RESEARCH LETTER

The *olsA* gene mediates the synthesis of an ornithine lipid in *Pseudomonas aeruginosa* during growth under phosphate-limiting conditions, but is not involved in antimicrobial peptide susceptibility

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Abstract

Pseudomonas aeruginosa responds to phosphate limitation by inducing the expression of phosphate transport systems, phosphatases, hemolysins and a DNase, many of which are important for virulence. Here we report that under phosphate-limiting conditions, *P. aeruginosa* produces a phosphate-free ornithine lipid (OL) as the primary membrane lipid. The *olsBA* (PA4350-PA4351) genes were highly induced under phosphate-limiting conditions. The production and structure of the OL was confirmed by MS, revealing diagnostic fragment ions and mainly C16 : 0 and C18 : 1 dialkyl chains. It was shown that *olsA* is required for production of these lipids and genetic complementation of the *olsA*::*lux* mutant restored OL production. Studies in other bacteria have correlated increased resistance to antimicrobial peptides with the production of OLs. Here it was demonstrated that resistance to antimicrobial peptides increased under phosphate-limiting conditions, but OLs were not required for this increased resistance. OL production was also not required for virulence in the *Caenorhabditis elegans* infection model. The production of OLs is a strategy to reduce phosphate utilization in the membrane, but mutants unable to produce OLs have no observable phenotype with respect to growth, antibiotic resistance or virulence.

Introduction

The response to phosphate limitation in *Pseudomonas aeruginosa* is diverse and includes the expression of phosphate acquisition systems, hemolysins, catalase, an alternative type II secretion system phosphatases, phenazines, pyoverdine, PQS and several auxiliary regulatory systems (Ostroff *et al.*, 1989; Hassett *et al.*, 1992; Ball *et al.*, 2002; Lewenza *et al.*, 2005; Jensen *et al.*, 2006; Zaborin *et al.*, 2009). We identified an extracellular DNase that is expressed and secreted under phosphate-limiting conditions and is required for utilizing extracellular DNA as a nutrient source of phosphate (Mulcahy *et al.*, 2010). There is accumulating evidence that phosphate limitation is an environmental challenge faced during an infection and therefore many of the phosphate-regulated virulence factors are likely important *in vivo* (Frisk *et al.*, 2004). Phosphate limitation occurs as a result of surgical injury to the gastrointestinal tract and leads to the induction of phosphate-regulated virulence factors in *P. aeruginosa* (Long *et al.*, 2008).

Another adaptation to phosphate-limiting conditions is the production of membrane lipids with non-phosphate-containing head groups. *Sinorhizobium (Rhizobium) meliloti* produces ornithine lipids (OL) that use ornithine as the head group instead of the more typical phosphate-containing head groups of most bacterial lipids (Weissenmayer *et al.*, 2002; Gao *et al.*, 2004). OLs are produced by multiple *Pseudomonas* species (Wilkinson, 1970; Kawai *et al.*, 1988). In *Pseudomonas fluorescens*, the production of OLs under phosphate-limiting conditions correlated with increased

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resistance to antimicrobial peptides, and it was suggested that OL function in resistance to antimicrobial peptides (Dorrer & Teuber, 1977). Upon binding of cationic peptides to negatively charged groups in bacterial membranes, peptides aggregate in the outer membrane and break the membrane integrity, leading to cell death. Resistance mechanisms to this class of antimicrobials often involve modifications that protect the bacterial membrane from peptide damage. In *P. aeruginosa*, these modifications include aminoarabinose modification of lipid A phosphates on lipopolysaccharide in the outer membrane (Gooderham & Hancock, 2009), while in Gram-positive bacteria, modifications are targeted to phospholipids and teichoic acid in the cell wall (Peschel, 2002). All modifications mask the negative surface charge, which reduces the binding of cationic antimicrobial peptides to the membrane.

We previously identified a number of phosphate-regulated genes by screening a library of mini-Tn*5-lux* mutants for genes that were strongly induced under phosphate-limiting conditions (Lewenza *et al.*, 2005). Among these genes, we identified PA4351 as a phosphate-regulated gene and a member of the two-gene operon PA4350–PA4351 (Lewenza *et al.*, 2005). In this report, we confirm the identity of the *P. aeruginosa olsBA* homologs, and demonstrate that OLs are a novel membrane lipid produced under phosphate-limiting conditions that have no role in resistance to cationic antimicrobial peptides or virulence.

