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1 **Pseudolysogeny and sequential mutations build multiresistance to virulent bacteriophages in**

2 *Pseudomonas aeruginosa*

3

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22

23 **Abstract**

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

41 **INTRODUCTION**

42 *Pseudomonas aeruginosa* is a bacterium frequently found in the environment and often associated
43 with human infections in clinical settings. This species displays an important genome plasticity due
44 in large part to horizontal gene transfer of genomic islands and mobile elements, but also to *de novo*
45 mutations (Spencer *et al.*, 2003). Bacteriophages are key actors in diversification of *P. aeruginosa*
46 by selecting for resistant mutants, and in turn adapting to new bacterial genotypes in a coevolution

47 arm race (Brockhurst *et al.*, 2005; Dennehy, 2012). A large variety of *P. aeruginosa* bacteriophages
48 has been isolated, some showing a wide host range. However, several studies have illustrated that
49 from 6 to 10% of genetically different clinical *P. aeruginosa* strains were not lysed by currently
50 known phages (Essoh *et al.*, 2013). Spontaneous mutations responsible for phage resistance are
51 frequently related to alterations in the phage receptor (Hyman & Abedon, 2010; Labrie *et al.*, 2010).
52 In *P. aeruginosa*, adsorption mutants are principally affected in type IV pili, often used as receptors
53 by podoviruses (Φ -KMV) (Chibeu *et al.*, 2009) and siphoviruses, or in lipopolysaccharide (LPS), a
54 major virulence factor (King *et al.*, 2009; Lam *et al.*, 2011) involved in the binding of myoviruses.
55 We and others showed that large chromosomal deletions could be selected in bacteria resistant to
56 single or multiple phages. Such deletions encompassed genes involved in fimbriae, outer membrane
57 proteins or LPS components (Latino *et al.*, 2014; Le *et al.*, 2014; Tanji *et al.*, 2008). Resistance to
58 phages due to modified type IV pili has consequences on bacterial motility by affecting twitching, a
59 form of solid surface translocation (Chiang & Burrows, 2003). Type IV pili allow the adherence of
60 *P. aeruginosa* cells to the host epithelium, and also play a role in biofilm formation (Bucior *et al.*,
61 2012; Klausen *et al.*, 2003; O'Toole & Kolter, 1998). The mechanism of resistance associated with
62 the loss of pili may drive an increase in *P. aeruginosa* diversity and strongly reduce infectivity
63 (Brockhurst *et al.*, 2005; Hahn, 1997). Scanlan *et al.* showed that coevolution of phages and
64 bacteria leads to the emergence of a complex population of cells with mutations that sometimes
65 increase bacterial fitness but also constrain evolution (Scanlan *et al.*, 2015).

66 It is generally accepted that the outcome of virulent bacteriophage infection is a lytic cycle leading
67 to bacterial death, whereas temperate phages can either perform a lytic cycle or lysogenize their
68 host. Pseudolysogeny is a third state, most frequently described for temperate phages as an
69 intermediate between the lytic cycle and lysogeny, allowing the bacteria to survive infection (Ripp
70 & Miller, 1997; Ripp & Miller, 1998). Pseudolysogeny was first described as an unstable
71 interaction which is not productive and eventually resolves into true lysogeny or virulent growth

72 (Baess, 1971). Los et al. (Los *et al.*, 2003) demonstrated that *Escherichia coli* phage T4 can form
73 pseudolysogens in starved, slowly growing cells. They showed that superinfection of the host by
74 another T-even phage was responsible for lysis inhibition (LIN) (Bode, 1967) caused by the T4rI
75 gene product (Bode, 1967). Later, pseudolysogeny was defined as a stage in the bacteriophage
76 development, without multiplication of the genome, allowing subsequent restart and resumption of
77 the virus cycle (Los & Wegrzyn, 2012). In *P. aeruginosa*, pseudolysogeny was documented in
78 slowly growing cells with two phages responsible for generalized transduction, F116 a temperate
79 phage and UT1 a virulent phage (Ripp & Miller, 1997; Ripp & Miller, 1998). The role played by
80 pseudolysogeny in the emergence of bacterial mutants has not been demonstrated. Early work by
81 Demerec and Fano described mutants of *E. coli* obtained on agar medium following infection by
82 seven different phages (T1 to T7) (Demerec & Fano, 1945). The authors noted that phages were
83 present for a long time after they re-isolated resistant colonies, and finally obtained mutants
84 showing different patterns of cross-resistance. A high frequency of what were likely double-mutants
85 was observed, but the authors were not able at that time to identify the genetic changes that
86 conferred the heritable cross-resistance.

