* encodes a glycosyl transferase (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, while Ab05 selected mutations in genes involved in type IV pilus synthesis. The number and variety of observed mutations was very high confirming that the procedure used to isolate the variants allowed for selection of independent events.

**The observed mutations are responsible for modifying the phage receptor**

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 type IV pilus, LPS and alginate biosynthesis. The motility of 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 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 after reaching an absorbance at 600 nm ($A_{600}$) of 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 were

346 extracted in PAO1-04 altered in *wzy* and *migA*, and PAO1-07 altered in *wzy*, and were analysed by
347 polyacrylamide gel electrophoresis. Fig. 5 displays the banding profiles compared to that of
348 $PAO1_{Or}$, showing absence of the A- and B- bands, as well as modifications in the proportion of core
349 and core +1 bands. PAO1-04 possessed only the core +1 oligosaccharide form. In contrast, variant
350 PAO1-07 possessed both bands in equal amounts, whereas $PAO1_{Or}$ had a small proportion of core +
351 1. Absence of core oligosaccharide in PAO1-04 was likely a consequence of the mutation in *migA*.
352 Finally to confirm that the observed *mucA* mutations were responsible for the mucoid phenotype,
353 we tested whether the mutants could be complemented by the WT gene. A full *mucA* amplicon was
354 cloned into an expression vector which was then introduced into PAO1-02, PAO1-06 and PAO1-
355 13. In the three cases the transformants were no longer showing a mucoid appearance, whereas the
356 vector alone was not reversing the mucoid phenotype. In addition, the *mucA* transformants
357 recovered normal susceptibility to all phages.

358 **Persistence of phage DNA in pseudolysogens**

359 In eleven variants, phage DNA represented a proportion of 2 to 85% of sequencing reads. The very
360 high proportion of phage DNA in some samples could only be explained by the presence of free
361 phages, inside bacteria, and/or attached to cells. To confirm that phage DNA was present inside
362 bacteria, we performed another genome sequencing of PAO1-17 and PAO1-20 at the P3
363 purification step, after treatment of the bacterial pellet with DNaseI and the virucide, followed by
364 three washing steps. The results showed that 1.6% of reads still corresponded to Ab09 in PAO1-17,
365 and 52% to Ab17 in PAO1-20, similarly to what was observed in the first sequencing analysis
366 (Table 2). A search for hybrid reads between phage and bacteria genomes did not bring any
367 significant result, indicating that the phage DNA was not inserted into the bacterial chromosome,
368 and therefore we refer to these bacteria as pseudolysogens. Interestingly, the three variants in which
369 no chromosomal mutation could be found, PAO1-30, PAO1-32 and PAO1-34, possessed large
370 amounts of phage DNA: PAO1-30 immune to Ab05, Ab17 and Ab27 contained high levels of Ab05

371 DNA; PAO1-32 and PAO1-34, immune to Ab09, Ab17 and Ab27, contained Ab27 DNA.
372 Intermediate resistance profiles observed in some variants were correlated with the existence of a
373 mixed population of wild type and mutated bacteria, and with the presence of phage DNA.

374 In the sequenced samples in which a high proportion of sequence reads derived from phage DNA
375 were present, it was possible to assemble the full phage genome sequence. This led to the
376 identification of several single nucleotide differences in tail fiber genes, as compared to the parental
377 genotype. In three pseudolysogens obtained independently, an Ab05 tail fiber gene displayed two
378 SNPs. By PCR and sequencing, we could also observe these SNPs in a fraction of the phages used
379 to select for resistant bacteria (Fig. S4). Similarly a single SNP was observed in an Ab17 tail fiber
380 gene from variants PAO1-20 and PAO1-22, and in the ribosome binding site of an Ab27 tail gene
381 from variants PAO1-24, PAO1-32 and PAO1-34. This might reflect the selection of phage variants
382 by strain $PAO1_{Or}$, possibly affecting the capacity of the bacteria to resist phages. However, we
383 could not see any differences in binding to the host or plating efficiency with these phage
384 genotypes, as compared to the parental genotype.

