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

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Pseudolysogeny and sequential mutations build multiresistance to virulent bacteriophages in *Pseudomonas aeruginosa* (PAO1 strain representative PAO1 Or is LN871187. All sequence reads have been deposited in the European Nucleotide Archive (ENA) project number PRJEB9838. Available with the online Supplementary Material. Three supplementary tables and seven supplementary figures are provided on the paper's website. For more information, please visit www.microbiologyresearch.org.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a bacterium frequently found in the environment and often associated with human infections in clinical settings. This species displays an important genome plasticity due in large part to horizontal gene transfer of genomic islands and mobile elements, but also to *de novo* mutations (Spencer et al., 2003). Bacteriophages (phages) are key actors in diversification of *P. aeruginosa* by selecting for resistant mutants and, in turn, adapting to new bacterial genotypes in a coevolution arms race (Brockhurst et al., 2005; Dennehy, 2012). A large variety of *P. aeruginosa* phages have been isolated, some showing a wide host range. However, several studies have illustrated that 6–10% of genetically different clinical *P. aeruginosa* strains were 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 pilus, alginate, LPS and O-antigen. Half of the variants possessed changes in homopolymer tracts responsible for frameshift mutations and these phase variation mutants were shown to be unstable. Eleven double mutants were detected. The presence of free phage DNA was observed in association with exclusion of superinfection in half of the variants and no chromosomal mutation could be found in three of them. Upon further growth of these pseudolysogens, some variants with new chromosomal mutations were recovered, presumably due to continuous evolutionary pressure.

**Coevolution between bacteriophages (phages) and their prey is the result of mutualistic interactions. Here, we show that pseudolysogeny is a frequent outcome of infection by virulent phages of *Pseudomonas aeruginosa* and that selection of resistant bacterial mutants is favoured by continuous production of phages. We investigated the frequency and characteristics of *P. aeruginosa* strain PA01 variants resisting infection by different combinations of virulent phages belonging to four genera. The frequency of resistant bacteria was $10^{-6}$ for single phage infection and $10^{-8}$ 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 PA01 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 possessing changed in homopolymer tracts responsible for frameshift mutations and these phase variation mutants were shown to be unstable. Eleven double mutants were detected. The presence of free phage DNA was observed in association with exclusion of superinfection in half of the variants and no chromosomal mutation could be found in three of them. Upon further growth of these pseudolysogens, some variants with new chromosomal mutations were recovered, presumably due to continuous evolutionary pressure.**

**The GenBank/EMBL/DDBJ accession number for the DNA sequence of the PA01 strain representative PA01 Or is LN871187. All sequence reads have been deposited in the European Nucleotide Archive (ENA) project number PRJEB9838.**

**Three supplementary tables and seven supplementary figures are available with the online Supplementary Material.**
(Brockhurst et al., 2005; Hahn, 1997). Scanlan et al. (2015) showed that coevolution of phages and bacteria leads to the emergence of a complex population of cells with mutations that sometimes increase bacterial fitness but also constrain evolution.

It is generally accepted that the outcome of virulent phage infection is a lytic cycle leading to bacterial death, whereas temperate phages can either perform a lytic cycle or lysogenize their host. Pseudolysogeny is a third state, most frequently described for temperate phages as an intermediate between the lytic cycle and lysogeny, allowing the bacteria to survive infection (Ripp & Miller, 1997, 1998). Pseudolysogeny was first described as an unstable interaction that is not productive and eventually resolves into true lysogeny or virulent growth (Baess, 1971). Los et al. (2003) demonstrated that Escherichia coli phage T4 can form pseudolysogens in starved, slowly growing cells. They showed that superinfection of the host by another T-even phage was responsible for lysis inhibition (Bode, 1967) caused by the T4Rl gene product. Later, pseudolysogeny was defined as a stage in the phage development, without multiplication of the genome, allowing subsequent restart and resumption of the virus cycle (Los & Wegzyn, 2012). In P. aeruginosa, pseudolysogeny was documented in slowly growing cells with two phages responsible for generalized transduction: F116 (a temperate phage) and UT1 (a virulent phage) (Ripp & Miller, 1997, 1998). The role played by pseudolysogeny in the emergence of bacterial mutants has not been demonstrated. Early work by Demerec & Fano (1945) described mutants of E. coli obtained on agar medium following infection by seven different phages (T1–T7). The authors noted that phages were present for a long time after they reisolated resistant colonies and finally obtained mutants showing different patterns of cross-resistance. A high frequency of what were likely double mutants was observed, but the authors were not able at that time to identify the genetic changes that conferred the heritable cross-resistance.

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

**METHODS**

**Bacterial strains and phages.** A single colony of P. aeruginosa PA01, a reference strain originating from a patient (Stover et al., 2000) and propagated in the laboratory for several years, was isolated in Abidjan (Côte d’Ivoire), have been described in detail in Essoh et al. (2015). PA01 LPS and type IV pilus transposon mutants were obtained from the P. aeruginosa Mutant Library (http://www.gs.washington.edu/labs/manoii/libraryindex.htm).