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

92 **METHODS**

93 **Bacterial strains and phages.** A single colony of *P. aeruginosa* PAO1, a reference strain
94 originating from a patient (Stover *et al.*, 2000) and propagated in the laboratory for several years,
95 was cultivated for storage at -80°C and for genome extraction and sequencing. This representative,
96 thereafter called PAO1_{Or} (where Or stands for Orsay), was used to isolate phage-resistant mutants.

97 Two podoviruses, vB_PaeP_PAO1_Ab05 (Ab05) and vB_PaeP_C2-10_Ab09 (Ab09), and two
98 myoviruses, vB_PaeM_PAO1_Ab17 (Ab17) and vB_PaeM_PAO1_Ab27 (Ab27) representing four
99 different genera were used in this study, alone, or combining a podovirus with a myovirus, or in a
100 cocktail of all four phages. These phages, isolated in Abidjan (Côte d'Ivoire), have been described
101 in detail in (Essoh *et al.*, 2015). PAO1 LPS and type IV pilus transposon mutants were obtained
102 from “The *P. aeruginosa* Transposon Mutant Library” (grant #NIH P30DK089507), UW Genome
103 Sciences, USA.

104 **Isolation of phage-resistant bacteria.** Bacteria were inoculated at a 600 nm absorbance (A_{600}) of
105 0.01 into glass vials with aeration, containing 5 ml of Luria broth (LB) medium, and grown (37°C,
106 shaking at 180 rotations per minute (rpm)) to an A_{600} of 0.2. Infections were performed at a
107 multiplicity of infection (MOI) of 0.1. Infections on solid medium used a 10 μ l inoculum of the
108 bacterial culture (2×10^6 colony forming units (CFU)) mixed with 10 μ l of a suspension, containing
109 either a single phage genera, a cocktail of two, or a cocktail of all four phages (10^5 plaque forming
110 units (PFU) for each phage). Ten μ l of SMG (saline magnesium gelatin) phage buffer (SMG
111 comprises NaCl at 5.8 g l⁻¹, MgSO₄ at 2 g l⁻¹, 1 M Tris-HCl, and gelatin at 0.1 g l⁻¹ [pH 8.0]) were
112 used in negative controls. The mixture was kept for 15 min at room temperature (RT), before being
113 poured on a fresh LB agar plate (1.5% wt/vol agar) with 4 ml of soft agar (0.7% wt/vol agar) and
114 incubated at 37°C for 3 days. Because no stable resistant variants were obtained with the solid assay
115 for phage Ab27, alone or associated with Ab05, liquid infection was also performed when using
116 Ab27. Bacteria were infected during the log phase (A_{600} of 0.6) at an MOI of 0.001 each 24 h for a
117 total of three infections. Thereafter the surviving bacteria were plated onto LB agar plates.

118 **Calculation of the frequency of resistance.** An overnight culture of *P. aeruginosa* PAO1_{Or} was
119 used to inoculate fresh medium to an A_{600} of 0.1. Bacterial cultures in the late log phase (A_{600} of
120 about 1, equivalent to 10^9 bacteria per ml, determined by titrating the bacteria), were 10-fold
121 serially diluted. One hundred μ l of each dilution were mixed with 10 μ l (about 10^6 PFU) of a single

122 phage suspension or a mixture of two or four phages as described above. The samples were kept for
123 15 min at RT and then poured on fresh LB agar plates using 4 ml of soft agar. Plates were inverted
124 and incubated at 37°C for 24 h. The frequency of resistance was calculated considering that all the
125 colonies growing on the plates after 24 h of incubation were resistant to phages used for the
126 infection. The divisor was the number of plated bacteria.

127 **Phage susceptibility assay.** Aliquots (500 µl) from the liquid culture of variants (A_{600} of 0.8 to 1.2)
128 were mixed with 6 ml of 0.7% wt/vol LB agar and poured onto a square LB 1.5% wt/vol agar plate.
129 Five dilutions (10^{10} , 10^9 , 10^8 , 10^7 , 10^6 PFU ml⁻¹) from a progenitor stock of each phage were spotted
130 (10 µl) onto the soft agar layer, incubated at 37°C overnight, and inspected for plaque formation.
131 The resistance of the mutants against the phage was expressed as EOP (efficiency of plating) using
132 PAO1_{Or} as a control.