Materials and methods

Strains and growth conditions

Pseudomonas aeruginosa PAO1 was used as the wild-type strain and *olsA*::*lux* mutant (75_C4) was previously constructed as part of a mini-Tn*5-lux* mutant library (Lewenza *et al.*, 2005). *Pseudomonas aeruginosa* was grown in basal medium 2 (BM2) as described previously (Mulcahy *et al.*, 2010). The phosphate source was a 2 : 1 mixture of K_2HPO_4 and KH_2PO_4. Low phosphate levels were defined as BM2 supplemented with 400 μM phosphate or less, and high phosphate medium was supplemented with 800 μM phosphate or more. Gene expression (bioluminescence) assays were performed in microplate assays as described previously (Mulcahy *et al.*, 2010).

Lipid detection and purification

For rapid lipid analysis, total cell lipids were extracted from 5 mL cultures that were grown overnight in BM2 medium. Cells from overnight cultures were pelleted by centrifugation and extracted with 100 μL of chloroform : methanol (1 : 2) to extract total lipids. The organic extract was spotted onto Al SigG/UV thin layer chromatography (TLC) plates (Whatman). TLC was performed in a solvent consisting of 1-propanol : chloroform : ethyl acetate : methanol : water (25 : 25 : 25 : 10 : 5.9). Total lipids were visualized by exposing the TLC plate to iodine vapor and amino group-containing lipids were visualized by spraying with the ninhydrin reagent (Sigma). For large-scale purification of OLs (~0.5 mg), large volume cultures were grown under phosphate limitation, extracted using the Bligh–Dyer protocol (Bligh & Dyer, 1959) and separated on TLC as described above. The suspected OL product was scraped and extracted from the silica and dried for MS analysis.

Capillary electrophoresis (CE)–MS Analysis of Lipids

Mass spectra were acquired using a 4000 QTrap mass spectrometer (Applied Biosystems/Sciex, Concord, ON, Canada) coupled to a Prince capillary electrophoresis system (Prince Technologies, the Netherlands). CE separation was obtained on a 90 cm length of bare fused-silica capillary (365 μm OD × 50 μm ID) with CE–MS coupling using a liquid sheath-flow interface and isopropanol : methanol (2 : 1) as the sheath liquid. An organic buffer consisting of 2 : 1 $CHCl_2$: MeOH with 50 mM ammonium acetate was used for all experiments in the positive and negative ion modes. Structural confirmation by CID MS/MS in positive and negative ion modes was performed with a collision energy of 55 eV. Precursor-ion scanning for the *m*/*z* 115 ornithine b-ion unique to this class of lipids was carried out in the positive-ion mode with a collision energy of 65 eV. Because precursor-ion scanning gives the advantage of specificity in observing ions, which gives rise to very specific fragments (*m*/*z* 115 in this case), the resolution settings on the scanning quadrupole (quadrupole 1) of the instrument were turned to low for increased sensitivity, with quadrupole 3 (which transmits only the 115.0 ion) set at unit resolution. Hence, the masses observed with precursor ion scans shown in the text are average masses, whereas masses observed with full-scan MS were acquired with unit mass resolution, resulting in monoisotopic masses being recorded for all ions. This is the reason for masses observed with precursor scans being systematically higher by approximately 0.7 a.m.u. from those observed with full-scan MS.

PCR amplification and cloning of *olsA*

PCR amplification of *olsA* was performed using *P. aeruginosa* genomic template DNA, Phusion High-Fidelity DNA Polymerase (Finnzymes) and the primers olsA-F4 (5′ ggaattCAAGATCTGCGGCGAGCCTTG) and olsA-R2 (5'cgggatc CTTGCCGATCAACGTGATCATG). The 1.06-kb PCR *olsA* product was EcoRI–BamHI digested and cloned into the medium copy vector pUCP22 under the control of the *lac* promoter. This construct (p*olsA*) was transformed into the *olsA*::*lux* mutant using 30 μg mL^{-1} gentamicin for selection.

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DNA sequencing confirmed the sequence identity of the cloned *olsA* gene.