**Isolation of phage-resistant bacteria.** Bacteria were inoculated at OD600 0.01 into glass vials containing 5 ml Luria broth (LB) medium, and grown with aereration (37°C with shaking at 180 r.p.m.) to OD600 0.2. Infections were performed at m.o.i. 0.1. Infections on solid medium used a 10 µl inoculum of the bacterial culture (2 x 10⁸ c.f.u.) mixed with 10 µl suspension containing either a single phage genus, a cocktail of two phages or a cocktail of all four phages (10⁶ p.f.u. for each phage). An aliquot of 10 µl SMG (saline magnesium gelatin) phage buffer (5.8 g NaCl l⁻¹, 2 g MgSO₄ l⁻¹, 2 mL Tris/HCl and 0.1 g gelatin l⁻¹, pH 8.0) was used in negative controls. The mixture was kept for 15 min at room temperature, before being poured on a fresh LB agar plate (1.5 % (w/v) agar) with 4 ml soft agar (0.7 % (w/v) agar) and incubated at 37 °C for 3 days. As no stable resistant variants were obtained with the solid assay for phage Ab27, alone or associated with Ab05, liquid infection was also performed when using Ab27. Bacteria were infected during the exponential phase (OD600 0.6) at m.o.i. 0.001 each 24 h for a total of three infections. Thereafter the surviving bacteria were plated onto LB agar plates.

**Calculation of the frequency of resistance.** An overnight culture of P. aeruginosa PA01Or, was used to inoculate fresh medium to OD600 ~1, equivalent to 10⁶ bacteria ml⁻¹, determined by titrating the bacteria) were 10-fold serially diluted. Aliquots of 100 μl of each dilution were mixed with 10 μl (~10⁶ p.f.u.) of a single phage suspension or a mixture of two or four phages as described above. The samples were kept for 15 min at room temperature and then poured on fresh LB agar plates using 4 ml soft agar. Plates were inverted and incubated at 37 °C for 24 h. The frequency of resistance was calculated considering that all the colonies growing on the plates after 24 h of incubation were resistant to phages used for the infection. The divisor was the number of plated bacteria.

**Phage susceptibility assay.** Aliquots (500 μl) from the liquid culture of variants (OD600 0.8–1.2) were mixed with 6 mL 0.7 % (w/v) LB agar and poured onto a square LB 1.5 % (w/v) agar plate. Five dilutions (10⁻⁰, 10⁻¹, 10⁻², 10⁻³ and 10⁻⁶ p.f.u. mL⁻¹) from a progenitor stock of each phage were spotted (10 μl) onto the soft agar layer, incubated at 37 °C overnight and inspected for plaque formation. The resistance of the variants against the phage was expressed as the efficiency of plating using PA01Or as a control.

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

**Adsorption assay.** An overnight bacterial culture was diluted to OD600 0.1–0.6 and left to equilibrate at 37 °C. Approximately 10⁶ phages were added to 1 ml diluted bacterial culture (1·6·10⁶ bacteria). At a fixed time point, 50 μl mixture was transferred to a 1.5 ml conical centrifuge tube containing 940 μL LB medium and 10 μl chloroform. The suspension was vortexed for 3 s and centrifuged in order to pellet the phages adsorbed on the bacterial surface. Then, 10 μl unadsorbed phage suspension was titrated. Phage adsorption was expressed as the percentage of the initial amount of phage employed for the infection that did not adsorb to the bacterial surface after 16 min (time necessary for adsorption of the four phages).
Phenotypic assays. A planktonic culture of strain *P. aeruginosa* PAO1, prepared from a single colony off a fresh LB agar plate, was used as a reference in all experiments. To test for haemolytic activity, 10 μl overnight culture of phage-resistant mutants (OD₆₀₀ 2) was spotted onto sheep blood [5 % (w/v)] agar and plates were incubated for 24 h at 37 °C. For twitching motility assessment, 1 μl overnight bacterial culture (OD₆₀₀ 2) was inoculated between the agar and the plastic surface of LB 1.5 % (w/v) agar plates. The diameter of the motility zone around the inoculation site was measured after 24 h incubation at 37 °C. LPS was purified using the method of Hitchcock & Brown (1983). In order to normalize the samples for the subsequent gel analysis, a similar amount of lyophilized bacteria was disrupted in lysis buffer (1 M Tris, 2 % SDS, 4 % β-mercaptoethanol and 10 % glycerol) prior to LPS extraction. The LPSs were resolved by electrophoresis on a 15 % SDS-polyacrylamide gel and the band pattern was visualized using the silver staining method (Fomsgaard et al., 1990).