133 **Virucide assay.** The protocol described by de Siqueira et al. (de Siqueira *et al.*, 2006) was used to
134 prepare a virucide solution from Chinese black tea leaves. The phage-containing bacteria were
135 treated for 10 min with 3 volumes of virucide, followed by centrifugation, washing with phosphate-
136 buffered-saline (PBS) and incubation at 37°C for 30 min with 50 µg ml⁻¹ DNaseI. Then total
137 bacterial DNA was purified.

138 **Adsorption assay.** An overnight bacterial culture was diluted to an A_{600} of 0.1-0.6 and left to
139 equilibrate at 37°C. Approximately 10^6 phages were added to 1 ml of the diluted bacterial culture
140 (1×10^8 to 6×10^8 bacteria). At a fixed time point, 50 µl of the mixture were transferred to a 1.5 ml
141 conical centrifuge tube containing 940 µl of LB medium and 10 µl of chloroform. The suspension
142 was vortexed for 5 sec and centrifuged in order to pellet the phages adsorbed on the bacterial
143 surface. Then, 10 µl of the unadsorbed phage suspension was titrated. Phage adsorption was
144 expressed as the percentage of the initial amount of phage employed for the infection that did not
145 adsorb to the bacterial surface after 16 min (time necessary for adsorption of the four phages).

146 **Phenotypic assays.** A planktonic culture of strain *P. aeruginosa* PAO1_{Or} prepared from a single
147 colony of a fresh LB agar plate was used as a reference in all experiments. To test for hemolytic
148 activity, 10 µl of an overnight culture of phage-resistant mutants (A_{600} of 2) were spotted onto
149 Sheep blood (5% wt/vol) agar, and plates were incubated for 24 h at 37°C. For twitching motility
150 assessment, one µl of an overnight bacterial culture (A_{600} of 2) was inoculated between the agar and
151 the plastic surface of LB 1.5% wt/vol agar plates. The diameter of the motility zone around the
152 inoculation site was measured after 24 h incubation at 37°C. Lipopolysaccharides (LPS) were
153 purified using the method of Hitchcock and Brown (Hitchcock & Brown, 1983). In order to
154 normalize the samples for the subsequent gel analysis, a similar amount of lyophilized bacteria was
155 disrupted in lysis buffer (Tris 1M, 2% SDS, 4% β-mercaptoethanol and 10% glycerol), prior to LPS
156 extraction. The LPS were resolved by electrophoresis on a 15% SDS-polyacrylamide gel, and the
157 band pattern was visualized using the silver staining method (Fomsgaard *et al.*, 1990).

158 **Biofilm formation.** 96-wells microtiter plates (Greiner) containing LB were inoculated with an
159 overnight bacterial culture (A_{595} of ≈ 0.1) and incubated at 37 °C for 48 h. Before proceeding with
160 biofilm quantification, the A_{595} was recorded. The wells were washed three times with PBS, 200 µl
161 of 0.1% wt/vol crystal violet was added and the plate was kept for 30 min at RT. The unattached
162 crystal violet was washed three times with PBS and then the remaining biomass was quantified by
163 re-suspending it into 200 µl of absolute ethanol. The A_{595} was then divided by the A_{595} value
164 measured for planktonic bacteria in each well to account for the difference in growth rates of the
165 mutants.

166 **Colony lift and hybridization.** A circular Nylon N+ membrane (Nytran) was applied on the agar
167 plate on which fifty-two colonies had been plated. After 5 min, the membrane was lifted using
168 forceps and treated successively for 2 min with NaOH 0.4 N twice, Tris 1 M pH 7.5 twice, 2X
169 Saline Sodium Citrate (SSC) twice. After this, the membrane was dried on Whatman filter paper
170 and kept at 20°C until use.

171 Pre-hybridization was performed at 65°C for 4 h with 2 ml of hybridization buffer (Church &
172 Gilbert, 1984) per membrane. The probe was labeled using the Megaprime™ kit (GE Healthcare
173 Amersham) and hybridization was performed overnight at 65°C in hybridization buffer. Washes
174 were done successively with 2XSSC and 0.1% wt/vol SDS, 0.5XSSC and 0.1% wt/vol SDS,
175 0.2XSSC and 0.1% wt/vol SDS.

