A carrier state is established in *Pseudomonas aeruginosa* by phage LeviOr01, a newly isolated ssRNA levivirus

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### Abstract

ssRNA bacteriophages are very abundant but poorly studied, particularly in relation to their effect on bacterial evolution. We isolated a new *Pseudomonas aeruginosa* levivirus, vB_PaeL_PcyII-10_LeviOr01, from hospital waste water. Its genome comprises 3669 nucleotides and encodes four putative proteins. Following bacterial infection, a carrier state is established in a fraction of the cells, conferring superinfection immunity. Such cells also resist other phages that use type IV pili as a receptor. The carrier population is composed of a mixture of cells producing phage, and susceptible cells that are non-carriers. Carrier cells accumulate phage until they burst, releasing large quantities of virions. The continuous presence of phage favours the emergence of host variants bearing mutations in genes involved in type IV pilus biogenesis, but also in genes affecting lipopolysaccharide (LPS) synthesis. The establishment of a carrier state in which phage particles are continuously released was previously reported for some dsRNA phages, but has not previously been described for a levivirus. The present results highlight the importance of the carrier state, an association that benefits both phages and bacteria and plays a role in bacterial evolution.

### INTRODUCTION

Members of the family *Leviviridae* are single-stranded RNA (ssRNA) viruses infecting Gram-negative bacteria. These are distributed into two genera, *Levivirus* (type species MS2) and *Allolevivirus* (type species Q-beta) [1–3]. Leviviruses have an icosahedral capsid (diameter 25–30 nm) and use pili as primary receptors. The first levivirus, described by Loeb and Zinder, was a male-specific coliphage [4], whereas other members of the *Leviviridae* were shown to bind to type IV pili [5]. Commonly, the genome of these phages encodes four proteins, a replicase, a coat protein, a maturase that binds to the RNA and is packaged with the genome, and a lysis protein. The lysin is short, hydrophobic and capable of crossing the cytoplasmic membrane [6]. Two *Levivirus* members have been shown to multiply in *Pseudomonas aeruginosa*, PP7 [7, 8] and PRR1, a broad host-range phage infecting different species of Gram-negative bacteria [9, 10]. Recently the isolation of three new *P. aeruginosa*-specific leviviruses was reported, but their genome sequence was not published [11].

Interactions between leviviruses and their hosts and the mechanisms of bacterial resistance to these phages remain poorly characterized. Brockhurst et al. [12] investigated variants of *P. aeruginosa* resisting phage P04 and observed both unpiliated and hyperpiliated mutants. Hyperpiliated nonretractile *P. aeruginosa* mutants that are resistant to phage P04 were previously described [13]. Leviviruses have not been shown to induce a carrier state, which refers to non-lysogenic bacteria producing phages inside the cell without releasing them after the duration of the life cycle, and displaying tolerance to superinfection [14]. This phenomenon has been observed frequently with dsRNA viruses, such as the *Pseudomonas syringae* pathovar phaseolicola cystovirus φ6 [15, 16]. A proportion of *P. syringae* pv phaseolicola mutants resisting phage φ6 infection produced phages and large amounts of intracellular phage particles in some cells, suggesting that these cells were still lysis-defective. The carrier state life cycle is also a common association of some dsDNA phages with their host, such as *Campylobacter jejuni* [17, 18]. A proportion of bacteria are immune, but the presence of a subpopulation of sensitive cells appears to sustain the phage population. Thus, some cells can be cured of the phage upon cell division, while others maintain the viral genome without being lysed [19]. It has also been proposed that only a subpopulation of the host is...
initially susceptible to phage infection. Phenotypic alterations have been shown to be associated with this bacteriophage carrier state, or so-called pseudo-lysogeny [20, 21]. We recently described P. aeruginosa dsDNA lytic phages that are capable of establishing pseudolysogeny in starved cells [22]. In addition we showed that the maintenance of the phage in a mixed population of carriers and sensitive non-carriers eventually resulted in the selection of resistant bacterial mutants [22].

Here, we describe a new P. aeruginosa levivirus, vB_PaeL_PcyII-10_LeviOr01 (LeviOr01), and show that it is capable of inducing a stable carrier state. This leads to the constant evolution of the bacterial population and the emergence of mutants.

