Fine structure analysis of lipopolysaccharides in bacteriophage-resistant *Pseudomonas aeruginosa* PA01 mutants

Libera Latino,¹ Martine Caroff¹,² and Christine Pourcel¹,*

**Abstract**

*Pseudomonas aeruginosa* lipopolysaccharides (LPS) serve as primary receptors for many bacteriophages and, consequently, their biosynthesis is frequently affected in phage-resistant mutants. We previously isolated phage-resistant PA01 mutants using three different phages, and showed that they were affected in the synthesis of LPS. Here we have investigated in detail the effect of mutations in seven genes involved in different steps of the production of core and oligosaccharide chains. The band profile of purified LPS was analysed by PAGE, and we further characterized the O-chains and core structures by MALDI mass spectrometry (MS). Mild LPS extraction conditions and native LPS MS analyses helped unveil lipid A molecular species with three phosphate residues in the close vicinity of the already highly charged inner-core region. No other MS direct analysis has allowed this peculiarity to be demonstrated for native lipid A high-molecular-weight molecular species, in normal growth conditions and without involving separation techniques. The present results shed light on the possible interactions between the phages and the LPS structures in the early phase of infection.

**INTRODUCTION**

Lipopolysaccharides (LPS) are the major components of the outer leaflet of the external membrane of Gram-negative bacteria, playing crucial roles in bacterial attachment to substrates and bacterial defence mechanisms [1, 2]. LPS contribute substantially to *Pseudomonas aeruginosa* virulence [3]. LPS are made up of three distinct structural elements: (1) the lipid A, a glyco-phospho-lipid anchored in the outer membrane; (2) a core oligosaccharide linked to lipid A through a 3-deoxy-d-manno-oct-2ulosonic acid (Kdo)-specific molecule; and (3) antigenic polysaccharide chains, the O-antigen, comprising variable numbers of repetitive units made up of up to eight sugars, and substituting the core. In *P. aeruginosa* two distinct O-antigen chains are synthesized, the A- and B-chains, giving the A and B bands upon SDS-polyacrylamide gel electrophoresis analysis [4]. The A-chain, also called the common polysaccharide antigen (CPA), is a homopolymer of D-rhamnose (D-Rha) arranged in trisaccharide repeated units [5]. The B-chain is a heteropolymer with repeated units of three to five distinct sugars, whose nature depends on the cluster of genes defining it. Cores on which A- and B-chains are attached are called ‘capped cores’ [6]. The presence of O-antigen makes the smooth-type LPS, while molecules containing only the lipid A and core oligosaccharides are called rough-type LPS, referring to the dry aspect of the corresponding bacterial colonies [7]. LPS containing the core plus a single O-unit are called ‘semi-rough’ [8].

In *P. aeruginosa*, more than 20 serotypes defined by different O-antigens have been described, and the clusters of genes responsible for this biosynthesis have been identified [9]. In the reference strain *P. aeruginosa* PA01 (serotype O5) the O-antigen is synthetized by the *wzy/wzx* pathway [4, 10]. Wzy is a glycosyltransferase that is responsible for linking groups of three sugars to form the B-chains [10]. Mutations in this gene do not affect the synthesis of the A-chains, which depends on an ABC-transporter pathway [11, 12]. Clusters of genes necessary for the synthesis of the lipid A and the inner and outer core (on which the A- and B-chains are attached) are located at different positions on the genome. Numerous glycosyl transferases are involved in this process. In addition, structural analyses have shown the presence of phosphate on the inner core, which is essential for outer-membrane integrity [13, 14].

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**Abbreviations:** CPA, common polysaccharide antigen; DHB, dihydroxybenzoic acid; D-Rha, D-rhamnose; Kdo, 3-deoxy-d-manno-octulosonic acid; LPS, lipopolysaccharide; MS, mass spectrometry; SR, semi-rough.