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

186 **Gene cloning and expression.** PCR amplicons were cloned into the pUCP24 plasmid, a generous
187 gift of Dr. Schweizer (West *et al.*, 1994). This is a shuttle vector which replicates in *E. coli* and in
188 *P. aeruginosa*, and contains a multiple cloning site downstream lacZα. The PAO1 *mucA* gene was
189 PCR-amplified using oligonucleotides mucA_Clon_F_Bam
190 5'TGGGATCCCGAGAAGCCTGACACAGC3' and mucA_Clon_R_Hind
191 5'GAAAGCTTACCGCCATCAGGCTGCCA3', which included restriction sites for *BamHI* and
192 *HindIII*. The amplicons were digested with *BamHI* and *HindIII*, ligated into the vector similarly
193 digested and transformed into *E. coli*, in which replication of pUCP24 is optimal (West *et al.*,
194 1994). A selected recombinant was then used to transform *P. aeruginosa* strains by electroporation
195 using the fast protocol described by Choi *et al.* (Choi *et al.*, 2006). Transformants were selected

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

201 **Whole genome sequencing.** Ten µg purified bacterial DNA was sent for draft whole genome
202 Illumina sequencing to the IMAGIF platform (CNRS, Gif sur Yvette, France). Libraries were made
203 from sheared fragments of DNA with a mean size of 900 bp, and 250 bp paired-end reads were
204 produced. One million up to 5 million reads were obtained corresponding to a 40-200 fold average
205 coverage. The mutations were identified by comparison with the genome of the PAO1_{Or} sequence
206 using native GeneiousR9 tools default parameters (Biomatters, New Zealand). The Geneious
207 mapper with the "Medium-Low Sensitivity/Fast" parameter option was used to map the reads of
208 each variant against the PAO1_{Or} genome. The "Find Variations/SNPs" analysis was used with the
209 parameter "Minimum Variant Frequency" set to 0.25. When a SNP or an indel was identified,
210 sequencing reads mapping in the mutated gene plus 1 kb on both sides were recovered, reassembled
211 and the contig was aligned with the PAO1_{Or} genome. This allowed the precise localisation of short
212 deletions. Mutations were confirmed by PCR amplification of the affected gene and Sanger
213 sequencing (Beckman-Cogenics).

214 *De novo* assembly of phage reads was done with GeneiousR9 native assembler using the Medium-
215 Low Sensitivity/Fast parameter.

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

220 **RESULTS**

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

222 Our goal was to evaluate the frequency and diversity of PAO1_{Or} mutants emerging from infection
223 with phages belonging to different genera, used alone or in cocktails. We hypothesized that each
224 phage may select for specific mutations. Four different virulent phages displaying various host
225 ranges (Essoh *et al.*, 2015) were used, alone or in combination of two or four. On PAO1_{Or}, Ab05
226 (Φ KMV-like phage), Ab09 (N4-like phage) and Ab17 (KPP10-like phage) produced clear plaques,
227 whereas Ab27 (PB1-like phage) produced tiny, turbid plaques. First we investigated the nature of
228 the four phages primary receptor by testing the susceptibility of two PAO1 transposon mutants,
229 affected in type IV pili (*pilA* mutant) or LPS-O antigen (*algC* mutant) synthesis genes. Ab05 was
230 not capable of growing on a type IV pilus mutant, as previously reported for most Φ KMV-like
231 phage (Ceysens *et al.*, 2011) whereas growth of Ab09, Ab17 and Ab27 was restricted on the LPS
232 defective mutant. Then we designed an experimental procedure to allow for phage amplification
233 and isolation of independent resistant mutants. For this purpose, PAO1_{Or} was infected at an MOI of
234 0.1 (one phage for ten bacteria) by single phage or cocktails, before plating the bacteria in soft agar
235 on LB solid medium. The cocktails consisted of a 1:1 mixture of Ab09 and Ab17 or Ab05 and
236 Ab27, and a 1:1:1:1 mixture of Ab09, Ab17, Ab05 and Ab27. Complete lysis was obtained in 8 h,
237 with the exception of dispersed insensitive bacteria which, after 72 h, produced colonies with
238 different morphologies (Fig. 1a). We calculated the frequency of surviving cells to be 3.2×10^{-5} for
239 single phage infection, 4×10^{-6} for double infection and 3.8×10^{-6} for multiple infections. Colonies of
240 variable shape, size and appearance were picked from seven independent experiments and were
241 purified by three re-isolation steps, in order to ensure that a pure population was obtained. A single
242 colony was recovered after the third re-isolation step (P3) and used to inoculate an overnight culture
243 which was then stored at -80°C in glycerol. This stock was later used for genomic DNA purification
244 and to perform further tests (Fig. 1a). The majority of PAO1_{Or} variants recovered after Ab05, Ab09
245 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 Hosseinidou et
249 al. (Hosseinidou *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