RESULTS

Phage characteristics

A bacteriophage was isolated from hospital sewage water, producing clear plaques of variable size with a halo on the clinical P. aeruginosa strain PcyII-10 (Fig. S1, available with the online Supplementary Material), and turbid plaques on strains C9-11, PcyII-11, PcyII-36 and PcyII-57. In a drop assay using dilutions of the phage suspension, no plaque formation was observed on 20 additional strains, including the laboratory-adapted reference strains PAO1 Or and PA14 Or (Table S1). Examination of the virions by transmission electron microscopy (TEM) revealed a small icosahedral capsid with a size of about 30 nm with no tail, which is characteristic of Leviriviridae (Fig. 1) [7].

The phage genome was extracted from a purified phage preparation and analysed in a non-denaturing agarose gel; a major species migrating as a 1800 bp DNA fragment was then detected (Fig. S2). It was degraded by RNase A and when the sample was denatured before electrophoresis, a band was observed migrating a little faster than the 3000 bp DNA size marker. These results suggested that the phage genome was a highly folded ssRNA molecule, in agreement with the characteristics of leviriviruses genomes [23]. The purified RNA was reverse-transcribed and the cDNA was sequenced. Quality-controlled trimmed reads were assembled into a single linear contig of 3669 nucleotides at a mean coverage of 15 287-fold. A BLASTN search using the nucleotide sequence did not produce an alignment with known genomes, but homologies could be detected at the protein level with phage PP7. According to the nomenclature proposed by Kropinski et al. [24], the phage was called vB_PaeL_PcyII-10_LeviOr01 or LeviOr01.

Fig. 2(a) shows a comparison of the gene organisation for LeviOr01, three leviriviruses and Q-beta, an allolevivirus. The LeviOr01 genome displayed three putative genes, starting with a methionine codon and encoding a 463 amino acid (aa) maturation protein, a 129 aa coat protein and a 544 aa RNA replicase. In addition, there was an open reading frame (ORF) encoding a putative hydrophobic 72 aa lysin that overlaps the end of the coat protein and starts with a leucine codon. Fig. 2(b) shows the phylogenetic trees produced by alignment of the three major proteins, confirming the highest relatedness between LeviOr01 and PP7. The LeviOr01 maturase, coat protein and replicase displayed, respectively, 50, 55.5 and 56.5 % identity with those of phage PP7. Alignment of the putative lysin with the lysin gene of other leviriviruses revealed only a few stretches of amino acids in common with PRR1 and PP7 proteins.

Infection cycle

Infections of bacteria in the logarithmic growth phase were performed in Luria broth (LB) at multiplicities of infection (m.o.i.) of 0.001, 0.01, 0.1 and 1, and phage titres were determined after 7 and 20 h. When compared to the non-infected control, only a slight reduction in the optical density (OD600 nm) was recorded, commencing 5 h after infection, with lysis observed after 20 h in all the infected cultures. The absence of a clear drop in the culture density after 7 h may be explained if not all of the added phage adsorbed to the cells, leaving some bacteria uninfected. The phage titres at m.o.i. of 0.001, 0.01, 0.1 and 1 were respectively 5×10⁷, 2×10⁸, 9×10⁹ and 6×10⁹ plaque-forming units (p.f.u.) ml⁻¹ at 7 h after infection, and 6×10⁹, 1×10¹⁰, 4×10¹⁰ and 2×10¹⁰ p.f.u. ml⁻¹ at 20 h after infection. A one-step growth assay at an m.o.i. of 0.01 was performed in order to determine the phage growth characteristics (Fig. 3). The results indicated a biphasic adsorption of the phage, a fast adsorption step lasting less than 10 min, a detachment step and a secondary adsorption that occurred at 20 min. The eclipse period was estimated to be about 40 min. The biphasic adsorption may be explained by the existence of an initial reversible state, followed by irreversible adsorption and injection of the phage genome.