Two supplementary figures are available with the online Supplementary Material.
Many *P. aeruginosa* bacteriophages, as well as R-pyocins, have been shown to be dependent on LPS for binding to their receptors [15]. Some phages were used to isolate LPS-defective mutants, such as E79 [16], binding to the core [17] and pls27, a rough-LPS-specific phage [18]. We recently derived LPS mutants of strain PA01 resistant bacteriophages Ab09, Ab17 and Ab27, belonging to three different genera [19]. Eight genes were affected (wzy, wzz2, wbpl, migA, dnpA, wapH, algC and pgI) from a total of 11 mutants. Seven different mutations were found in wzy, with five of them resulting in the synthesis of a truncated protein, and two resulting in amino acid substitutions. The mutations in dnpA and pgI had not previously been described.

Here, we have investigated these mutants in more detail, and analysed the structure of the LPS. For this we adopted new extraction procedure conditions, which allowed a more precise definition of the LPS core structure in *P. aeruginosa*.

**METHODS**

**Bacterial strains**

*P. aeruginosa* PAO1Or phage-resistant mutants have been described in [19].

**Lipopolysaccharides extraction**

Bacteria were grown overnight in Luria–Bertani (LB) media at 37 °C and then killed by treatment with 2 % formaldehyde for 15 h at 4 °C, followed by three washings with PBS. In order to normalize the samples for the subsequent gel analysis, a similar amount of lyophilized bacteria was disrupted in lysis buffer (Tris 1M, 2 % SDS, 4 % β-mercaptoethanol and 10 % glycerol) prior to treatment with 50 µg ml⁻¹ proteinase K for 2 h at 50 °C. When the LPS had to be fully purified for chemical analysis, an extraction method with isobutyric:1M ammonium hydroxide was performed [20], followed by DNase I, RNase A and proteinase K treatments, and then extraction with mixtures of solvents as previously described [21].

**Polyacrylamide gel electrophoresis (SDS-PAGE)**

Whole-cell samples and purified LPS were analysed on 15 % polyacrylamide gels with a 4 % stacking gel [22]. The LPS were stained using the Tsai and Frash method [23].

**MALDI negative-ion mass spectrometry**

MALDI-TOF analyses were performed on a Shimadzu Axima Performance system time-of-flight mass spectrometer (IZBC, Université Paris Sud, France) in the linear negative mode. The ion-accelerating voltage was set at 20 kV. Dihydroxybenzoic acid (DHB) (Sigma chemical Co., St Louis, USA) was used as a matrix. A few micrograms of lipid A were dissolved in a mixture of chloroform:methanol:water (3 : 1.5 : 0.25, v:v:v) at 1 µg µl⁻¹, and desalted with a few grains of ion-exchange resin (Dowex 50W-X8) (H⁻) in an Eppendorf tube. When the LPS samples were analysed, water was used for the sample and the matrix solutions. A 1 µl aliquot of the solution was deposited on the target and covered with an equal amount of the matrix suspended at 10 µg µl⁻¹ in the same mixture of solvents or water [24]. Different ratios between the samples and DHB were tested when necessary. *Bordetella pertussis* LPS was used as an external standard.

**RESULTS**

**Analysis of the LPS banding pattern in phage-resistant mutants**

We previously described a collection of *P. aeruginosa* PAO1Or variants with mutations in seven genes involved in the LPS-biosynthesis pathway, and showing different levels of resistance toward three lytic phages: Ab09, a podovirus, and Ab17 and Ab27, two myoviruses [19, 25]. Table 1 displays the function of the proteins expressed by the seven mutated genes, and their role in LPS biosynthesis. In order to identify the effect of the different mutations, the LPS of 11 mutants were extracted and then analysed by SDS-PAGE, using PAO1Or as a control (Fig. 1a). The presence/absence of A-chains, B-chains and different forms of core oligosaccharides in the phage-resistant mutants analysed in this study are summarized in Table 2, and described in detail in the following paragraphs.