Antibiotic killing and outer membrane permeability assays

Kill curves were performed as described previously (McPhee *et al.*, 2003) to determine the kinetics of polymyxin B killing of mid-logarithmic phase cultures grown in low and high phosphate BM2-glucose media. Minimal inhibitory concentrations (MIC) were assessed using the standard broth microdilution method in cultures grown in low and high phosphate BM2-glucose media (Wiegand *et al.*, 2008). The outer membrane permeability of polymyxin B-treated cells was measured using the 1-N-phenylnapthylamine (NPN) fluorescence assay (Hancock & Wong, 1984).

Caenorhabditis elegans infection model

Caenorhabditis elegans infections were performed as described previously with minor modifications (Powell & Ausubel, 2008). *Pseudomonas aeruginosa* strains were grown in Luria–Bertani for 18 h at 37 °C. Nine 3-μL drops of these overnight cultures were placed on each SK agar plates, which were incubated for 24 h at 37 °C and 24 h at room temperature. The plates were then stored at 4 °C until use. Cold plates were allowed to re-equilibrate to room temperature before transferring 30 wild-type L4 worms onto each plate. There were three plates (90 worms total) per *P. aeruginosa* strain and the killing kinetics were measured in two separate experiments. Live worms were counted every 24 h. At 48 h, worms were transferred to new SK plates of *P. aeruginosa* to avoid the confounding effects of progeny. Plates were incubated at 25 °C for the duration of the infections.

Results

PA4351 (*olsA*) expression under phosphate-limiting growth conditions

We previously screened a mini-Tn*5-lux* mutant library in *P. aeruginosa* to identify genes regulated by phosphate limitation. This approach led to the identification of PA4351, which has been annotated as being similar to 1-acyl-sn-glycerol-3-phosphate acyltransferase and shares modest identity (34.5% with six gaps) with the *S. meliloti* OL biosynthesis gene *olsA* (Weissenmayer *et al.*, 2002). The neighboring gene *PA4350* is 34.9% identical to nine gaps compared with *S. meliloti olsB*. In *S. meliloti*, the biosynthesis of ornithine involves two steps: formation of lyso-OL from ornithine by the OlsB 3-hydroxyacyl-AcpP-dependent acyltransferase activity and the acylation of lyso-OL by OlsA to form OL (Weissenmayer *et al.*, 2002; Gao *et al.*, 2004). There is a degree of sequence identity between PA4350-PA4351 and *olsBA* (~35%), and these genes were previously proposed as *P. aeruginosa olsBA* homologs (Gao *et al.*, 2004).

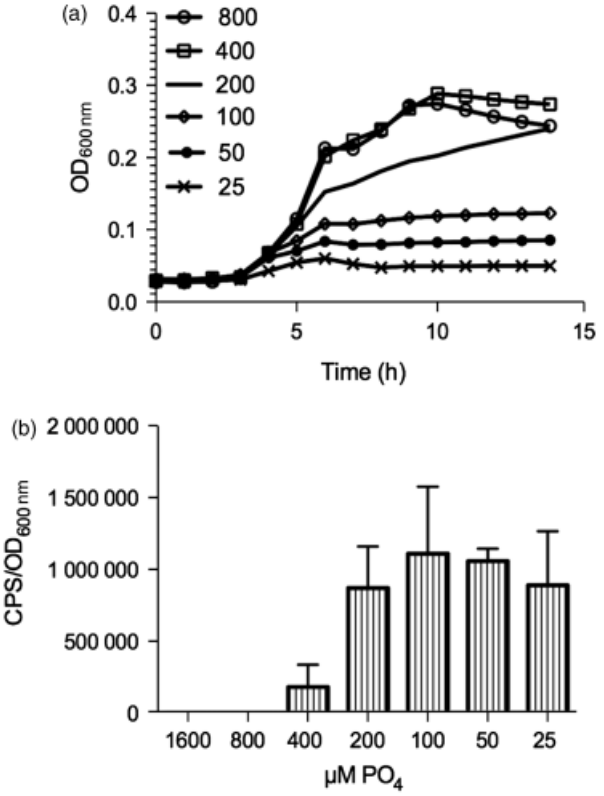

Fig. 1. The OL biosynthesis gene *olsA* is induced under phosphate-limiting conditions. (a) The wild-type PAO1 grows optimally in BM2-succinate containing 400 μM of phosphate or higher, but growth is limited in a concentration-dependent manner when the phosphate concentration is reduced below 400 μM. Varying phosphate concentrations are indicated. (b) Gene expression from the *olsA*::*lux* transcriptional fusion was measured every 20 min throughout growth at various concentrations of phosphate. The values shown are the average values from triplicate experiments of the maximum levels of gene expression during mid-log growth ($t = 8$ h), and error bars represent the SD. Gene expression is normalized to growth ($CPS/OD_{600\,nm}$).