Fig. 1. Morphology of LeviOr01 virions. Transmission electron micrograph of virions stained with 2 % potassium phosphotungstate. Scale bar, 50 nm.
Characteristics of phage-resistant variants

To investigate the nature of the mechanisms employed by the bacteria to resist LeviOr01 infection, we analysed independent variants surviving phage infection. For this, 2 × 10^6 PcyII-10 colony forming units (c.f.u.) were infected at an m.o.i. of 0.1 and plated in soft agar on LB solid medium. After 3 days, approximately 50 colonies with different morphologies grew over the mostly lysed bacteria; 10 were selected and purified by three successive replatings of a single well-separated colony. At passage three, several variants (C7, C8, C14 and C19) still displayed colonies of different sizes and morphologies. One colony was grown in 2 ml LB overnight for storage and phenotypic analyses. Surprisingly, all of the variants produced phages in the culture supernatant, as shown with a rapid assay (see the Methods section) for variants C9 and C19 in Fig. S3(a, b) displays the growth curve of four variants and the parental strain PcyII-10, showing that at the beginning of the exponential growth phase no significant difference was seen between the control and the phage-resistant variants. Then growth stopped, but the cells did not appear to lyse, although phage particles were found in the supernatant at titres of 1 × 10^7, 2 × 10^6, 1 × 10^6 and 2 × 10^5 p.f.u. ml^{-1}, for C7, C9, C14 and C19, respectively. These results are similar to those observed with phage φ6 [25]. Variants C9 and C19, respectively low and high phage producers, were selected for additional analysis. On LB agar, as shown in Fig. 4, C9 formed homogeneous colonies with a dark centre and clear edges (flower-like), with a tendency to aggregate to form large patches. In contrast, C19 formed colonies with different sizes and shapes, with some appearing almost transparent in the microscope (Fig. 4).

Persistence of phages in resistant bacteria

To evaluate the percentage of cells producing phages at different passages, C9 and C19 at passage three were streaked onto the surface of an agar LB plate and grown for 24 h at 37 °C. Then individual colonies were picked and transferred, first onto an LB plate, and then onto a double-agar-layer plate of PcyII-10 bacteria. None of the 52 tested C9 colonies showed evidence of phage production, suggesting that only a fraction of the population was responsible for sustaining phage growth at passage three. For C19, we observed that all the colonies were surrounded by a halo of lysis on the

Fig. 2. Comparison of five viral genomes belonging to Leviviridae. (a) Gene organization of the four leviviruses, LeviOr01, PP7, MS2, PRR1, and of allolevivirus Q-beta. The maturase, coat protein, lysin and replicase are shown with yellow, blue, green and red arrows, respectively. (b) UPGMA trees obtained from CLUSTALW alignments of three proteins.

Fig. 3. One-step growth experiment. A logarithmic scale was used for the y-axis. The primary adsorption phase is likely followed by phage detachment and a second adsorption phase, and then the eclipse and rise periods.
PcyII-10 lawn (Fig. S4a). A phage-producing colony from C19 was picked and streaked onto a fresh agar LB plate and the same test was repeated with new and independent colonies. Extensive heterogeneity was still observed regarding the shape and size of the colonies, and some no longer produced phage. It appeared that very small colonies always produced phage, whereas some of the large ones were cured. A total of eight passages (P8) from a phage-positive colony were performed for C19 and 90% of the colonies were still positive for the presence of phage (Fig. S4b), suggesting that a stable carrier state was established. Analysis of bacterial sections by EM at passage P8 showed that many contained phage, with some cells being packed with large amounts of viral particles and significantly enlarged, with the typical appearance of spheroplasts (Figs 5 and S5). The cell wall seemed to be intact in carriers before the burst released the phage. This suggests that ‘lysis’ is brought about through osmotic shock, as deduced from the observation by Bradley [7]. We tested the resistance of C9 and C19 to phage LeviOr01 and to eight additional phages belonging to seven genera. All of these phages use type IV pili or LPS as receptors (Table 1). Variants C9 and C19 were resistant to LeviOr01 and type IV pili-dependent phages. The efficiency of plating was unchanged, but the plaques were smaller for Ab27, whereas Ab09 and Ab22 showed reduced plating efficiency (Fig. S6, Table 2). Because we expected pili to be involved in the binding of the phage to cells, we analysed the capacity of phage-resistant variants to perform swarming and twitching, two modes of bacterial motility. Swarming is dependent on both flagella and type IV pili, as well as the presence of rhamnolipids [26–28], whereas twitching is dependent on the presence of type IV pili [29]. Variant C19 exhibited defects in twitching and swarming motility, whereas variant C9 could swarm, although, interestingly, with two waves as compared to the dendritic appearance of PcyII-10 (Fig. S7). We performed adsorption tests, but the continuous production of phage by carriers prevented an evaluation of the capacity of the phage to bind to its receptor.