**Wzy mutants**

PAO1-04 and PAO1-07, possessing the same wzy mutation, displayed the same banding pattern characterized by the complete absence of O-specific antigen and the presence of CPA. PAO1-04, with an additional mutation in migA, appeared to have converted all the core oligosaccharide into the core +1 unit form, a semi-rough (SR) profile. PAO1-01, PAO1-36_1 and PAO1-20_1 produced higher amounts of CPA, which was of the core +1 unit form. The wzy mutations induced a frameshift resulting in synthesis of a truncated protein of 74 and 54 amino acids in PAO1-01 and PAO1-36_1, respectively. Interestingly, in PAO1-20_1 the mutation in wzy involved a nucleotide substitution producing a Wzy protein with an amino acid substitution at position 42 (Ser → Phe), and this change allowed the bacteria to become completely resistant to phages Ab09, Ab17 and Ab27.

An amino acid substitution was also identified in the PAO1-37 Wzy protein. In this case, an aspartate residue (position 286) was replaced by a glycine. The presence of this mutation seemed not to impede the production of the O-antigen and core oligosaccharide, but an increase in the amount of the core +1 unit form, A-chains, and very long B-chains could be observed, compared to the wild-type PAO1Or. Thus it is possible that the mutated protein played a direct role in the bacterial resistance to phages.

The Wzy mutants, when transformed with an expression plasmid bearing the wild-type wzy gene, recovered their susceptibility to phages, except PAO1-20_1 and PAO1-37 (data not shown), suggesting competition between the mutated and wild-type proteins in these two mutants.

**wzz2 mutant**

PAO1-03, possessing a frameshift mutation in wzz2, presented a normal banding pattern concerning A-chains and short and...
long B-chains, but lacked the very long B-chains. The same results were obtained by Daniels and colleagues when they knocked out \textit{wzz2} in \textit{P. aeruginosa PAO1} [26]. The SR core +1 unit form, as well as all the O-antigen chains possessed by this variant, seemed to be present in higher amounts as compared to \textit{PAO1\textsubscript{0r}}. Consequently the lack of very long chains appeared to affect the infection by phages Ab09 and Ab27, reducing their plating efficiency.

\textbf{Pgi, WapH, DnpA and WbpL mutants}

An amino acid substitution, replacing a threonine with a proline in the \textit{pgi} gene of \textit{PAO1-10}, reduced the production of short B-chains, whereas long and very long chains were not affected. Three forms of core oligosaccharides were produced: the amount of the SR core +1 unit form was comparable to that of \textit{PAO1\textsubscript{0r}}, whereas the band of the uncapped core form was less intense and a band corresponding to the inner core could also be observed. Phages Ab09 and Ab17 produced turbid plaques on \textit{PAO1-10}, but with the same efficiency with which they produced clear plaques on \textit{PAO1\textsubscript{0r}}, which may indicate a lower growth rate. In contrast, the plating efficiency was reduced for phage Ab27 on the same variant.

\textit{PAO1-18} was shown to be completely resistant to phages Ab17 and Ab27, and to be partially resistant (reduced plating efficiency) to phage Ab09. It possessed a mutation in \textit{wapH} resulting in an amino acid substitution (Arg \rightarrow Leu). The banding pattern was compatible with the presence of long and very long chains, but the core oligosaccharide was reduced to the inner-core form, and neither the uncapped nor the SR core +1 unit forms could be detected.

The inner-core oligosaccharide was also the only form present in the \textit{PAO1-19} LPS. The \textit{dnpA} gene of this mutant produced a 67-amino-acid protein, instead of the wild-type 472-amino-acid protein, leading to a complete lack of A-