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

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

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

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

Gene cloning and expression. PCR amplicons were cloned into the pUCP24 plasmid, a generous gift of Dr Schweizer (West et al., 1994). This is a shuttle vector which replicates in *E. coli* and in *P. aeruginosa*, and contains a multiple cloning site downstream from lacZα. The PAO1 mucA gene was PCR-amplified using oligonucleotides mucA_Clone_F_Bam (5’-TGGGATCCCGAGAAGCCTGACACG-3’) and mucA_Clone_R_Hind (5’-GAAAGCTTACGGCAATCCGGCTGCCA-3’), which included restriction sites for BamHI and HindIII. The amplicons were digested with BamHI/HindIII, ligated into the similarly digested vector and transformed into *E. coli*, in which replication of pUCP24 is optimal (West et al., 1994). A selected recombinant was then used to transform *P. aeruginosa* strains by electroporation using the fast protocol described by Choi et al. (2006). Transformants were selected using 10 μg gentamicin ml⁻¹, and the presence of the plasmid was verified by PCR amplification using a mucA forward oligonucleotide mucA_int_F (5’-AGCAGGATAGCTCGGAGAC-3’) and a plasmid reverse oligonucleotide pUCP24_MCS_R (5’-GGCCTCCATTGCATTACGGC-3’). The colony aspect was observed under the stereomicroscope. The transformants were then tested for their susceptibility to the four phages.

Whole-genome sequencing. Purified bacterial DNA (10 μg) was sent for draft whole-genome Illumina sequencing to the IMAGIF platform (CNRS, Gif-sur-Yvette, France). Libraries were made from sheared fragments of DNA with a mean size of 900 bp and paired-end reads of 250 bp were produced. Between 1 and 5 million reads were obtained corresponding to a 40- to 200-fold mean coverage. The mutations were identified by comparison with the genome of the PAO1Or sequence using native Geneious R9 tools default parameters (Biomatters). The Geneious mapper with the ‘Medium-Low Sensitivity/Fast’ parameter option was used to map the reads of each variant against the PAO1Or genome. The ‘Find Variations/SNPs’ analysis was used with the parameter ‘Minimum Variant Frequency’ set to 0.25. When an SNP or an indel was identified, sequencing reads mapping in the mutated gene plus 1 kb on both sides were recovered, reassembled and the contig was aligned with the PAO1Or genome. This allowed the precise localization of short deletions. Mutations were confirmed by PCR amplification of the affected gene and Sanger sequencing (Beckman-Cogenics).

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

RESULTS

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

Our goal was to evaluate the frequency and diversity of PAO1Or mutants emerging from infection with phages belonging to different genera, used alone or in cocktails. We hypothesized that each phage may select for specific mutations. Four different virulent phages displaying various host ranges (Essoh et al., 2015) were used, alone or in combinations of two or four. On PAO1Or, Ab05 (ϕKMV-like phage), Ab09 (N4-like phage) and Ab17 (KPP10-like phage) produced clear plaques, whereas Ab27 (PB1-like phage) produced tiny, turbid plaques. First, we investigated the nature of the primary receptor of the four phages by testing the susceptibility of two PAO1 transposon mutants, affected in type IV pili (pilA mutant) or LPS O-antigen (algC mutant) synthesis genes. Ab05 was not capable of growing on a type IV pilus mutant, as previously reported for most ϕKMV-like phages (Ceyssens et al., 2011), whereas growth of Ab09, Ab17 and Ab27 was restricted on the LPS-defective mutant. We then designed an experimental procedure to...
allow for phage amplification and isolation of independent resistant mutants. For this purpose, PAO1Or was infected at m.o.i. 0.1 (one phage for 10 bacteria) by single phage or cocktails, before plating the bacteria in soft agar on LB solid medium. The cocktails consisted of a 1:1 mixture of Ab09 and Ab17 or Ab05 and Ab27, and a 1:1:1:1 mixture of Ab09, Ab17, Ab05 and Ab27. Complete lysis was obtained in 8 h, with the exception of dispersed insensitive bacteria which, after 72 h, produced colonies with different morphologies (Fig. 1a). We calculated the frequency of surviving cells to be $3.2 \times 10^{-5}$ for single phage infection, $4 \times 10^{-6}$ for double infection and $3.8 \times 10^{-6}$ for multiple infection. Colonies of variable shape, size and appearance were picked from seven independent experiments and were purified by three reisolation steps, in order to ensure that a pure population was obtained. A single colony was recovered after the third reisolation step (P3) and used to inoculate an overnight culture which was then stored at $-80 \, ^\circ C$ in glycerol. This stock was later used for genomic DNA purification and to perform further tests (Fig. 1a). The majority of PAO1Or variants recovered after Ab05, Ab09 and Ab17 single or multiple infections were ‘tolerant’ to at least one phage. Tolerance was defined as the capacity to survive the phage infection, whether this was due to a lack of receptor or to any other mechanism. In contrast, none of the surviving bacteria recovered from infection with Ab27 alone turned out to be stably tolerant to Ab27. A similar observation was made by Hosseinidoust et al. (2013a) who failed to isolate bacteria resistant to phage E79, another PB1-like phage.