271 inside the bacteria or adsorbed on the surface by treating two of the variants with a virucide (tea
272 decoction) and DNaseI digestion, followed by several washings of the bacteria pellet. Phage DNA
273 was still present in large amounts in the bacteria, as shown by semi-quantitative PCR reaction (Fig.
274 S2 shows PA01-20 and PA01-32), suggesting that the phage genome was maintained in an
275 episomal state: lysogeny was not likely as these phages are believed to be strictly lytic, based on
276 their genome characteristics, and because the amount of phage DNA appears to be in large excess
277 over that of the bacterial DNA (see dedicated paragraph below). The results obtained suggest that
278 some bacterial cells might contain in the order of 100 phage genomes.

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

284 **A wide range of chromosomal mutations is selected by phages**

285 To identify *de novo* mutations, it was necessary to sequence the genome of the parental PAO1_{Or}
286 strain, prepared from the culture used to derive phage-tolerant variants. The PAO1_{Or} sequencing
287 reads were mapped against the sequence of the reference PAO1 (NC_002516) strains, allowing the
288 assembly of the full genome and identification of differences (Fig. S3 and Table S3). These
289 differences included a large inversion between rRNA sequences (positions 727 255 to 4 788 575),
290 the presence of a copy of filamentous Pfl prophage in PAO1_{Or} at position 5 242 103 to 5 254 164,
291 and 63 SNPs or short indels events. As expected, some of these differences, including the inversion
292 and the Pfl prophage plus a number of the SNPs and indels were previously reported by
293 Klockgether et al. (Klockgether *et al.*, 2010). Others were specific to the PAO1_{Or} sub line.

294 The sequencing reads from each of the 23 whole-genome sequenced phage-tolerant variants were
295 mapped against the PAO1_{Or} genome showing a uniform distribution with a mean coverage of 40-

296 200 fold and only a few places with low coverage and relatively poor quality sequence, common to
297 all variants. In variants PAO1-30, PAO1-32 and PAO1-34, no chromosomal mutation could be
298 detected. In fourteen variants a single chromosomal mutation was identified. Six variants were
299 double mutants. Coverage at these genetic alterations was always at least 40, and up to 250 fold.
300 PAO1-02, with a mucoid phenotype, was analyzed by PCR using a candidate gene approach and
301 this allowed to find a frameshift mutation in the *mucA* gene. Table 2 displays the position and
302 nature of the mutations, as well as the percentage of sequencing reads containing a particular
303 mutation. In several variants, reads corresponding to both the wild-type (WT) and mutated sequence
304 could be found, indicating that the cell population was mixed. This indicates that an unstable
305 mutation has reverted back. Three different kinds of genetic alterations were found, phase variation,
306 deletions of 10 bp or more, and nucleotide substitutions. Fourteen genes were affected. The *wzy*
307 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)
308 to G(5) at position 1 977 338, were found in six, one and other two mutants, respectively. Seven
309 different frameshift mutations due to a single nucleotide insertion or deletion in homopolymer tracts
310 resulted, in six cases, in early termination of protein synthesis. In the last case, PAO1-06, deletion
311 of a T in a stretch of four Ts in the *mucA* gene suppressed normal termination of translation
312 resulting in the production of a longer MucA protein fused with the beginning of MucB. Deletions
313 were found in three type IV pili structural genes, *pilY1*, *pilQ* and *pilJ*: *pilY1* was missing ten bp in
314 PAO1-37 and 109 bp in PAO1-33, *pilQ* was missing 19 bp in PAO1-26 and 555 bp in PAO1-20,
315 whereas *pilJ* was missing 11 bp in PAO1-35. A 213 bp deletion was detected in the PAO1-22 *algC*
316 gene. The deletions either caused a frameshift and the creation of a premature stop codon, or deleted
317 an internal domain. Eight variants showed a single nucleotide substitution. All mutations were
318 confirmed by PCR amplification and Sanger sequencing.