385 **Stability of the pseudolysogen state**

386 Viable phages were released by pseudolysogens, sometimes at high titers, during overnight culture
387 in LB medium. This suggested that a portion of the bacterial population could achieve a productive
388 viral cycle. To evaluate the dynamics inside pseudolysogen colonies, we measured the percentage
389 of bacteria containing phage DNA and producing viable phages, and evaluated for how long phage
390 DNA was maintained. For this purpose, 1 µl of some bacterial strains from the frozen P3 stock were
391 spread on LB agar ($P3_0$). Fifty-two colonies were picked and deposited successively on an LB agar
392 plate, then on an LB agar plate covered with a lawn of soft agar containing $PAO1_{Or}$ (see PAO1-30
393 replatings as an example in Fig. 6). After incubation at 37°C for 24 h, a lysis zone could be seen
394 around some colonies on the lawn of $PAO1_{Or}$. One such phage-producing colony from the LB agar
395 plate was streaked onto a new LB plate and the procedure was repeated. The fraction of

pseudolysogenic cells contained in a single colony varies from 4 up to 100 percent. 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 ten 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, -22, -24, -26, -33, -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 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 double *wzy-pilY1* mutants, devoid of Ab05 and displaying

421 resistance to Ab05 and Ab27. In other variants, the phage susceptibility profile changed when
422 additional colony re-isolation steps were performed, and new mutations could be found upon
423 sequencing candidate genes (Table 4). In the mucoid variant PAO1-17_1 devoid of phage Ab09, a
424 new *mucA* frameshift mutation (a deletion of a single C in a stretch of five Cs present in the WT
425 strain) was identified in about half of the sequenced PCR products, resulting in superimposition of
426 two sequencing profiles (Fig. S5a). PAO1-20_1 and PAO1-22_1 acquired additional mutations in
427 *wzy*, providing resistance to LPS-dependent phages. PAO1-24_1, devoid of Ab27 DNA was shown
428 to resist all four phages whereas the PAO1-24 progenitor was susceptible to both Ab09 and Ab17
429 (Table 1). The original *pilR* mutation in PAO1-24 (Table 2) was confirmed through PCR and DNA
430 sequencing. Surprisingly, sequencing of a *wzy* PCR amplicon showed that the original insertion of
431 an additional A in a stretch of seven As residues in the WT *wzy* gene was replaced by a deletion of
432 one A, resulting in a frameshift and early stop. Similarly to the *mucA* mutation in PAO1-17_1, the
433 sequencing profile showed the superimposition of a wild type and mutated profile (Fig. S5b).
434 PAO1-25_1 and PAO1-36_1, devoid of Ab05,were sequenced, and mutations were found in *pilR*,
435 and in *wzy* and *pilC*, respectively. All the new mutations were confirmed by Sanger sequencing of
436 the PCR amplification products.

437 Colony re-isolation was also performed for the three pseudolysogens for which no chromosomal
438 mutation could be observed, PAO1-30 (Ab05 infection), -32, -34 (Ab05 and Ab27 co-infection)
439 (Table 1). PAO1-30_1 devoid of Ab05 still resisted Ab05. Three genes involved in type IV pilus
440 assembly were PCR-analysed in a candidate gene approach, and a new *pilQ* mutation was identified
441 showing a substitution of a T by a G causing a threonine to proline mutation (Table 4). In contrast,
442 PAO1-32_1 and PAO1-34_1, devoid of Ab27 DNA, recovered full susceptibility to all phages, and
443 Ab27-resistant mutants were not obtained. This confirmed that Ab27 conferred the observed
444 superinfection exclusion in the P3 variant and that it was not selecting mutants on both solid and
445 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 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 to the control $PAO1_{Or}$ (Fig. S7).

## DISCUSSION

### Pseudolysogeny is a major factor in 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 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. 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 when the large majority of bacteria has been lysed and high amounts of phages are present, thus resembling the LIN control observed in T4. We propose a model in which a pseudolysogenic cell, which may contain more than 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 (CSLC) 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 bacteriophages and resistant mutants is higher than the total amount of WT susceptible bacteria allowing bacteriophages 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 homogenous population of cells or if there are sectors in which phage activation is favored, 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) bacteriophages. The genes appear to be involved in the 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 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/arm 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). In some assays where prey and predators are left to evolve for a long time two possible outcomes were described. 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 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 fiber 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 behavior of these phages as compared to the parental ones 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 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 & Ounjai, 2014), and therefore phage-host relationships can be seen as not 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 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 & Schmidt, 1964).