In total, 32 PAO1Or variants were retained and tentatively distributed into five groups according to their phage susceptibility pattern, evaluated by the efficiency of plating (Tables 1 and S2). The 13 Group 1 variants displayed normal susceptibility only to phage Ab05. The six Group 2 variants showed intermediate susceptibility patterns to the different phages. The five Group 3 variants were resistant only to phage Ab05. The four Group 4 variants displayed full resistance to phages Ab05 and Ab27, and reduced susceptibility to phages Ab09 and Ab17, characterized by the production of small plaques instead of large, clear plaques. Four variants resisting all four phages constituted Group 5. In Group 2, a mucoid phenotype was stably observed for PAO1-02, PAO1-06 and PAO1-13, whereas PAO1-17 continuously produced two types of colonies on solid LB media, some with a smooth appearance as for the control PAO1Or strain and others surrounded by an irregular transparent edge (Fig. 1b). This phenotype

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**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 $-80 \, ^\circ C$. (b) Colony morphotype of PAO1Or, PAO1-06 and PAO1-17.
**Table 1.** Clustering of phage-tolerant variants according to their resistance pattern against the four phages used in the present study

<table>
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<th>Phage resistance*</th>
<th>Phage DNA†</th>
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*S, Completely susceptible; R, completely resistant; I, reduced efficiency of plating; s, normal efficiency of plating but small and turbid plaques.

†Detection by PCR.

§Infection performed with all four phages.

§Mucoid variant.

may be related to an observed decrease in swarming capacity (data not shown). In addition, mucoid colonies appeared after several days of growth. When replated, the PAO1-17 colonies surrounded by a transparent edge again produced both types of colonies, whereas the others stably maintained their phenotype. Growth on sheep blood agar plates showed the existence of new phenotypic characteristics for some variants as compared with the parental PAO1Or strain (Fig. S1). Variants PAO1-02, PAO1-13 and PAO1-17 lacked the haemolytic ability displayed by the WT PAO1Or but, interestingly, the colonies of PAO1-13 presented zones of reversion to the WT phenotype.

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

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

### A wide range of chromosomal mutations is selected by phages

To identify de novo mutations, it was necessary to sequence the genome of the parental PAO1Or strain, prepared from the culture used to derive phage-tolerant variants. The PAO1Or sequencing reads were mapped against the sequence of the reference PAO1 (GenBank accession number NC_002516), allowing the assembly of the full genome and identification of differences (Fig. S3 and Table S3). These differences included a large inversion between rRNA sequences (position 727253–4788575), the presence of a copy of filamentous Pf1 prophage in PAO1Or at position 5242103–5254164 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. (2010). Others were specific to the PAO1Or subline.

The sequencing reads from each of the 23 whole-genome sequenced phage-tolerant variants were mapped against the PAO1Or genome, showing a uniform distribution with a mean coverage of 40- to 200-fold and only a few places with low coverage and relatively poor quality sequence, common to all variants. In variants PAO1-30, PAO1-32 and PAO1-34, no chromosomal mutation could be detected. In 14 variants, a single chromosomal mutation was identified. Six variants were double mutants.
The nucleotide substitutions. Fourteen genes were affected: phase variation, deletions of the mucA gene. Table 2 displays the position and nature of the mutations, as well as the percentage of sequencing reads containing a particular mutation. In several variants, reads corresponding to both the WT and mutated sequence could be found, indicating that the cell population was mixed. This indicated that an unstable mutation had reverted back. Three different kinds of genetic alterations were found: phase variation, deletions of ≥10 bp and nucleotide substitutions. Fourteen genes were affected. The wzy mutations A(7)→A(8) at position 1 976 849 as well as A(7)→A(6) at the same position, and G(6)→G(5) at position 1 977 338, were found in six, one and two other mutants, respectively. Seven different frameshift mutations due to a single nucleotide insertion or deletion in homopolymer tracts resulted, in six cases, in early termination of protein synthesis. In the last case, PAO1-06, deletion of a T in a stretch of four Ts in the mucA gene suppressed normal termination of translation resulting in the production of a longer MucA protein fused with the beginning of MucB. Deletions were found in three type IV pili structural genes: pilY1, pilQ and pilJ. pilY1 was missing 10 bp in PAO1-37 and 109 bp in PAO1-33, pilQ was missing 19 bp in PAO1-26 and 555 bp in PAO1-20, whereas pilJ was missing 11 bp in PAO1-35. A 213 bp deletion was detected in the PAO1-22 algC gene. The deletions either caused a frameshift and the creation of a premature stop codon or deleted an internal domain. Eight variants showed a single nucleotide substitution. All mutations were confirmed by PCR amplification and Sanger sequencing.