**Chromosomal mutations selected by LeviOr01**

Whole-genome sequencing of C9 and C19 variants was performed and the sequences were compared to that of PcyII-10. In order to detect changes that were linked to phage resistance, DNA extracted from the parental PcyII-10 culture used to select variants was also re-sequenced. C9 was shown to possess a single mutation (A→T) in the type IV pilus synthesis gene pilQ, with a valine to glutamine change at residue 309 (Table 2). The product of pilQ forms the outer-membrane secretin channel through which type IV pili are secreted [30, 31]. No mutation was found in the C19 genome, suggesting that the insensitivity of these variants was due to a carrier state, although the precise mechanism remains unclear.

To test whether the continuous production of phage by the carrier cells would select for new mutations, as previously observed for DNA phages [22], we analysed the susceptibility of different C19 colonies obtained at P8 to phage. The majority of non-carriers (as tested by absence of phage release) formed normal colonies and were susceptible to phage LeviOr01. C19-A1 was an exception, as it formed large colonies, did not secrete phage and was resistant to LeviOr01 and other type IV pilus-dependent phages (Table 2). Whole-genome sequencing showed that C19-A1 possessed a mutation in the type IV pilus synthesis gene pilB [position 5068188 SNP: A→C (Asp→Ala)]. The product of pilB, an accessory protein, is an ATPase that powers pilus extension. Therefore the observed mutation is probably responsible for inhibition of type IV pilus biosynthesis [32]. C19-5 and C19-C, with a small colony (SC) phenotype, secreted LeviOr01 phage particles, and formed aggregates when grown in LB, while their motility was strongly reduced. Interestingly, they were resistant to all of the tested phages, regardless of whether they used type IV pilus or LPS as receptors (Table 2). The C19-C genome was sequenced and two mutations were found. The first was in wbpL [position 1944187 InDel + G: G(7)→G(8) giving a frameshift], a gene involved in LPS O antigen biosynthesis. The second mutation was found in vfr [position 682677 SNP: T→A
Table 1. Bacteriophage characteristics

<table>
<thead>
<tr>
<th>Phage name</th>
<th>Short name</th>
<th>Related phage*</th>
<th>Putative receptor†</th>
<th>Reference‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>vB_PaeL_PcyII-10_LeviOr01</td>
<td>LeviOr01</td>
<td>PP7</td>
<td>Type IV pilus</td>
<td>This work</td>
</tr>
<tr>
<td>vB_PaeP_PAO1_Ab05</td>
<td>Ab05</td>
<td>PhiKMV</td>
<td>Type IV pilus</td>
<td>[44]</td>
</tr>
<tr>
<td>vB_PaeP_PAO1_Ab12</td>
<td>Ab12</td>
<td>PhiKMV</td>
<td>Type IV pilus</td>
<td>[44]</td>
</tr>
<tr>
<td>vB_PaeS_SCH_Ab26</td>
<td>Ab26</td>
<td>PA73</td>
<td>Type IV pilus</td>
<td>[44]</td>
</tr>
<tr>
<td>vB_PaeP_PAO1_phiC725A</td>
<td>phiC725A</td>
<td>FI16</td>
<td>Type IV pilus</td>
<td>[45]</td>
</tr>
<tr>
<td>vB_PaeS_PcyII10_pfi140A</td>
<td>pfi140A</td>
<td>D3112</td>
<td>Type IV pilus</td>
<td>UP</td>
</tr>
<tr>
<td>vB_PaeC_C2-10_Ab09</td>
<td>Ab09</td>
<td>N4</td>
<td>LPS</td>
<td>[44]</td>
</tr>
<tr>
<td>vB_PaeM_PAO1_Ab27</td>
<td>Ab27</td>
<td>PB1</td>
<td>LPS</td>
<td>[44]</td>
</tr>
<tr>
<td>vB_PaeP_C2-10_Ab22</td>
<td>Ab22</td>
<td>LU224</td>
<td>UN</td>
<td>[44]</td>
</tr>
</tbody>
</table>

*Indicates the reference bacteriophage which shows the highest level of sequence similarity.
†Reference comes from lack of growth on bacterial mutants affected in the production of either type IV pilus or LPS.
‡UP, unpublished.