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

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

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

335 To confirm that the observed mutations were responsible for affecting the bacteriophage receptor,
336 we investigated the phenotype of the three classes of mutants affected in type IV pilus, LPS and
337 alginate biosynthesis. The motility of the variants was evaluated by performing a twitching assay on
338 semisolid agar. Compared to the PAO1_{Or} control, the diameter of the twitching zone was
339 significantly reduced in all variants, but the strongest effect was observed with those bearing a
340 mutation in Pil genes and/or resisting Ab05 infection (Fig. 3). PAO1-32 and PAO1-34 were also
341 affected in twitching although no Pil mutations could be observed, but this was likely related to a
342 continuous cell death due to phage production. Indeed upon culture in LB broth the cells lysed
343 totally after reaching an absorbance at 600 nm (A_{600}) of 0.8. Inhibition of twitching was
344 accompanied by a decrease in biofilm formation, except for PAO1-20 and PAO1-06 (Fig. 4). This
345 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 μ l of some bacterial strains from the frozen P3 stock were
391 spread on LB agar (P3₀). 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

396 pseudolysogenic cells contained in a single colony varies from 4 up to 100 percent. In PAO1-30 the
397 pseudolysogenic state was observed up to 10 replatings (Table 3). Hybridization with phage DNA
398 probes in a colony lift experiment confirmed that bacteria not releasing phages were devoid of
399 phage DNA, thus excluding the presence of colonies able to maintain phage DNA without releasing
400 functional phage particles (data not shown).

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

407 **Continuous evolution of bacteria from pseudolysogens**

408 A mixture of bacterial WT and mutant reads was clearly observed in PAO1-17, -22, -24, -26, -33, -
409 37, after three purification steps, always accompanied by phage DNA (Table 2). This, added to the
410 high frequency of double mutants, suggested that immunity provided by the phage in a
411 pseudolysogenic state allowed survival and subsequent emergence of mutations. To investigate this
412 hypothesis, we tested whether new mutations would appear in response to the pressure imposed by
413 phages. We went back to the -80°C stocks of seven variants (P3), isolated new colonies, replated
414 them and tested for the presence of phage DNA by PCR, until a colony devoid of phage DNA was
415 obtained (Table 4). Susceptibility to the four phages was evaluated in these cured colonies, and the
416 mutations previously identified by whole genome sequencing were searched by PCR and Sanger
417 sequencing. Different situations existed when phage DNA was no longer present. The Group 3
418 PAO1-26 variant *pilQ* microdeletion was found in about two thirds of the colonies re-isolated after -
419 80°C storage, and it was associated with resistance to Ab05. Similarly, upon re-isolation of PAO1-
420 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.

446 In summary, it appeared that pseudolysogenic colonies continuously evolved due to the production
447 of new functional phage particles that selected for new phage-resistant variants. Eventually, all
448 variants possessed mutations in one of the pilus type IV assembly genes, and, as expected, the
449 ability of phages to adsorb on their surface (Fig. S6) and the twitching motility of these variants
450 were defective when compared to the control PAO1_{Or} (Fig. S7).

451 **DISCUSSION**

452 **Pseudolysogeny is a major factor in selection of mutants**

453 In our experimental model, pseudolysogeny appears to be a frequent outcome of infection by the
454 four virulent phages, providing immunity to the bacteria, and allowing emergence of mutations in
455 genes involved in receptor synthesis. In the present investigation, we might even underestimate the
456 frequency of pseudolysogeny as we started the analyses after three replatings for purification
457 purposes. The frequency of single mutants was on the order of one per 10⁵ plated bacteria but,
458 surprisingly, we observed that double mutants could be recovered at a frequency of 10⁻⁶, which is
459 far higher than expected if these were present at the onset of infection. We show that selection of a
460 second mutation takes place in pseudolysogenic colonies that can constitute a reservoir for
461 bacteriophages exerting a permanent pressure on the bacteria. Many controlled studies have
462 demonstrated the role of starvation and slow growth in the establishment of pseudolysogeny. In
463 contrast, pseudolysogeny in rich medium is not understood (Los & Wegrzyn, 2012; Ripp & Miller,
464 1998). Being in the inner part of a colony might mimic starvation and slow growth conditions,
465 whereas cells in direct contact with the agar medium would be in a rich medium context.