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

**Observed mutations are responsible for modifying the phage receptor**

To confirm that the observed mutations were responsible for affecting the phage receptor, we investigated the phenotype of the three classes of mutants affected in type IV pilius, LPS and alginate biosynthesis. The motility of the variants was evaluated by performing a twitching assay on semisolid agar. Compared with the PAO1Or control, the diameter of the twitching zone was significantly reduced in all variants, but the strongest effect was observed with those bearing a mutation in pil genes and/or resisting Ab05 infection (Fig. 3). PAO1-32 and PAO1-34 were also affected in twitching although no pil mutations could be observed, but this was likely related to a continuous cell death due to phage production. Indeed upon culture in LB broth the cells lysed totally...
Table 2. Mutations identified in phage-tolerant variants according to their comparison with the reference PAO1<sub>Or</sub>

<table>
<thead>
<tr>
<th>Group</th>
<th>PAO1 variant</th>
<th>Phage resistance*</th>
<th>Position on PAO1&lt;sub&gt;Or&lt;/sub&gt;</th>
<th>Mutation</th>
<th>Locus tag</th>
<th>Protein alteration</th>
<th>Mutation</th>
<th>Phages</th>
<th>Bacteria</th>
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<td>1</td>
<td>01 SRRR</td>
<td>1976849</td>
<td>A(7)→A(8)&lt;sup&gt;†&lt;/sup&gt; 1977338</td>
<td>G(6)→G(5) wzy</td>
<td>74/438 aa&lt;sup&gt;‡&lt;/sup&gt;</td>
<td>4736958 100 0 99</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>04 SRRR</td>
<td>1977338</td>
<td>G(6)→G(5) wzy</td>
<td>migA</td>
<td>Arg→His</td>
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<td></td>
<td></td>
</tr>
<tr>
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<td>1977338</td>
<td>G(6)→G(5) wzy</td>
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<td>100 0 98</td>
<td></td>
<td></td>
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<tr>
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<td></td>
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<tr>
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<td>15 sRRR</td>
<td>1986619</td>
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<td></td>
<td>19 SIRR</td>
<td>5632885</td>
<td>G→C</td>
<td>dnpA</td>
<td>67/472 aa</td>
<td>100 24 [Ab09]&lt;sup&gt;§&lt;/sup&gt;</td>
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<td>1976849</td>
<td>A(7)→A(8) wzy</td>
<td>74/438 aa</td>
<td>100 0 99</td>
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<tr>
<td></td>
<td>22 ssRR</td>
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<td>73 [Ab27] 25</td>
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<td>2</td>
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<td></td>
<td>06 SISS</td>
<td>4683359</td>
<td>T(4)→T(3) mucA</td>
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<td>Thr→Pro</td>
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<td>4683943</td>
<td>T→C mucA ... algU&lt;sup&gt;¶&lt;/sup&gt;</td>
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<tr>
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<td>1977343</td>
<td>C→A</td>
<td>wzy</td>
<td>220/348 aa</td>
<td>47 2 [Ab09] 97</td>
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<td>3</td>
<td>26 RSSS</td>
<td>5689432</td>
<td>19 bp pilQ</td>
<td>180/714 aa</td>
<td>81 2 [Ab05] 97</td>
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<td>74/438 aa</td>
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<td></td>
<td>30 RssR</td>
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<td></td>
<td>37 RSII</td>
<td>5103099</td>
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<td>1977570</td>
<td>A→G</td>
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<td>555 bp pilQ</td>
<td>529/714 aa</td>
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<td>5095650</td>
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<td>334/445 aa</td>
<td>97 11 [Ab17] 88</td>
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<td>1976849</td>
<td>A(7)→A(8) wzy</td>
<td>74/438 aa</td>
<td>49 2 [Ab27] 98</td>
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<td>35 RRRR</td>
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<td>11 bp pilJ</td>
<td>501/1161 aa</td>
<td>100</td>
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<td></td>
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<tr>
<td></td>
<td>36 RRRR</td>
<td>1976849</td>
<td>A(7)→A(8) wzy</td>
<td>74/438 aa</td>
<td>100</td>
<td></td>
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</table>