The different assays show that, depending on the bacteriophage 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. (Hosseinidoust *et al.*, 2013a) described such phenotypes induced by two phages which use type IV pilus 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 favors 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 (TTSS) (Augustin *et al.*, 2007). In addition, changes in O-polysaccharide expression in PAO1 affects 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 favored 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 were 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 (Abedon *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 (Hosseinidoust *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. On the other hand, some phages driving evolution toward loss of virulence could be favored if they exist (Leon & Bastias, 2015). Another concern is the potential role of bacteriophages in horizontal transfer, which could be favored 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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## Table and figure legends

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

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

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

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

**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.

**Fig. 2: Phage absorption on sixteen PAO1 variants devoid of phage DNA.** On the y-axis is reported the percentage of unabsorbed phages at 16 min after infection. The standard deviation is the result of three independent assays.

**Fig. 3: Twitching motility of original (P3) phage-tolerant variants.** 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.

**Fig. 4: Biofilm formation assay of original (P3) phage-tolerant variants**. The amount of bacteria bound to the wells is evaluated by measuring the $A_{595}$ of crystal violet resuspended in ethanol. The standard deviation is the result of three independent assays.

**Fig. 5: SDS-PAGE of Proteinase K-treated samples.** The position of the core, the A-band and the B-band of the O-antigen were identified according to the work of Islam et al. (Islam *et al.*, 2013). *, very long B-chains, **, long B-chains and ***, short B-chains.

**Fig. 6: Evaluation of pseudolysogeny persistence in PAO1-30**. Fifty-two colonies recovered from the P3 stock (left panel) were simultaneously plated (small horizontal arrows) using a sterilized pipette tip on LB agar (center panel) and on $PAO1_{Or}$ embedded in soft agar overlay (right panel). The clear zone around the bacterial colony in the right panel is due to phage lysis of the indicator bacteria. The percentage of colonies producing phages (pseudolysogens) is indicated on the right side. In red is circled the colony that was chosen and replated at each re-isolation step (in this case from $P3_0$ to $P3_4$) because of its ability to release phage-particles and lyse $PAO1_{Or}$).

**Fig. 7: 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.

777 Single mutants (left) or double mutants (right) selected by bacteriophage (phage A) resulted from
778 the activation of a lytic cycle in a wild-type pseudolysogen or in a pseudolysogen already
779 containing a mutation, previously selected by another phage using a different receptor for infection
780 (phage B), respectively.

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784 **Table 1**

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| | Infection | PAO1 variant | Phage resistance | | | | Phage DNA‡ |
|---|---|---|---|---|---|---|---|
| | | | Ab05 | Ab09 | Ab17 | Ab27 | |
| Group 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 |
| Group 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 |
| Group 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 |
| Group 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 |
| Group 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 | - |

786 *infection performed with the four phages
787 † mucoid variant
788 ‡detection by PCR
789 S, completely susceptible
790 R, completely resistant
791 I, reduced efficiency of plating
792 s, normal efficiency of plating but small and turbid plaques
793