466 We observe that pseudolysogeny is established in a situation when the large majority of bacteria has
467 been lysed and high amounts of phages are present, thus resembling the LIN control observed in T4.
468 We propose a model in which a pseudolysogenic cell, which may contain more than 100 phage
469 genome copies according to the phage burst size, forms, after several rounds of division, a colony
470 containing bacteria cured of the phage and bacteria in which the phage lytic cycle is resumed,

471 producing new phages (Fig. 7). The cured bacteria become prey for further amplification and
472 production of new pseudolysogens in which phage growth is stalled. This interaction between
473 phages and bacteria is reminiscent of the carrier state life cycle (CSLC) observed in different
474 systems (Siringan *et al.*, 2014). However, in the present study the phage/host equilibrium is not
475 stable. The appearance of pseudolysogenic cells could occur when the amount of bacteriophages
476 and resistant mutants is higher than the total amount of WT susceptible bacteria allowing
477 bacteriophages to be protected against extinction. The relative efficiency of reactivation of the
478 phage cycle and production of cured bacteria determines the duration of the pseudolysogeny stage.
479 It will be interesting to perform *in situ* analyses to check whether the colony is a homogenous
480 population of cells or if there are sectors in which phage activation is favored, and to follow
481 fluctuations of free phage concentrations within a single colony.

482 A lack of immunity to superinfection, mediated by immunity genes in temperate phages is supposed
483 to differentiate true lysogeny from pseudolysogeny (Wommack & Colwell, 2000). The present
484 pseudolysogens demonstrate inhibition of superinfection by the same phage and, more interestingly,
485 by phages of different genera, which bind to different receptors. Immunity genes have been found
486 in *E. coli* T4 (*imm*) (Lu & Henning, 1989) and P1 (*sim*) (Maillou & Dreiseikelmann, 1990)
487 bacteriophages. The genes appear to be involved in the successful injection of phage DNA into the
488 cell. This mechanism could account for inhibition of phage infection by phages using different
489 receptors, but there is no evidence of such genes in our phages at the current time. Further
490 experiments are needed to understand at which stage phage multiplication is inhibited.

491 **Red Queen dynamics/arm race coevolution**

492 Studies performed in chemostats have addressed the coevolution dynamics of phage and bacteria in
493 controlled growth conditions (Betts *et al.*, 2014; Buckling & Rainey, 2002). In some assays where
494 prey and predators are left to evolve for a long time two possible outcomes were described. In the
495 Arms race, the fittest genotype survives and this limits the diversity, whereas in the Red Queen

496 dynamics, frequency-dependent selection leads to constant production of new mutants, thus
497 maintaining diversity (Dennehy, 2012). In our assay, which takes place in a micro community, after
498 several rounds of coevolution, the population of free phages fluctuates, to the extent that they may
499 seem to almost disappear within the colony. A large diversity of resistant mutants is selected, and
500 eventually the colony will be phage-free. Reversion to WT phenotype is observed for alginate and
501 LPS mutants so that new preys will emerge. We observed, with three phages, the presence of new
502 phage genotypes in pseudolysogens, all three showing one or two SNPs in a tail fiber gene. The
503 mutations were present in a subpopulation of phages used to derive the resistant mutants, and may
504 have been selected during coevolution of phages and bacteria. No particular behavior of these
505 phages as compared to the parental ones could be demonstrated, such as plaque morphology, and
506 growth characteristics. However it is possible that these phages are capable of inducing a
507 pseudolysogenic stage at a higher frequency as compared to the ancestral phage. Our results
508 confirm that success in infection is not sufficient for phage survival, as phages are dependent upon
509 the survival of their host population (Chaturongakul & Ounjai, 2014), and therefore phage-host
510 relationships can be seen as not merely parasitic but as mutualistic (Williams, 2013).

511 **Cross-resistance and reversibility of mutants**

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

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

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

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

542 A total of 25 components are involved in the type IV pilus biogenesis (Kim *et al.*, 2006). In the
543 present small scale investigation we observed ten mutations affecting five genes. Half of the
544 mutations are irreversible deletions which contrast with the high frequency of reversible phase
545 variation mutations in LPS. This suggests that the fitness cost of such mutants would rapidly lead to

546 their elimination, and that phages using type IV pili as receptors should be favored for phage
547 therapy. Several studies have investigated the effect of type IV pilus mutations and phage
548 resistance. Interestingly, phage F6, a dsRNA cystovirus of *Pseudomonas syringae* pathovar
549 *phaseolicola* selects for several types of mutants that differ in the number of type IV pili expressed
550 per cell, but none of the mutated genes were known to be directly involved in type IV pilus
551 expression (Sistrom *et al.*, 2015).