<table>
<thead>
<tr>
<th>Gene</th>
<th>Protein</th>
<th>Localization</th>
<th>Function</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>\textit{wzy}</td>
<td>Polymerase</td>
<td>Transmembrane</td>
<td>Elongation of B-chain</td>
<td>[10]</td>
</tr>
<tr>
<td>\textit{wbpL}</td>
<td>Ligase</td>
<td>Cytoplasm</td>
<td>Attachment of the first oligosaccharide to the core</td>
<td>[5]</td>
</tr>
<tr>
<td>\textit{wzz2}</td>
<td>Polysaccharide copolymerase</td>
<td>Transmembrane</td>
<td>Chain-length regulator</td>
<td>[10]</td>
</tr>
<tr>
<td>\textit{pgi}</td>
<td>Glucose-6-phosphate isomerase</td>
<td>Cytoplasm</td>
<td>Convert D-glucose 6-phosphate in D-fructose 6-phosphate</td>
<td>[4]</td>
</tr>
<tr>
<td>\textit{wapH}</td>
<td>Glucosyltransferase</td>
<td>Cytoplasm</td>
<td>(Putative) capping of the core</td>
<td>[34]</td>
</tr>
<tr>
<td>\textit{dnpA}</td>
<td>de-N-acetylase</td>
<td>Cytoplasm</td>
<td>(Putative) modification of the core</td>
<td>[33]</td>
</tr>
<tr>
<td>\textit{migA}</td>
<td>Rhamnosyltransferase</td>
<td>Cytoplasmic membrane</td>
<td>Outer-core biosynthesis</td>
<td>[40, 41]</td>
</tr>
</tbody>
</table>

\textbf{Table 1.} Role of the analysed mutated genes, as reported in the literature.
We sought to describe in more detail the LPS composition of MALDI-TOF analyses the same two major lipid A molecular species at uncapped core oligosaccharide form. The four strains presented phages, lacked A- and B-chains and possessed a wild-type LPS extract for the three mutants (Fig. S1, available in the online Supplementary Material.). In contrast, PAO1-15, a WbpL mutant resisting the three phages, lacked A- and B-chains and possessed a wild-type uncapped core oligosaccharide form.

**MALDI-TOF analyses**

We sought to describe in more detail the LPS composition of PAO1<sub>18</sub>, and three interesting variants, Wzy mutants PAO1-20_1 and PAO1-37, and Pgi mutant PAO1-10. To separate the different LPS fractions (A-band, B-band, S-, R- and SR-forms) present in a ‘classical’ extract, we set up new sequential extraction conditions by selecting several ratios for the isobutyric : 1M ammonium hydroxide extraction reagent, resulting in different solubility capacities that were efficient for the different LPS molecular species. The method, applied to PAO1<sub>18</sub>, separated the rough-type LPS (fraction 1) from the A- and B-chain molecular species (Fig. 1b). MALDI-TOF analysis was performed on fraction 2 and on the total LPS extract for the three mutants (Fig. S1, available in the online Supplementary Material.). The four strains presented the same two major lipid A molecular species at m/z=1447 and m/z=1348, corresponding respectively to five fatty acids, with 2 N-acyl-glucosamine (GlcNAc), two phosphate residues, and to the same molecular species without phosphate (H<sub>2</sub>P<sub>2</sub>O<sub>7</sub>), and (H<sub>3</sub>P<sub>2</sub>O<sub>7</sub>). A detailed analysis of the lipid A structure is given in the following paragraph. The lipopolysaccharide region displayed molecular species of small sizes, which was compatible with the pattern observed in the lower part of the polyacrylamide gel (Fig. 1a). PAO1-10 showed LPS of shorter molecular masses, as compared to the other samples (around m/z=2600), which could correspond to the inner-core species. The polysaccharide at m/z=2055, observed in PAO1<sub>18</sub>, fraction 2, might correspond to a LPS molecular species with a hexa-acyl lipid A with two 10:0 (3-OH) plus two Kdo derivatives.