*Resistance pattern is reported in order against phages Ab05, Ab09, Ab17 and Ab27. S, completely susceptible; R, completely resistant; I, reduced efficiency of plating; s, normal efficiency of plating but small and turbid plaques.
†Number of repeated nucleotides indicated in parentheses.
‡Length of the mutated protein/WT.
§Phage found by sequencing indicated in square brackets.
¶Mutation found by PCR and Sanger sequencing of the mucA gene.
¶¶Intergenic region indicated by ellipsis (…).
after reaching OD_{600} 0.8. Inhibition of twitching was accompanied by a decrease in biofilm formation, except for PAO1-20 and PAO1-06 (Fig. 4). This may be due to the existence of a mixed population of bacteria in these variants. The LPS extracted from PAO1-04 (wzy and migA double mutant) and PAO1-07 (wzy mutant) were analysed by PAGE. Fig. 5 displays the banding profiles compared with that of PAO1\textsubscript{Or}, showing absence of the A and B bands, as well as modifications in the proportion of core and core +1 bands. PAO1-04 possessed only the core +1 oligosaccharide form. In contrast, variant PAO1-07 possessed both bands in equal amounts, whereas PAO1\textsubscript{Or} had a small proportion of core +1. Absence of core oligosaccharide in PAO1-04 was likely a consequence of the mutation in \textit{migA}. Finally, to confirm that the observed \textit{mucA} mutations were responsible for the mucoid phenotype, we tested whether the mutants could be complemented by the WT gene. A full \textit{mucA} amplicon was cloned into an expression vector, which was then introduced into PAO1-02, PAO1-06 and PAO1-13. In the three cases, the transformants no longer showed a mucoid appearance, whereas the vector alone did not reverse the mucoid phenotype. In addition, the \textit{mucA} transformants recovered normal susceptibility to all phages.

**Persistence of phage DNA in pseudolysogens**

In 11 variants, phage DNA represented 2–85 % of sequencing reads. The very high proportion of phage DNA in some samples could only be explained by the presence of free phages, inside bacteria, and/or attached to cells. To confirm that phage DNA was present inside bacteria, we performed further genome sequencing of PAO1-17 and PAO1-20 at the P3 purification step, after treatment.
of the bacterial pellet with DNase I and the virucide, followed by three washing steps. The results showed that 1.6% of reads still corresponded to Ab09 in PAO1-17 and 52% to Ab17 in PAO1-20, similar to what was observed in the first sequencing analysis (Table 2). A search for hybrid reads between phage and bacteria genomes did not produce any significant result, indicating that the phage DNA was not inserted into the bacterial chromosome, and therefore we refer to these bacteria as pseudolysogens. Interestingly, the three variants in which no chromosomal mutation could be found, i.e. PAO1-30, PAO1-32 and PAO1-34, possessed large amounts of phage DNA: PAO1-30, immune to Ab05, Ab17 and Ab27, contained high levels of Ab05 DNA; PAO1-32 and PAO1-34, immune to Ab09, Ab17 and Ab27, contained Ab27 DNA. Intermediate resistance profiles observed in some variants were correlated with the existence of a mixed population of WT and mutated bacteria, and with the presence of phage DNA.

In the sequenced samples in which a high proportion of sequence reads derived from phage DNA were present, it was possible to assemble the full phage genome sequence. This led to the identification of several single nucleotide differences in tail fibre genes, as compared with the parental genotype. In three pseudolysogens obtained independently, an Ab05 tail fibre gene displayed two SNPs. By PCR and sequencing, we could also observe these SNPs in a fraction of the phages used to select for resistant bacteria (Fig. S4). Similarly, a single SNP was observed in an Ab17 tail fibre gene from variants PAO1-20 and PAO1-22, and in the ribosome-binding site of an Ab27 tail gene from variants PAO1-24, PAO1-32 and PAO1-34. This might reflect the selection of phage variants by strain PAO1Or, possibly affecting the capacity of the bacteria to resist phages. However, we could not see any differences in binding to the host or plating efficiency with these phage genotypes, as compared with the parental genotype.

Stability of the pseudolysogen state

Viable phages were released by pseudolysogens, sometimes at high titres, during overnight culture in LB medium. This suggested that a portion of the bacterial population could achieve a productive viral cycle. To evaluate the dynamics inside pseudolysogen colonies, we measured the percentage of bacteria containing phage DNA and producing viable phages, and evaluated for how long phage DNA was maintained. For this purpose, 1 μl of some bacterial strains from the frozen P3 stock was spread on LB agar (P30). In total, 52 colonies were picked and deposited successively on an LB agar plate, then on an LB agar plate covered with a lawn of soft agar containing PAO1Or (see PAO1-30 replatings as an example in Fig. 6). After incubation at 37 °C for 24 h, a lysis zone could be seen around some colonies on the lawn of PAO1Or. One such phage-producing colony from the LB agar plate was streaked onto a new LB plate and the procedure was repeated. The fraction of pseudolysogenic cells contained in a single colony varied from 4 up to 100%. In PAO1-30, the pseudolysogenic state was observed up to 10 replatings (Table 3). Hybridization with phage DNA probes in a colony lift experiment confirmed that bacteria not releasing phages were devoid of phage DNA, thus excluding the presence of colonies able to maintain phage DNA without releasing functional phage particles (data not shown).