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## Table 2

| | PAO1 variant | Phage resistance[†] | Position on $PAO1_{Or}$ | Mutation | Locus tag | Protein alteration | % of reads representing | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Mutation | Phage | Bacteria |
| Group 1 | **01** | S R RR | 1 976 849 | A(7) → A(8) | *wzy* | 74/438 aa | 100 | 0 | 98 |
| | **04** | S R R R | 1 977 338 | G(6) → G(5) | *wzy* | 224/438 aa | 100 | 0 | 99 |
| | | | 4 736 958 | G → A | *migA* | Arg→ His | 100 | | |
| | **07** | S R R R | 1 977 338 | G(6) → G(5) | *wzy* | 224/438 aa | 98 | 0 | 98 |
| | **14** | s s R R | 1 976 849 | A(7) → A(8) | *wzy* | 74/438 aa | 100 | 0 | 98 |
| | **15** | s R R R | 1 986 619 | G(9) → G(8) | *wbpL* | 88/339 aa | 100 | 0 | 99 |
| | **18** | s I R R | 5 634 829 | C → A | *wapH* | Arg→Leu | 99 | 0 | 99 |
| | **19** | S I R R | 5 632 885 | G → C | *dnpA* | 67/472 aa | 100 | 24 [Ab09] | 74 |
| | **21** | s I R R | 1 976 849 | A(7) → A(8) | *wzy* | 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) | *mucA* | 146/194 aa | * | * | * |
| | **03** | S I S I | 4 487 654 | C(5) → C(6) | *wzz2* | 228/443 aa | 100 | 0 | 98 |
| | **06** | S I S S | 4 683 359 | T(4) → T(3) | *mucA* | 253/194 aa | 100 | 0 | 99 |
| | **10** | s s s I | 5 327 357 | A →C | *pgi* | Thr→ Pro | 99 | 0 | 99 |
| | **13** | s s S s | 4 683 943 | T → C | *mucA…algU* | - | 100 | 0 | 99 |
| | **17** | s R s R | 1 977 343 | C → A | *wzy* | 220/438 aa | 47 | 2 [Ab09] | 97 |
| Group 3 | **26** | R S S S | 5 689 432 | 19 bp | *pilQ* | 180/714 aa | 81 | 2 [Ab05] | 97 |
| Group 4 | **24** | R s s R | 1 976 849 | A(7) → A(8) | *wzy* | 74/438 aa | 40 | 24 [Ab27] | 72 |
| | | | 5 095 901 | G → C | *pilR* | Arg→ Pro | 98 | | |
| | **30** | R S I I | - | - | - | - | 0 | 40 [Ab05] | 57 |
| | **37** | R s s R | 5 103 099 | 10 bp | *pilY1* | 816/1161 aa | 67 | 3 [Ab05] | 96 |
| | | | 1 977 570 | A → G | *wzy* | Asp →Gly | 100 | | |
| Group 5 | **20** | R R R R | 5 688 665 | 555 bp | *pilQ* | 529/714 aa | 100 | 35 [Ab17] | 63 |
| | **22** | R R R R | 5 095 650 | C(2) → C(1) | *pilR* | 334/445 aa | 97 | 11 [Ab17] | 88 |
| | | | 6 005 075 | 213 bp | *algC* | 797/868 aa | 63 | | |
| | **33** | R R R R | 1 976 849 | A(7) → A(8) | *wzy* | 74/438 aa | 49 | 2 [Ab27] | 98 |
| | | | 1 976 848 | A(7) → A(6) | *wzy* | 54/438 aa | 49 | | |
| | | | 5 102 164 | 109 bp | *pilY1* | 501/1161 aa | 100 | | |
| | **35** | R R R R | 451455 | 11 bp | *pilJ* | 751/682 aa | 100 | 0 | 98 |
| | | | 1 976 849 | A(7) → A(8) | *wzy* | 74/438 aa | 100 | | |

[†] Resistance pattern is reported in order against phage Ab05, Ab09, Ab17 and Ab27
* mutation found by PCR and Sanger sequencing of the *mucA* gene
… intergenic region
/ length of the mutated protein over the wild type
( ) number of repeated nucleotide
[ ] phage found by sequencing
aa, aminoacids
S, completely susceptible
R, completely resistant

I, reduced efficiency of plating
s, normal efficiency of plating but small and turbid plaques

811 **Table 3**

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| **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 |

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## Table 4

| $PAO1_{Or}$ variant | Resistance pattern* | Sequencing method | Position on $PAO1_{Or}$ | Mutation | Mutation event | Locus tag | Protein alteration |
|---|---|---|---|---|---|---|---|
| **17_1** | I R R R | PCR candidate gene approach | 4 683 508 | C(5) → C(4) | F | *mucA* | 146/194 aa |
| | | | 1 977 343 | C → A | TV | *wzy* | 220/438 aa |
| **20_1** | R R R R | Illumina | 1 976 837 | C → T | TS | *wzy* | Ser→Phe |
| | | | 5 688 664 | 555 bp | D | *pilQ* | 529/714 aa |
| **22_1** | R R R R | Illumina | 1 976 848 | A(7) → A(8) | F | *wzy* | 74/438 aa |
| | | | 5 095 649 | C(2) → C(1) | F | *pilR* | 334/445 aa |
| **24_1** | R R R R | PCR candidate gene approach | 1 976 848 | A(7) → A(6) | F | *wzy* | 54/438 aa |
| | | | 5 095 900 | G → C | TV | *pilR* | Arg→ Pro |
| **25_1** | R S S S | Illumina | 5 096 064 | A → C | TV | *pilR* | Thr→ Pro |
| **30_1** | R S S S | PCR candidate gene approach | 5 688 968 | T → G | TV | *pilQ* | Thr→ Pro |
| **36_1** | R R R R | Illumina | 1 976 848 | A(7) → A(6) | F | *wzy* | 54/438 aa |
| | | | 5 071 804 | G → A | TS | *pilC* | Arg→ His |

* Resistance pattern is reported in order against phage Ab05, Ab09, Ab17 and Ab27
/ length of the mutated protein over the wild type
aa, aminoacids
S, completely susceptible
R, completely resistant
I, reduced efficiency of plating
F, frameshift
D, deletion
TS, transition
TV, transversion