Growth and gene expression were measured in BM2 media containing a range of phosphate concentrations between 1600 and 50 μM phosphate (Fig. 1). As the concentration of phosphate decreased, growth was limited in a concentration-dependent manner (Fig. 1a). Gene expression was monitored from the *olsA*::*lux* transcriptional fusion throughout growth at all phosphate concentrations. The *olsA* gene was not expressed in BM2 media containing 800 μM phosphate or more, but was strongly induced in BM2 media with 400 μM phosphate or less (Fig. 1a). The growth kinetics of the *olsA* mutant showed only a slight delay before entering the log phase of growth relative to the parent strain, but there was no significant effect on the growth rate or the final yield of growth after 18 h (data not shown). Given the modest identity to the

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S. meliloti olsBA genes and the below-described requirement for PA4351 in OL production, we named these genes *olsB* and *olsA*, respectively, in *P. aeruginosa*.

OL production under phosphate-limiting conditions

Total lipids were extracted from cultures grown under phosphate-rich and phosphate-limiting conditions, and analyzed by one-dimensional TLC. Figure 2 shows the TLC profiles of wild-type PAO1 and the *olsA*::*lux* mutant. In *P. aeruginosa* PAO1, the major lipid produced under phosphate-rich conditions is likely phosphatidylethanolamine based on a similar mobility of control phosphatidylethanolamine. However, a novel lipid species was produced under phosphate-limiting conditions, along with a significant reduction in phosphatidylethanolamine (Fig. 2). This novel lipid band was detected with iodine staining of total lipids (data not shown) and by ninhydrin staining for amino group-containing lipids (Fig. 2a). In the *olsA*::*lux* mutant grown under phosphate-limiting conditions, there was no production of this novel phosphate-regulated lipid species and a corresponding increase in phosphatidylethanolamine production (Fig. 2a). The TLC profiles of both strains under phosphate-rich conditions were similar, where phosphatidylethanolamine was the predominant lipid in the membrane (Fig. 2a). The *olsA* gene was cloned into a medium copy plasmid and introduced into the *olsA*::*lux* mutant, which restored the production of OLs under phosphate-limiting growth conditions (Fig. 2b).

To determine the identity of the novel phosphate-regulated lipid, this band was purified from the TLC plate and analyzed by MS. A positive-ion mode electrospray analysis of the purified lipid revealed major signals at 625, 651 and 665 *m*/*z* (Fig. 3a). Using the 115 *m*/*z* ion characteristic of ornithine (Geiger *et al.*, 1999; Aygun-Sunar *et al.*, 2006), it was possible to determine which of the observed signals corresponded to OLs according to the general structure shown in the inset in Fig. 3b. A cluster of signals from 598 to 706 *m*/*z* all contained the 115 *m*/*z* ion, strongly implicating the three major signals and several minor less intense signals as molecular ions of OLs.

Further confirmation for the presence of a cluster of OLs with varying acyl chains was achieved by MS/MS analysis of each of the major molecular ions. From the molecular anion signal, it is possible to determine the total number of carbon atoms in the two acyl chains and the number of unsaturated bonds (or cyclopropane rings); in the case of the 623.4 molecular anion signal, this corresponded to 32 : 0 (Fig. 4a). A major signal occurs upon cleavage of the terminal fatty acid (see inset) that is characteristic of the OL structure. Cleavage of the terminal fatty acid in Fig. 4a produced a 255 *m*/*z* fatty acyl anion of 16 : 0, and the expected signal of 367 was a dominant cleavage product. From these data, it is evident that the amido