(Thr→Ser]), encoding a receptor for cAMP that controls the production of many virulence factors [33]. C19-C showed a lack of A- and B-chains of LPS, as observed by PAGE analysis, whereas clone C19-A1 produced normal LPS (Fig. 6). In order to check whether small-colony variants spontaneously arising from PcyII-10 would hold similar mutations, we picked two such variants and found that their susceptibility to phage was unchanged. The genome of one small-colony variant was sequenced and we found no genomic difference as compared to the parental strain.

**DISCUSSION**

LeviOr01 is a new PP7-like levivirus, whose putative lysis gene appears to overlap mostly with the end of the coat gene. Infection of PcyII-10 by LeviOr01 in LB medium did not lead to cell lysis after 7 h, although phage was present in the culture medium. Lysis was observed after 20 h, together with high phage production, and thus whether LeviOr01 possesses a functional lysis, and how its expression is regulated, remain unknown.

We investigated the mechanism of resistance to phage LeviOr01 and found that the establishment of a carrier state was a frequent outcome of infection, since the majority of tolerant bacteria happened to produce phage. Upon EM examination we observed bacteria densely packed with phage, but the appearance was different from the crystal formations described in *Esherichia coli* infected with phage f2 [34], or *P. aeruginosa* infected with PP7 [7]. In the latter two cases such an aggregation of phage particles was observed at the end of the infection phase when cells were lysing. Notably, such non-lysogenic perpetuation of phage has been described under different names – persisting bacteriophage infection, pseudolysogeny and carrier state – and they probably represent different virus-host associations [35]. Hunter [36] first described the carrier state in lactic streptococci as a symbiotic association of bacteria and phage [36]. This consists of a mixture of bacteria and viruses that are in a quite stable equilibrium and is observed in different bacterial species [37, 38]. We showed that the carrier state could be maintained stably in some PcyII-10-LeviOr01-resistant variants, and that a minority of cells inside a colony became cured of the phages, whereas others still maintained phage production. The carrier state had never been described before for a levivirus, but is common for some cystoviruses. What we observed is reminiscent of the behaviour of phage φ6 carriers, in which phage-producing mutants appear to be lysis-defective [15]. It was suggested that a high concentration of intracellular virus-specific lytic enzyme was ultimately necessary to disrupt the cell membrane, causing the cell to burst and release phage. Some bacteria deriving from carrier C19 no longer produced phage and regained both their susceptibility to LeviOr01 and their motility. The finding that C19 apparently in the carrier state does not hold any chromosomal mutations indicates that the presence of phage is responsible in some way for ‘resistance’, not only to LeviOr01 but to other phages that use type IV pili as receptors. Several explanations can be proposed for the carrier state, one being a modification of bacterial metabolism, as previously shown in different studies. During the lytic cycle of MS2 in *E. coli*, the cell growth and synthesis of the cell wall are strongly reduced [39]. It was shown that host protein synthesis is suppressed as a result of a considerable change in the level of expression of ribosomal proteins and that there is competition between *E. coli* mRNA and MS2 RNA [40]. In PRR1-infected *P. aeruginosa*, there is a minimal effect on major biosynthesis pathways other than protein synthesis [41]. Comparative transcriptomic analyses of the parental compared to the carrier state would help to identify the genes involved in the regulation of the carrier state.

The continuous production of phage appears to favour the emergence of host mutants, which is similar to what we previously observed in *P. aeruginosa* PAO1 variants that were ‘resistant’ to DNA phages [22]. Whole-genome sequencing of LeviOr01-resistant variants revealed the presence of mutations that most probably were selected by the phage; however, we did not evaluate the frequency of spontaneous
The double agar overlay method was used for the first step of phage isolation and later for precise titration of phage. Phage isolation and analysis