Purified LPS were hydrolysed with acetic acid (1 % at 100 °C for 90 min) in order to cleave the core Kdo glycosidic linkage to lipid A and liberate the polysaccharides from the latter [27, 28]. The MALDI-TOF core oligosaccharide spectra are shown on Fig. 2. In the lower-mass region of the spectra, corresponding to band 1 in SDS-PAGE, masses were clearly observed in the whole PAO1-10 LPS sample spectrum. A major peak at m/z=1214 (band 1) could be interpreted as corresponding to one Kdo, two L-glycero-d-manno-heptose (Hep) [one carrying a phosphate group and the other a carbamoyl group (CONH<sub>2</sub>)], one galactosamine (GalN) and two glucose (Glc).

\[
\begin{align*}
\text{Glc} & \quad \text{CONH}_2 \quad \text{P} \\
\text{Glc} & \quad \text{GalN} \quad \text{Hep} \quad \text{Hep} \quad \text{Kdo} \\
\text{M} & = 1214 \text{ Da}
\end{align*}
\]

This structure was tentatively established according to the already-described core structures [7], and was accompanied by the presence of two vicinal corresponding peaks, at minus and plus one phosphate residue, appearing at m/z=1135 and 1295, respectively.

PAO1-37 and PAO1-20_1 LPS displayed higher molecular species corresponding to the masses expected for the core in the m/z=1800–2100 mass region (band 2), and core plus one and plus two O-chain units at plus 702u (band 3) and 1404u (band 4), respectively.

### Table 2. Structure of the LPS in the different mutants as analysed by PAGE

<table>
<thead>
<tr>
<th>PAO1 variant</th>
<th>Phage resistance*</th>
<th>Locus tag</th>
<th>Protein alteration†</th>
<th>A-chains†</th>
<th>B-chains†</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAO1</td>
<td>SSS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-04</td>
<td>RRR</td>
<td>wzy</td>
<td>224/438 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-07</td>
<td>RRR</td>
<td>wzy</td>
<td>224/438 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-01</td>
<td>RRR</td>
<td>wzy</td>
<td>74/438 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-36_1</td>
<td>RRR</td>
<td>wzy</td>
<td>54/438 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-20_1</td>
<td>RRR</td>
<td>wzy</td>
<td>224/438 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-37</td>
<td>ssR</td>
<td>wzy</td>
<td>74/438 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-03</td>
<td>ISI</td>
<td>wzy2</td>
<td>228/443 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-10</td>
<td>isi</td>
<td>pgi</td>
<td>Thr→Pro</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-18</td>
<td>IRR</td>
<td>wapH</td>
<td>Arg→Leu</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-19</td>
<td>IRR</td>
<td>dnpA</td>
<td>67/472 aa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1-15</td>
<td>RRR</td>
<td>wbpL</td>
<td>88/339 aa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

†The presence of the band is designated by X (similar band intensity to the wild-type variant) or x (less intense band than the wild-type variant).

‡Length of the mutated protein over the wild-type: aa, aminoacids.
PAO1\(_{\text{Or}}\)-fraction 2 LPS, displayed masses corresponding to the core and core +1 unit region.

**Detailed lipid A analysis**

In Fig. 3, the lipid A spectrum obtained by fragmentation in the MS (Fig. 3a) was compared to the same lipid A molecular species obtained by mild-acid hydrolysis followed by extraction in a mixture of solvents (Fig. 3b). Upon closer examination of the peaks in the lipid A region of the different spectra, a particular molecular species appeared at \(m/z=1616\) (1447+170u), corresponding to a hexa-acyl lipid A containing an additional 10:0(3-OH) fatty acid (170u). This molecular species can only correspond to a hexa-acyl lipid A carrying a 10:0(3-OH) at position C3 on the first GlcN, due to partial lack of O-deacylation by the PagL deacylase known to hydrolyse the ester-linked FA bond at the C-3 position of lipid A [29]. Small molecular species were observed at \(m/z=1616+80\), \(m/z=1696\) in the three spectra of PAO1-20 and PAO1-37, as well as the PAO1\(_{\text{Or}}\)-fraction. The level of phosphorylation was better visualized for the corresponding penta-acyl molecular species at \(m/z=1447+80=1527\), as illustrated schematically in Fig. 3a. The corresponding molecular species was less abundant in the PAO1-10 spectrum. A phosphate group was suggested to be located at position 1 on the reducing GlcN of the di-GlcN-di-P lipid A backbone, according to the literature concerning *Escherichia coli* lipid A, following the action of the LpxT enzyme with the participation of the carrier lipid undecaprenyl phosphate (C\(_{55}\)-P) [30]. However, this pyrophosphate group could also have been located at the distal GlcN moiety, as demonstrated with the *Pseudomonas* LpxT orthologue LpxTPa [31]. Being part of a pyrophosphate group, it would have been liberated under the same mild-acid hydrolysis conditions in both positions.