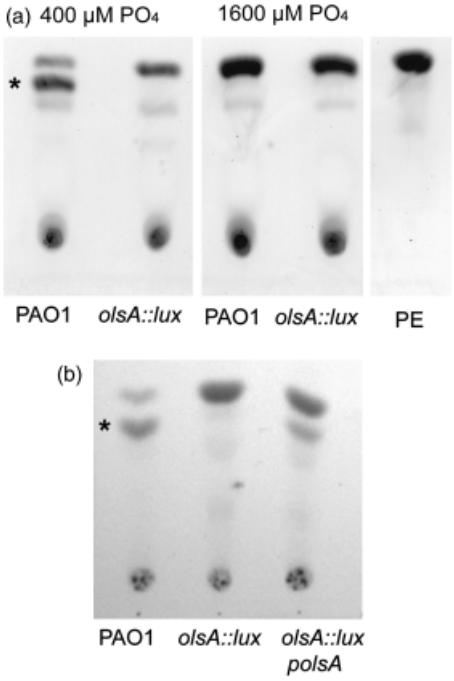

Fig. 2. TLC of total lipids from *Pseudomonas aeruginosa* wild type and *olsA*::*lux* detected with ninhydrin. (a) Under high-PO_4 conditions (1600 μM), the predominant lipid had a retention time similar to control phosphatidylethanolamine (PE) in both strains. Under low-PO_4 conditions (400 μM) there was a novel lipid species produced and a decrease in PE content. The novel lipid species under low-phosphate conditions (*) was absent in the *olsA*::*lux* mutant membrane extracts. (b) During growth in 200 μM phosphate, total lipids were stained with ninhydrin and the production of OLs was restored by complementation *in trans* with a plasmid encoding wild-type *olsA*.

chain must also be 16 : 0. Further, a 131 *m*/*z* cleavage product occurs as expected for OLs (Aygun-Sunar *et al.*, 2006).

Similar MS/MS analysis of the 649.6 *m*/*z* signal produced two major fragment ions of 367 and 393 *m*/*z*, indicating the occurrence of two OLs of the same mass (34 : 1). The fatty acid cleavage products were 255 and 281 *m*/*z* as expected for 16 : 0 and 18 : 1 chains, respectively, and illustrated as insets to Fig. 4b. A 131 *m*/*z* fragment typical of the ornithine ion is observed again. The third major OL anion was 663.6 *m*/*z* and could be assigned to the structure shown in the inset to Fig. 4c based on the 407, 255 and 301 *m*/*z* fragments. Thus, while the head group of these lipids is analogous to those observed for *S. meliloti*, the fatty acyl chains are quite different and rather typical of those observed for *Pseudomonas* phospholipids.

OLs are not required for antimicrobial peptide resistance or virulence

A previous study on *P. fluorescens* demonstrated a correlation between OL production and increased resistance to

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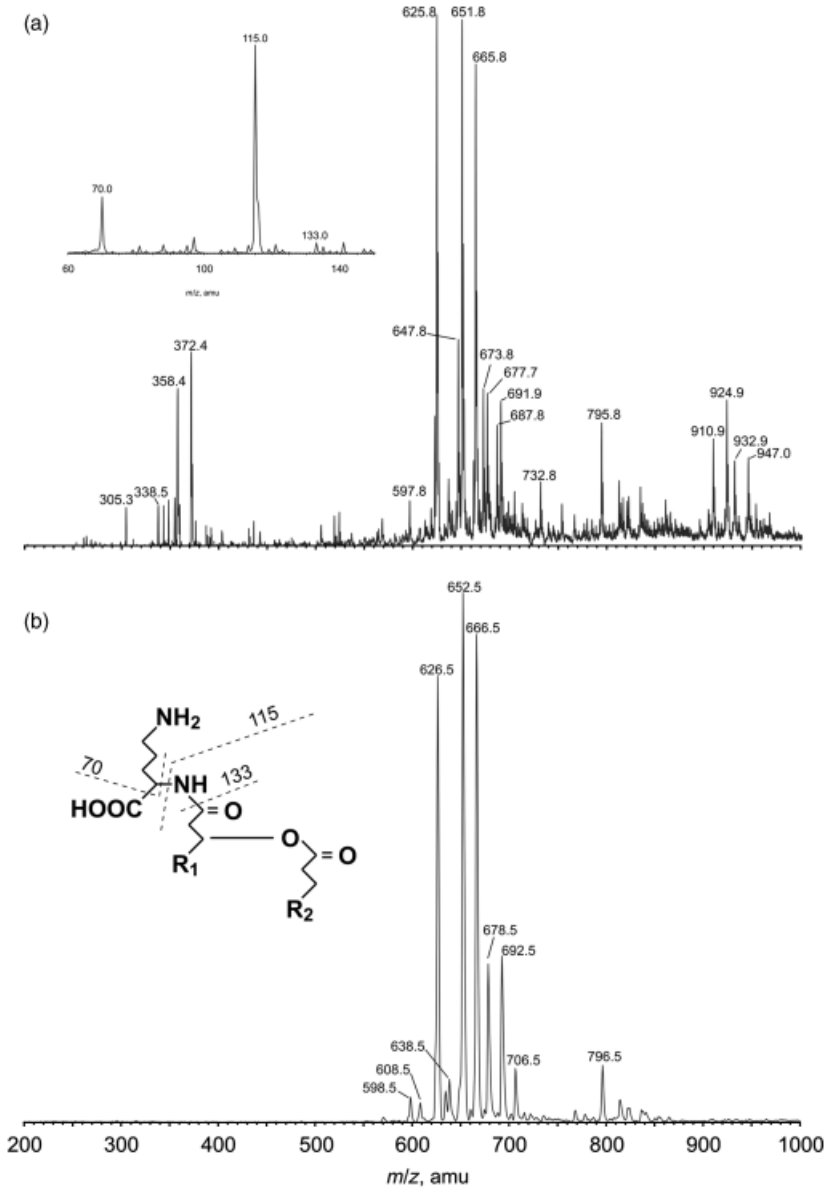