**Table 2. Characteristics of PcyII-10 and phage-resistant variants**

<table>
<thead>
<tr>
<th>Variants</th>
<th>Phage production *</th>
<th>LeviOr01</th>
<th>Ab05</th>
<th>Ab12</th>
<th>Ab26</th>
<th>phiC725A</th>
<th>phiF40A</th>
<th>Ab09</th>
<th>Ab27</th>
<th>Ab22</th>
<th>Swarming (mm)</th>
<th>Mutation</th>
<th>Mutation event †</th>
<th>Locus tag</th>
<th>Protein alteration §</th>
</tr>
</thead>
<tbody>
<tr>
<td>PcyII-10</td>
<td>0</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>35</td>
<td>WT</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C9</td>
<td>2 × 10⁴</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>S</td>
<td>S</td>
<td>35</td>
<td>A → T</td>
<td>TV</td>
<td>p pil Q</td>
<td>Val→Glu (aa309)</td>
</tr>
<tr>
<td>C19</td>
<td>2 × 10⁴</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>5</td>
<td>wt</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C19-A1</td>
<td>2 × 10⁴</td>
<td>R</td>
<td>I</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>S</td>
<td>I</td>
<td>I</td>
<td>35</td>
<td>A → C</td>
<td>TV</td>
<td>pil B</td>
<td>Asp→Ala (aa588)</td>
</tr>
<tr>
<td>C19-5</td>
<td>2 × 10³</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>I</td>
<td>R</td>
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<td>R</td>
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<td>3</td>
<td>ND</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>C19-C</td>
<td>2 × 10³</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
<td>R</td>
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<td>R</td>
<td>I</td>
<td>5</td>
<td>G7→G8</td>
<td>F</td>
<td>wbpL.</td>
<td>163/346 aa</td>
</tr>
</tbody>
</table>

* p.l.u. per ml of culture medium.
† Resistance pattern is expressed as S, completely susceptible; R, completely resistant; I, reduced efficiency of plating.
‡ F, frameshift; TV, transversion.
§ aa, aminoacids, /length of the mutated protein over the wild type.

**METHODS**

**Strains and media**

Strain PcyII-10 was isolated from a burn patient in Percy Hospital, Clamart, France. Its genome has been totally sequenced (Genome accession number: LT673656) and is presently under analysis (Pourcel et al., unpublished). The additional reference strains used in this study are listed in Table S1. Different phages used in this study are listed in Table 1 and were described in [44, 45]. LB medium supplemented with 2 mM CaCl₂ was used for bacterial growth and phage titration. Phage-free bacterial culture (PBS) supplemented with 2 mM CaCl₂ was used to preserve purified phage at 4°C.

**Phage isolation and analysis**

This work shows for the first time that a levivirus, a very simple virus expressing only four proteins, is capable of establishing a carrier state within its host, allowing its population to remain viable despite the presence of phage. Several studies have investigated the effect of mutations in pilus biosynthesis genes on phage resistance. Roine et al. reported that non-piliated Tn5 mutants of *P. syringae* pv. phaseolicola were resistant to infection by phage f6 [42]. In addition, it was shown that phage O6 selected for non-piliated or superpiliated mutants, although none of the mutated genes were known to be directly involved in type IV pilus expression [43].

This work, in contrast, shows that the presence of phage promotes the emergence of true mutants at a high rate, and with pleiotropic characteristics. The mutations in genes *pilB* and *pilQ* may affect normal access of the phage to the receptor or retraction of the pilus that brings the phage to the cell surface. PilQ residue 309 is part of the periplasmic N0 domain, required for PilF-mediated secretin assembly. Koo et al. (2018) showed that deletion of this domain suppresses twitching motility and sensitivity to bacteriophage PO4. PilB residue 388 mutated in variant C19-A1 is inside the Walker B domain of this ATPase, a key element driving pilus extension. The exact consequences of *pilB* and *pilQ* mutations deserve further analysis. Several studies have investigated the effect of mutations in pilus biosynthesis genes on phage resistance. Roine et al. reported that non-piliated Tn5 mutants of *P. syringae* pv. phaseolicola were resistant to infection by phage f6 [42]. In addition, it was shown that phage O6 selected for non-piliated or superpiliated mutants, although none of the mutated genes were known to be directly involved in type IV pilus expression [43].

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After centrifugation at 15 000 g with 10 % polyethylene glycol (PEG) 8000 overnight at 4 °C, the supernatant was recovered and the phages were precipitated 10-fold by centrifugation at 32 000 g for 30 min at 26 °C. The phage suspension was cleared of bacteria and debris by centrifugation through a 0.45 µm filter and ultracentrifugation for 45 min at 260 000 g in a swinging bucket rotor (Beckman XL-70, rotor SW41) to pellet the phage. The pellet was resuspended in 50 µl of PBS, 2 mM CaCl₂. To study the infection cycle, 10¹⁰ PcyII-10 c.f.u. were infected with 10⁴, 10⁵, 10⁶ or 10⁷ p.f.u. in 100 µl PBS before transfer into 10 ml LB and incubation at 37 °C. The phage present in the supernatant was titrated after 7 h and 20 h. A one-step growth assay was performed as previously described [48]. Briefly, an overnight culture of PcyII-10 was diluted to 10⁵ c.f.u. ml⁻¹ into a final volume of 5 ml in LB. Phages at 10⁵ p.f.u. ml⁻¹ were added and the mixture was incubated at 37 °C with shaking at 180 r.p.m. Immediately after infection and then every 5 min, 100 µl was collected and diluted 10-fold in cold LB containing a drop of chloroform. Phage present in the supernatant was titrated by the double agar overlay method. The experiment was repeated twice.