This pyrophosphate molecular species, known to be present in *E. coli* lipid A and a few other Gram-negative genera, was always very difficult to visualize by MS on LPS or lipid A, due to its high lability. Here, the corresponding lipid A molecular-species peak could be visualized in the different spectra a rare, if not unique, example. This was most probably possible because there was less LPS fragmentation into the mass spectrometer following mild extraction conditions.

**DISCUSSION**

**New Wzy mutants**

Islam and colleagues performed systematic site-directed mutagenesis of 83 periplasmic and cytoplasmic residues spanning the length of the Wzy protein of *P. aeruginosa* PAO1 (Wzy\(_{Pa}\)), in order to identify those of functional importance for the polymerization of O-antigen [32]. They reported that the amino acids shown to be important for Wzy\(_{Pa}\) function remained confined to periplasmic loops. The amino acid substitution Ser\(\rightarrow\)Phe (at position 42) observed in mutant PAO1-20 was not present among the described mutations. We show that it completely abolished the O-antigen B-chain synthesis, suggesting that change of a serine, a polar residue, to a phenylalanine, a hydrophobic residue, induces conformational changes that alter the protein function. The importance of the aspartate at position 286 was reported by Islam *et al.* [32]. Interestingly, PAO1-
37 with amino acid substitution Asp → Gly showed an increase in the total amount of long and very long B-chains compared to the wild-type banding pattern of PAO1_or with some defects in the production of short B-chains and alteration in the relative amount of core oligosaccharide and SR core +1 unit forms. Thus, the mutation may only partially repress the protein function.

**The truncated core: role of dnpA, wapH and pgi**

The LPS core oligosaccharide biosynthesis gene cluster encompasses 17 genes, including dnpA and wapH [33, 34]. In contrast to our observations, Liebens and colleagues [33] did not find significant changes in the LPS structure of a DnpA mutant, and they suggested that it was responsible for minor modifications of the LPS. Our results show that synthesis of a truncated protein (67/472 aa) has a dramatic effect in suppressing complete biosynthesis of the O-antigen, uncapped core and SR core +1 unit forms, leaving only the truncated form first described by De Lucia in Waap-depleted mutants [13]. These consisted of two Kdo, two L-glycero-d-manno-heptose (Hep) and one hexose, but were completely devoid of phosphate groups. The absence of Waap, a kinase responsible for the addition of a phosphate on the core unit, engenders the deep-rough phenotype [35]. Rough and deep-rough LPS mutants are found naturally and show reduced fitness [13]. As for the dnpA gene, the wapH is not well characterized and its role remains hypothetical. It was predicted to add the glucose II in (1→4)-linkage to N-(l-alanyl)-galactosamine (Fig. S2), and studies demonstrated that a WapH mutant was not completely devoid of O-polysaccharide and still produced some high-molecular-mass O-antigens [1, 36]. We show here that the
major LPS form in PAO1-18 is the truncated core, but unlike the DnpA mutant, some of the oligosaccharide chains may be A-chains. The Pgi mutant also possesses a truncated core form, together with core, SR core +1 and long O-chains. The MALDI-TOF analyses allowed us to describe these different forms in detail for the first time.