Fig. 3. Positive-ion mode electrospray mass spectra of the suspected OLs purified from *Pseudomonas aeruginosa* grown under low-phosphate conditions. (a) Positive-ion spectrum of OL fraction. (b) Positive-ion precursor spectrum showing molecular masses for lipids containing a 115 *m/z* fragment ion. The inset structure shows the formation of the 115/116 *m/z* ornithine ion, also reported for ornithine-lipids found in *Rhodobacter capsulatus* (Aygun-Sunar *et al.*, 2006).

polymyxin B under phosphate-limiting conditions (Dorrer & Teuber, 1977). It was proposed that the positively charged ornithine head group might prevent antimicrobial peptide binding to the membrane and thus limit the permeability of cationic antimicrobial peptides (Dorrer & Teuber, 1977). Resistance mechanisms to antimicrobial peptides often involve modifications of the membrane or surface that neutralize the negative charges in both Gram-negative and Gram-positive bacteria (Peschel, 2002; Gooderham & Hancock, 2009).

We wanted to determine if OL production was required for increased resistance to polymyxin B in *P. aeruginosa*. The polymyxin B resistance phenotype was determined for *P. aeruginosa* PAO1 wild-type and the *olsA*::*lux* mutant that were grown under high- and low-phosphate conditions. The killing kinetics of polymyxin B indicated that cells grown under low-phosphate conditions were 100-fold more resistant to polymyxin B killing than cells grown under high-phosphate conditions (Fig. 5a). However, OL production was not required for this increased resistance to polymyxin B under low-phosphate conditions (Fig. 5a). These data suggested that additional changes to the cell envelope were induced under limiting phosphate conditions that contributed to decreased membrane permeability to peptides. MIC assays were also performed with the *olsA*::*lux* mutant against a large panel of antibiotics that included polymyxin B, other cationic antimicrobial peptides and the cationic detergent chlorhexidine. OL production did not affect the resistance phenotype to any of the drugs tested (data not shown).