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

Continuous evolution of bacteria from pseudolysogens

A mixture of bacterial WT and mutant reads was clearly observed in PAO1-17, PAO1-22, PAO1-24, PAO1-26,
PAO1-33 and PAO1-37, after three purification steps, always accompanied by phage DNA (Table 2). This, added to the high frequency of double mutants, suggested that immunity provided by the phage in a pseudolysogenic state allowed survival and subsequent emergence of mutations. To investigate this hypothesis, we tested whether new mutations would appear in response to the pressure imposed by phages. We went back to the −80 °C stocks of seven variants (P3), isolated new colonies, replated them and tested for the presence of phage DNA by PCR, until a colony devoid of phage DNA was obtained (Table 4). Susceptibility to the four phages was evaluated in these cured colonies, and the mutations previously identified by whole-genome sequencing were searched by PCR and Sanger sequencing. Different situations existed when phage DNA was no longer present. The Group 3 PAO1-26 variant pilQ microdeletion was found in about two-thirds of the colonies reisolated after −80 °C storage and it was associated with resistance to Ab05. Similarly, upon reisolation of PAO1-37, ~50% of colonies were stable double wzy/pilY1 mutants, devoid of Ab05 and displaying resistance to Ab05 and Ab27. In other variants, the phage susceptibility profile changed when additional colony reisolation steps were performed and new mutations could be found upon sequencing candidate genes (Table 4). In the mucoid variant PAO1-17_1 devoid of phage Ab09, a new mucA frameshift mutation (a deletion of a single C in a stretch of five Cs present in the WT strain) was identified in about half of the sequenced PCR products, resulting in superimposition of two sequencing profiles (Fig. S5a). PAO1-20_1 and PAO1-22_1 acquired additional mutations in wzy, providing resistance to LPS-dependent phages. PAO1-24_1, devoid of Ab27 DNA, was shown to resist all four phages, whereas the PAO1-24 progenitor was susceptible to both Ab09 and Ab17 (Table 1). The original pilR mutation in PAO1-24 (Table 2) was confirmed through PCR and DNA sequencing. Surprisingly, sequencing of a wzy PCR amplicon showed that the original insertion of an additional A in a stretch of seven A residues in the WT wzy gene was replaced by a deletion of one A, resulting in a frameshift and early stop. Similar to the mucA mutation in PAO1-17_1, the sequencing profile showed the superimposition of a WT and mutated profile (Fig. S5b). PAO1-25_1 and PAO1-36_1, devoid of Ab05, were sequenced, and mutations were found in pilR, wzy and pilC, respectively. All the new mutations were confirmed by Sanger sequencing of the PCR amplification products.

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

In summary, it appeared that pseudolysogenic colonies continuously evolved due to the production of new functional phage particles that selected for new phage-resistant variants. Eventually, all variants possessed mutations in one
of the pilus type IV assembly genes, and, as expected, the
ability of phages to adsorb on their surface (Fig. S6) and
the twitching motility of these variants were defective
when compared with the control PAO1Or (Fig. S7).

DISCUSSION

Pseudolysogeny is a major factor in the selection of mutants

In our experimental model, pseudolysogeny appears to be a
frequent outcome of infection by the four virulent phages,
providing immunity to the bacteria and allowing emer-
gence 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 fre-
cuency of single mutants was of the order of 10
2
plated
bacteria but, surprisingly, we observed that double mutants
could be recovered at a frequency of 10
2
, which is far
higher than expected if these were present at the onset of
infection. We show that pseudolysogenic colonies consti-
tute a reservoir for phages that exert a permanent pressure
on bacteria, leading to selection of secondary mutations.

Many controlled studies have demonstrated the role of
starvation and slow growth in the establishment of pseudo-
lysogeny. In contrast, pseudolysogeny in rich medium is
not understood (Los & Wegrzyn, 2012; Ripp & Miller,