**Fine structure of the lipid A**
MALDI-TOF also allowed us to detect pyrophosphate groups substituting the reducing GlcN in the different *Pseudomonas* lipid A moieties obtained by LPS fragmentation. These molecular species have already been reported in *E. coli* and other genera, including *Pseudomonas*, and their presence was demonstrated by phosphate-labelled molecular species separated by thin-layer chromatography on lipid A biosynthesis intermediates [37]. They are usually too labile to be detected by MS methods. Their presence was, however, demonstrated in the low-mass region of different LPS, but not in the higher-mass region as obtained and shown here (Fig. 3) [30]. To the best of our knowledge, the present example is the first of its kind to be obtained directly on extracted LPS molecules in *Pseudomonas* lipid A high-molecular species. Another attempt to obtain such molecular species necessitated the improvement of culture conditions and their separation by ion-chromatography selection methods [31]. The accessibility and unusual detection of these molecular species in the present work were also certainly favoured by the use of the milder isobutyric–ammonium hydroxide extraction method, performed at room temperature [20], as opposed to the usual phenol–water procedure performed at 65 °C in acidic phenol conditions.

The high level of phosphorylation present in *P. aeruginosa* LPS with three phosphate groups on the lipid A GlcN disaccharide, added to the already-described high level of phosphorylation of the proximal core, makes this strategic region, close to the outer membrane, an amazingly charged area. If this region is usually rich in negative charges with the two phosphate groups present in lipids A and 1 or 2 phosphate groups substituting the core heptoses, in this case, at least two additional negative charges are present. These are known to favour interactions with receptors or other molecules, and could well be at the origin of phage attachment and adsorption to the bacterial membrane.

**LPS as a phage receptor**
Taking into consideration the different observations, we conclude that Ab09, Ab17 and Ab27 require the presence of B-chains to infect PAO1\(_{Or}\). More specifically, we think that Ab17 necessitates the presence of short B-chains to reach its receptor on the surface of the cell, since it is completely unable to infect variants lacking them, but it can infect PAO1-37 and PAO1-10 with the same efficiency, although producing smaller and more turbid plaques. In these two mutants, the short B-chains are present, even if in smaller quantities compared to the wild-type PAO1\(_{Or}\). Another observation that can support this theory is the fact that mutant PAO1-03, possessing a similar quantity of short B-chains to the wild-type PAO1\(_{Or}\), is normally susceptible to phage Ab17 infection.

Ab27 appears to require long B-chains for efficient infection. Unlike E79, another PB1-like phage, it does not seem to be capable of binding to the core and infecting mutants lacking A- and B-chains [16, 17]. Ultrastructural analyses showed that LPS extend 35–40 nm over the cell surface and that very long chains can extend up to 60 nm [38]. Ab27 possesses 50 nm-long tail fibres that may help the phage to reach the cell surface, after binding to long LPS chains. The results obtained with mutant PAO1-37 suggest that loop PL5 of the Wzy protein [10] may be part of the receptor for Ab27.

The molecule with which phage Ab09 interacts during the first steps of infection of *P. aeruginosa* PAO1\(_{Or}\) has still not been perfectly identified. It seems that Ab09 infection can occur when the short B-chains are not present, but only if the inner core is directly accessible on the bacterial surface, i.e. in the total absence of the O-antigen, such as in mutants PAO1-18 and PAO1-19. The uncapped core has been shown to be exposed and to mediate various interactions with the environment [38], and it was shown to serve as a receptor for phiPL27 podovirus [18].

The O-polysaccharides influence biofilm development and outer-membrane vesicle biogenesis [2]. In the context of increased antibiotic bacterial resistance, phagotherapy is presented as an alternative approach, although resistance to phages will also represent a challenge [39]. As both methods reach their limits when bacteria evolve and adapt to their environment, one can hypothesize that their complementarity could benefit different medical cases. A better knowledge of the mechanisms of bacterial resistance to phages is required and the present work provides a contribution to this domain by looking at phage–LPS interactions at the molecular level.

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**Conflicts of interest**
The authors declare that there are no conflicts of interest

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