Electron microscopy

To visualize phages by transmission electron microscopy (TEM), 5 µl of purified suspension was stained with 2 % potassium phosphotungstate (pH 7.0) and observed using JEM-100B operating at 80kV as previously described [49].

To observe intracellular phage particles, the bacteria were embedded in Epon resin, as previously described [50]. Briefly, bacteria were grown from a colony overnight in 2 ml of LB and the bacterial pellet was recovered after centrifugation. After fixation using 2.5 % glutaraldehyde, the cells were dehydrated with ethanol and acetone before being fixed in Epon low-viscosity agar (Oxford Instruments). The resin blocks were sliced into 90 nm sections. Before EM observation, staining was performed using uranyl acetate 2 % for 15 min, followed by three 5 min washes with water.

Genome purification

Phages were grown on solid LB medium and precipitated with PEG 8000 as described above. After centrifugation the pellet was resuspended in 200 µl PBS with 2 mM CaCl₂, and one chloroform extraction was performed. Then, 500 µl of TRI reagent (Sigma-Aldrich) and 100 µl chloroform were added. The mixture was shaken for 15 s and then left for 5 min at room temperature (RT). Centrifugation was performed at 15 000 g for 15 min, the supernatant was transferred to a new tube and 250 µl of isopropanol added. After 10 min at RT, centrifugation was performed at 15 000 g for 15 min, the supernatant was discarded and the pellet was washed with 1 ml 75 % ethanol. Finally, the pellet was dried and resuspended in 100 µl RNase- DNase-free water.

Isolation of resistant variants

We used the protocol previously employed to isolate and analyse bacteria resistant infection by DNA bacteriophages [22]. Bacteria were inoculated at a 600 nm absorbance (OD₆₀₀) of 0.01 into glass vials with aeration, containing 5 ml of LB medium, and grown at 37 °C with shaking at 180 r.p.m. to an OD₆₀₀ of 0.2. Infections were performed at an m.o.i. of 0.1. For infection on solid medium, a 10 µl suspension [46]. For faster evaluation of bacterial strain susceptibility to phages, a drop assay was performed by overlaying an LB agar plate (1.5 % wt/vol agar) with melted soft agar (0.7 % wt/vol agar) containing host bacteria, and after the agar had solidified, placing 5 µl of sequential phage dilutions onto the surface. A fast method to detect the production of phages in bacterial culture consisted of spreading bacteria in 2 ml of LB medium and 10 ml of PBS and shaken gently by hand for 1 min before incubation at 4 °C overnight. The agar, bacteria and debris were pelleted by centrifugation at 15 000 g for 20 min at 10 °C in a fixed-angle rotor (Sigma 2K15, rotor 12139). The supernatant was recovered and the phages were precipitated with 10 % polyethylene glycol (PEG) 8000 overnight at 4 °C. After centrifugation at 15 000 g for 20 min, the pellet was suspended in 1 ml of PBS supplemented with 2 mM CaCl₂. Three chloroform extractions were performed prior to filtration through a 0.45 µM filter and ultracentrifugation for 2 h at 260 000 g in a swinging bucket rotor (Beckman XL-70, rotor SW41) to pellet the phage.

Fig. 6. SDS-polyacrylamide gel electrophoresis of LPS extracted from PcyII-10, C19-A1 and C19-C. In order to clearly detect the different molecular species present in the LPS profiles, two electrophoreses were performed, using different quantities of samples, when looking at the long O-chains (top) and the shorter core LPS (bottom). We used 12.5 µg of suspended lyophilized bacteria for the top gel and 2.5 µg for the bottom one.