---

**Table 3.** Percentage of phage-producing colonies during replatings of some PAO1Or variants containing phage DNA

<table>
<thead>
<tr>
<th>Replating</th>
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<th>P3₁</th>
<th>P3₂</th>
<th>P3₃</th>
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</tr>
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<td>31</td>
<td>44</td>
<td>69</td>
<td>38</td>
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<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>30</td>
<td>96</td>
<td>35</td>
<td>23</td>
<td>19</td>
<td>62</td>
<td>25</td>
<td>100</td>
<td>100</td>
<td>88</td>
<td>2</td>
<td>0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>PAO1Or variant</th>
<th>Resistance pattern*</th>
<th>Sequencing method</th>
<th>Position on PAO1Or</th>
<th>Mutation</th>
<th>Mutation event</th>
<th>Locus tag</th>
<th>Protein alteration</th>
</tr>
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<tbody>
<tr>
<td>17_1</td>
<td>IRRR</td>
<td>PCR candidate</td>
<td>4683508</td>
<td>C(5)→C(4)†</td>
<td>Frameshift</td>
<td>mucA</td>
<td>146/194 aa‡</td>
</tr>
<tr>
<td>20_1</td>
<td>RRRR</td>
<td>Illumina</td>
<td>1977343</td>
<td>C→A</td>
<td>Transversion</td>
<td>wzy</td>
<td>220/438 aa</td>
</tr>
<tr>
<td>22_1</td>
<td>RRRR</td>
<td>Illumina</td>
<td>1976837</td>
<td>C→T</td>
<td>Transition</td>
<td>wzy</td>
<td>Ser→Phe</td>
</tr>
<tr>
<td>24_1</td>
<td>RRRR</td>
<td>PCR candidate</td>
<td>5688664</td>
<td>555 bp</td>
<td>Deletion</td>
<td>pilQ</td>
<td>529/714 aa</td>
</tr>
<tr>
<td>25_1</td>
<td>RSSS</td>
<td>Illumina</td>
<td>1976848</td>
<td>A(7)→A(8)</td>
<td>Frameshift</td>
<td>pilR</td>
<td>334/443 aa</td>
</tr>
<tr>
<td>30_1</td>
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<td>PCR candidate</td>
<td>5095649</td>
<td>C(2)→C(1)</td>
<td>Frameshift</td>
<td>pilR</td>
<td>54/438 aa</td>
</tr>
<tr>
<td>36_1</td>
<td>RRRR</td>
<td>Illumina</td>
<td>1976848</td>
<td>A(7)→A(6)</td>
<td>Frameshift</td>
<td>pilR</td>
<td>Arg→Pro</td>
</tr>
<tr>
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<td>PCR candidate</td>
<td>5095900</td>
<td>G→C</td>
<td>Transversion</td>
<td>pilR</td>
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<td>Frameshift</td>
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<td>Transition</td>
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<td>54/438 aa</td>
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</table>

*Resistance pattern is reported in order against phages Ab05, Ab09, Ab17 and Ab27. S, Completely susceptible; R, completely resistant; I, reduced efficiency of plating.
†Number of repeated nucleotides indicated in parentheses.
‡Length of the mutated protein/WT.
1998). Being in the inner part of a colony might mimic starvation and slow growth conditions, whereas cells in direct contact with the agar medium would be in a rich medium context.

We observe that pseudolysogeny is established in a situation where the large majority of bacteria have been lysed and high amounts of phages are present, thus resembling the lysis inhibition control observed in T4. We propose a model in which a pseudolyticogenic cell, which may contain >100 phage genome copies according to the phage burst size, forms, after several rounds of division, a colony containing bacteria cured of the phage and bacteria in which the phage lytic cycle is resumed, producing new phages (Fig. 7). The cured bacteria become prey for further amplification and production of new pseudolysogens in which phage growth is stalled. This interaction between phages and bacteria is reminiscent of the carrier state life cycle observed in different systems (Siringan et al., 2014). However, in the present study the phage/host equilibrium is not stable. The appearance of pseudolysogenic cells could occur when the amount of phages and resistant mutants is higher than the total amount of WT susceptible bacteria, allowing phages to be protected against extinction. The relative efficiency of reactivation of the phage cycle and production of cured bacteria determines the duration of the pseudolysogeny stage. It will be interesting to perform in situ analyses to check whether the colony is a homogeneous population of cells or if there are sectors in which phage activation is favoured and to follow fluctuations of free phage concentrations within a single colony.

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

**Red Queen dynamics/arms race coevolution**

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

**Cross-resistance and reversibility of mutants**

We showed that mutations selected by phages were often
frameshift mutations known as phase variation (Hen-
derson 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 mech-
anism 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 frame-
shift 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 oligo-
saccharide linker and a distal polysaccharide termed
O-antigen, in the form of A and B bands (Taylor et al.,
2013). Both WT and mutant forms of wzy and mucA
genes were simultaneously found in the presently described
mutants, suggesting that the mutation can reverse at a high
rate. Constant variations in LPS and alginate biosynthesis
pathways may help *P. aeruginosa* face aggression or
environmental changes. This might be one explanation
for the ‘colonial dissociation’ frequently observed with
*P. aeruginosa*, characterized by colonial differences of a
single strain (Zierdt & Schmidt, 1964).

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

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

Phage therapy is considered as a promising approach to
fight against antibiotic resistant strains (Abeden et al.,
2011). Either ready-made cocktails or ‘sur-measure’
phages will be used to treat patients, similar to what is
still done in several countries of Eastern Europe. It is
important to investigate the risks linked to the use of phages, particularly in the selection of bacterial mutants that could show deleterious characteristics (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. Alternatively, some phages driving evolution toward loss of virulence could be favoured if they exist (León & Bastías, 2015). Another concern is the potential role of phages in horizontal transfer, which could be favoured by the long-term maintenance of phage genomes inside the bacteria during pseudolysogeny. Additional experiments are needed to further investigate the fate of the phages and bacteria in a pseudolysogen interaction.

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