552 Phage therapy is considered as a promising approach to fight against antibiotic resistant strains
553 (Abedon *et al.*, 2011). Either readymade cocktails or "sur- measure" phages will be used to treat
554 patients, similarly to what is still done in several countries of Eastern Europe. It is important to
555 investigate the risks linked to the use of phages, particularly in the selection of bacterial mutants
556 that could show deleterious characteristics (Hosseinidoust *et al.*, 2013b), or drive the expression of
557 undesirable bacterial virulence factors (Olszak *et al.*, 2015). In a mouse model of *E. coli* gut
558 infection, it was proposed that virulent phages remained inside bacteria in a pseudolysogenic state,
559 therefore becoming resistant to degradation and allowing persistence of bacteria (Maura &
560 Debarbieux, 2012; Maura *et al.*, 2012). It would be interesting to know whether new variants
561 emerge in such experiments. On the other hand, some phages driving evolution toward loss of
562 virulence could be favored if they exist (Leon & Bastias, 2015). Another concern is the potential
563 role of bacteriophages in horizontal transfer, which could be favored by the long-term maintenance
564 of phage genomes inside the bacteria during pseudolysogeny. Additional experiments are needed to
565 further investigate the fate of the phages and bacteria in a pseudolysogen interaction.

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744 **Table and figure legends**

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

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

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

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

752

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

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

759 **Fig. 3: Twitching motility of original (P3) phage-tolerant variants.** Bacterial motility is
760 expressed as the diameter (mm) of the growth zone at the bottom of the agar plate. The standard
761 deviation is the result of three independent assays.

762 **Fig. 4: Biofilm formation assay of original (P3) phage-tolerant variants.** The amount of bacteria
763 bound to the wells is evaluated by measuring the A₅₉₅ of crystal violet resuspended in ethanol. The
764 standard deviation is the result of three independent assays.

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

768 **Fig. 6: Evaluation of pseudolysogeny persistence in PAO1-30.** Fifty-two colonies recovered from
769 the P3 stock (left panel) were simultaneously plated (small horizontal arrows) using a sterilized
770 pipette tip on LB agar (center panel) and on PAO1_{Or} embedded in soft agar overlay (right panel).
771 The clear zone around the bacterial colony in the right panel is due to phage lysis of the indicator
772 bacteria. The percentage of colonies producing phages (pseudolysogens) is indicated on the right
773 side. In red is circled the colony that was chosen and replated at each re-isolation step (in this case
774 from P3₀ to P3₄) because of its ability to release phage-particles and lyse PAO1_{Or}).

775 **Fig. 7: Model of pseudolysogeny evolution.** Continuous production of cured bacteria and release
776 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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	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 variant788 [‡] 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

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 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>pilYI</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
			1 976 848	A(7) → A(6)	<i>wzy</i>	54/438 aa	49		
	35	R R R R	5 102 164	109 bp	<i>pilYI</i>	501/1161 aa	100	0	98
451455			11 bp	<i>pilJ</i>	751/682 aa	100			
			1 976 849	A(7) → A(8)	<i>wzy</i>	74/438 aa	100		

797 [†] Resistance pattern is reported in order against phage Ab05, Ab09, Ab17 and Ab27798 * mutation found by PCR and Sanger sequencing of the *mucA* gene

799 ... intergenic region

800 / length of the mutated protein over the wild type

801 () number of repeated nucleotide

802 [] phage found by sequencing

803 aa, aminoacids

804 S, completely susceptible

805 R, completely resistant

806 I, reduced efficiency of plating
807 s, normal efficiency of plating but small and turbid plaques
808

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811 **Table 3**

812

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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816 **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	<i>mucA</i>	146/194 aa
			1 977 343	C → A	TV	<i>wzy</i>	220/438 aa
20_1	R R R R	Illumina	1 976 837	C → T	TS	<i>wzy</i>	Ser→Phe
			5 688 664	555 bp	D	<i>pilQ</i>	529/714 aa
22_1	R R R R	Illumina	1 976 848	A(7) → A(8)	F	<i>wzy</i>	74/438 aa
			5 095 649	C(2) → C(1)	F	<i>pilR</i>	334/445 aa
24_1	R R R R	PCR candidate gene approach	1 976 848	A(7) → A(6)	F	<i>wzy</i>	54/438 aa
			5 095 900	G → C	TV	<i>pilR</i>	Arg→Pro
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 candidate gene approach	5 688 968	T → G	TV	<i>pilQ</i>	Thr→Pro
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

817 * Resistance pattern is reported in order against phage Ab05, Ab09, Ab17 and Ab27

818 / length of the mutated protein over the wild type

819 aa, aminoacids

820 S, completely susceptible

821 R, completely resistant

822 I, reduced efficiency of plating

823 F, frameshift

824 D, deletion

825 TS, transition

826 TV, transversion

827

828