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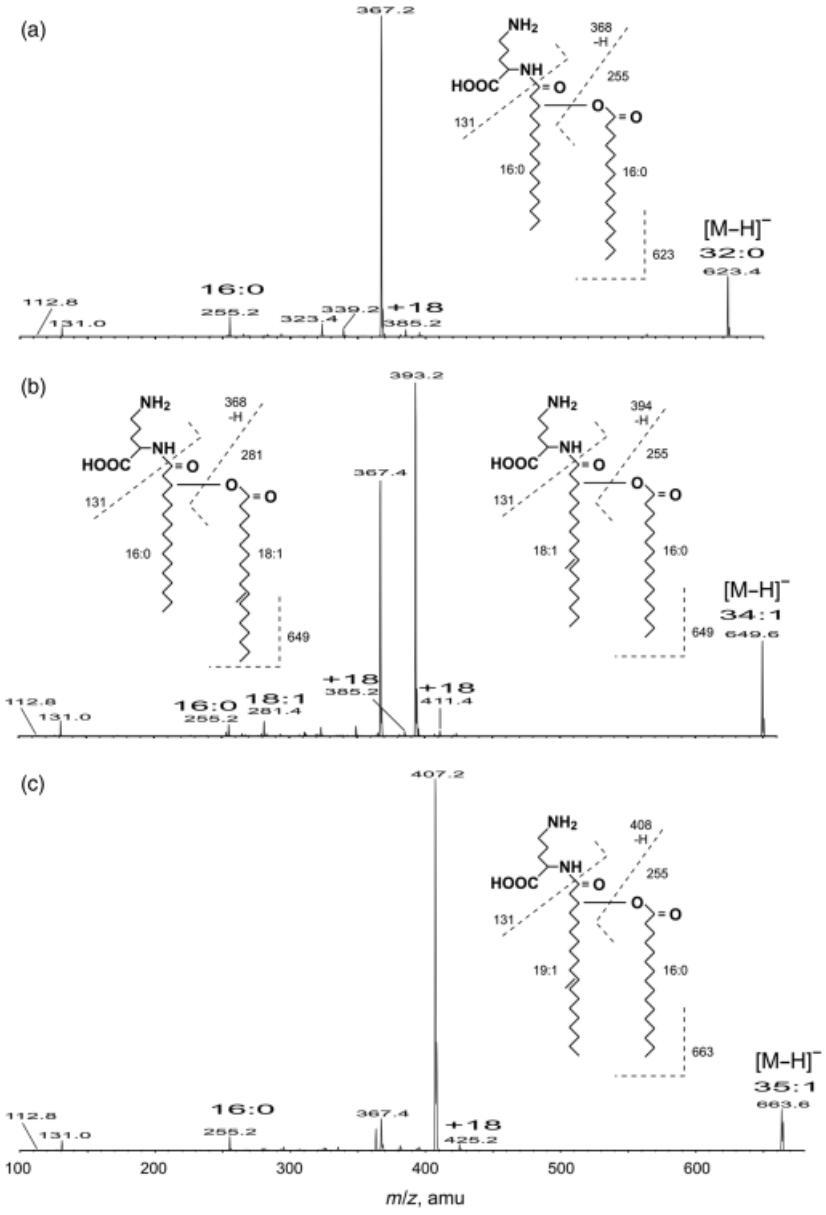

Fig. 4. Negative-ion electrospray MS/MS spectra of the three dominant molecular ion signals from OLs. (a) Major fragments of the 623.4 *m/z* molecular ion corresponding to a 32 : 0 OL; (b) major fragments of the 649.6 *m/z* molecular ion corresponding to a 34 : 1 OL; and (c) major fragments of the 663.6 *m/z* molecular ion corresponding to a 35 : 1 OL. The position of any unsaturation is currently unknown.

We used the NPN fluorescence assay to measure the incorporation of NPN into the outer membrane in polymyxin B-treated cells as a measure of outer membrane permeability. This assay measures the efficiency of self-promoted uptake across the outer membrane, a process that involves disruption of divalent cation-binding sites between adjacent lipopolysaccharide molecules on the surface of the outer membrane. Consistent with the kill curve data, PAO1 and *olsA*::*lux* outer membranes were equivalently susceptible to polymyxin B when grown under phosphate-rich conditions, which resulted in a greater amount of NPN incorporation into the membrane (Fig. 5b). Under phosphate-limiting conditions, there was restricted outer membrane permeability to NPN and under either conditions, the *olsA*::*lux* mutant resembled the parent strain, suggesting that OLs do not contribute to outer membrane permeability.

In a *C. elegans* infection model, worms that were fed *P. aeruginosa* lawns died from the production of multiple phosphate-regulated virulence factors that resulted in the 'red death' phenotype (Zaborin *et al.*, 2009). We tested the role of *olsA* in killing *C. elegans* by comparing the killing efficiency of worms fed with the wild-type PAO1 and *olsA*::*lux* strains. The *olsA* mutant was not impaired for killing *C. elegans* after 7 days postinfection (Fig. 5c).