To isolate the new phage, a sample of waste water was recovered from Armand Trousseau hospital in Paris (France) and, after filtration (Micropore, 0.45 µm filter), 200 µl were added to 10⁸ bacteria in 2 ml of LB medium and incubated overnight at 37 °C with shaking. The culture supernatant was cleared of bacteria and debris by centrifugation at 10 000 g for 20 min at 320 000 g in a swinging bucket rotor (Jouan GR412, rotor M-4). The presence of phage in the clarified medium was assessed by the double agar overlay method by mixing 10⁵ µl of the supernatant onto an LB agar plate and then overlaying it with melted soft agar containing 10⁸ bacteria.

After the agar had solidified, placing 5 µl of sequential phage dilutions onto the surface. A fast method to detect the production of phages in bacterial culture consisted of spreading bacteria in 2 ml of LB medium and 10 ml of PBS and shaken gently by hand for 1 min before incubation at 4 °C overnight. The agar, bacteria and debris were pelleted by centrifugation at 15 000 g for 20 min at 10 °C in a fixed-angle rotor (Sigma 2K15, rotor 12139). The supernatant was recovered and the phages were precipitated with 10 % polyethylene glycol (PEG) 8000 overnight at 4 °C. After centrifugation at 15 000 g for 20 min, the pellet was suspended in 1 ml of PBS supplemented with 2 mM CaCl₂.
inoculum of the bacterial culture (2×10⁶ c.f.u.) was mixed with 10 µl of a suspension containing 10⁵ p.f.u. The mixture was kept at RT for 15 min before being poured onto a fresh LB agar plate with 4 ml of melted soft agar and then incubated at 37°C for 3 days. Colonies growing over the phage lysis zone were picked and streaked onto an LB agar plate. After 24 h, colonies with different sizes and shapes were picked and streaked onto LB agar plates. This was repeated twice (passage three or P3) in order to obtain a pure population before storage and phenotypic tests were performed.

**Phenotypic assays**

For the type IV pilus-mediated twitching assay, one µl of overnight bacterial culture was inoculated between the agar and the plastic surface of 1.5 % LB agar plates. The diameter of the motility zone around the inoculation site was measured after 24 h incubation at 37°C. For the swarming assay, 2 µl of an overnight culture was loaded onto a fresh semi-solid agar plate (0.7 % agar) before the plate was incubated for 48 h at 37°C. LPS was purified using Hitchcock and Brown’s method [51]. In order to normalize the samples for the subsequent gel analysis, a similar amount of lyophilized bacteria was disrupted in lysis buffer (Tris 1M, 2 % SDS, 4 % β-mercaptoethanol and 10 % glycerol) prior to LPS extraction. LPS was resolved by electrophoresis on a 15 % SDS-polyacrylamide gel and the band pattern was visualized using the silver staining method [52].

**Whole-genome sequencing**

Two µg of purified phage RNA was sent to the IMAGIF MiSeq Illumina platform (CNRS, Gif sur Yvette, France) for draft whole-genome sequencing. CDNA was synthesized, a 385 bp average insert size library was constructed and 250 bp paired-end sequences were produced. Phage genome assemblies and other sequence analyses were performed using tools in Geneious R10 (Biomatters, New Zealand). The sequence was compared to those of phages MS2 (NC_001417), PP7 (NC_001528), PRR1 (NC_008294) and Q-beta (NC_001890). Alignments were performed using CLUSTALW and the trees were drawn with UPGMA.

Ten µg purified bacterial DNA was sent to the IMAGIF platform (CNRS, Gif sur Yvette, France) for draft whole-genome Illumina sequencing. Libraries were made from sheared fragments of DNA with a mean size of 900 bp and 250 bp paired-end reads were produced. The mutations were identified by comparison with the genome of the parental PcyII-10 sequence using tools in Geneious R10 (Biomatters, New Zealand) as previously described [22]. Mutations were confirmed by PCR amplification of the affected gene and Sanger sequencing (Beckman-Cogenics, UK).

**Nucleotide sequence accession number**

The complete sequence of *P. aeruginosa* phage LeviOr01 (accession number: LT821717) has been deposited in the European Nucleotide Archive (ENA) under project accession PRJEB18601, together with the sequencing reads of the phage and of the four phage-resistant PcyII-10 variants, C9, C19, C19-A1 and C19-C. The complete sequence of the parental PcyII-10 *P. aeruginosa* strain (accession number: LT673656), as well as the corresponding raw sequencing data, were deposited within project PRJEB18612. The data can be viewed from the ENA browser at http://www.ebi.ac.uk/ena/data/view/<accession number>.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

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