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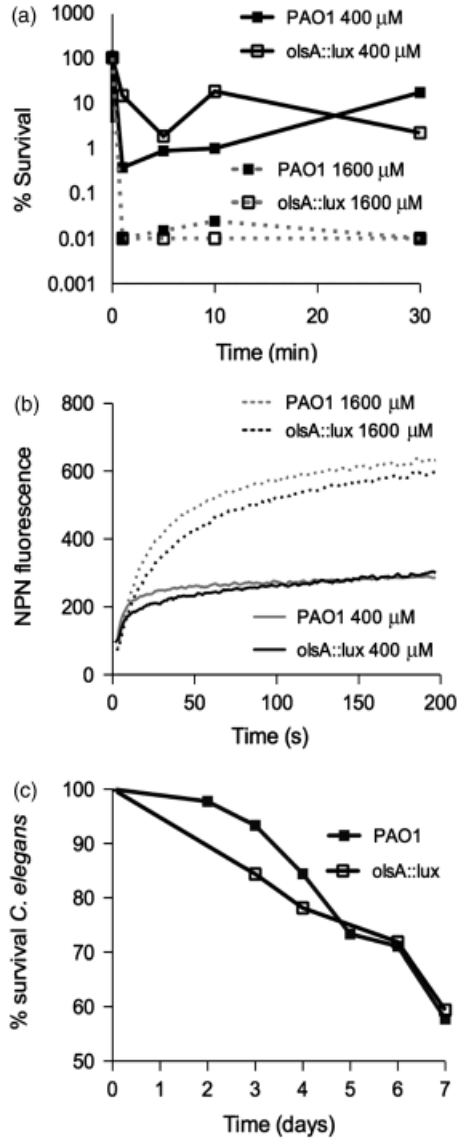

Fig. 5. Increased polymyxin B resistance in *Pseudomonas aeruginosa* grown under limiting phosphate conditions. (a) Killing kinetics after treatment of *P. aeruginosa* with 2 μg mL^{-1} polymyxin B. Cultures were pregrown in 1600 μM PO_4 (dashed lines) or 400 μM PO_4 (solid lines) before antibiotic treatment. (b) Outer membrane permeability was measured by monitoring the NPN fluorescence in cells treated with 0.8 μg mL^{-1} polymyxin B. The time zero point is after the addition of NPN and just before polymyxin B addition. Cultures were pregrown in 1600 μM PO_4 (dashed lines) or 400 μM PO_4 (solid lines) before antibiotic treatment. (c) Survival analysis of *Caenorhabditis elegans* feeding on *P. aeruginosa* wild-type PAO1 and the *olsA*::*lux* mutant.

Discussion

In this study we report the identification of the OL biosynthesis genes *olsBA* in *P. aeruginosa*. These genes are widely conserved among the genomes of the other *Pseudomonas* genomes annotated in the *Pseudomonas* Genome Database (Winsor *et al.*, 2009) and other Gram-negative bacteria (Geiger *et al.*, 2010), but have been studied only in two species of bacteria to date. The *P. aeruginosa olsBA* genes were strongly induced by phosphate limitation and are required for OL production. The production of a phosphate-free membrane lipid is a mechanism to adapt to phosphate-limiting conditions; however, we could not detect any major physiological consequence in a mutant unable to produce OLs. Despite limiting phosphate, the *olsA* mutant showed no significant growth defect, which is consistent with a report showing that only double mutants lacking OLs and diacylglyceryl-*N,N,N*-trimethylhomoserines have significant growth defects under these conditions (Lopez-Lara *et al.*, 2005).

We provide evidence that OLs do not contribute to antimicrobial peptide resistance, which contradicts the conclusions of an earlier study in *P. fluorescens* that showed a correlation between OL production and peptide resistance under phosphate-limiting conditions (Dorrer & Teuber, 1977). It was proposed that the positive charge of ornithine may prevent binding of cationic peptides to membranes (Dorrer & Teuber, 1977), but at neutral pH, OLs are zwitterionic, with a net neutral charge similar to phosphatidylethanolamine. However, we did observe increased resistance of cells grown in limiting phosphate and this is likely due to the remodeling of the outer membrane under phosphate limitation because the outer membrane was more impermeable to NPN incorporation after polymyxin B treatment (Fig. 5b). The most likely explanation, given that the NPN assay reflects self-promoted uptake across the outer membrane, is that cells grown in limiting phosphate incorporate less phosphate into the lipopolysaccharide in the outer membrane, and this may reduce peptide binding to the outer membrane. It is worth noting that under normal growth conditions, *P. aeruginosa* lipopolysaccharide contains 12–13 phosphate residues (Peterson *et al.*, 1985). Ingram *et al.* (2010) recently described a phosphatase that cleaves 1- and 4-phosphates from lipid A in *Rhizobium etli* and contributes to antimicrobial peptide resistance. Additional experiments are required to elucidate the mechanism of antimicrobial peptide resistance under phosphate-limiting conditions.

Acknowledgements

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