Modulation of quorum sensing in *Pseudomonas aeruginosa* through alteration of membrane properties

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Changes in the cellular envelope are major physiological adaptations that occur when micro-organisms encounter extreme environmental conditions. An appropriate degree of membrane fluidity is crucial for survival, and alteration of membrane lipids is an essential adaptive response. Emerging data suggest that microbial cells may recognize alterations in their membrane viscosity resulting from certain environmental changes as a trigger for adaptive cellular responses. In *Pseudomonas aeruginosa*, the quorum-sensing (QS) system involves a complex regulatory circuitry that coordinates the expression of genes according to a critical population density. Interestingly, it has been shown that the QS system of *P. aeruginosa* can also be activated by nutritional stress, independently of the cell density, and therefore may be part of a more general adaptive response to stressful environmental conditions. In order to examine the proposed link between membrane properties and stress signalling, the effects of genetically engineered alterations of the membrane phospholipid composition of *P. aeruginosa* PAO1 on the activation of the stringent response and the QS system were examined. The *lptA* gene encoding a functional homologue of PlsC, an *Escherichia coli* enzyme that catalyses the second step of the phospholipid biosynthesis pathway, was identified and disrupted. Inactivation of *lptA* altered the fatty acid profile of phospholipids and the membrane properties, resulting in decreased membrane fluidity. This resulted in a premature production of the QS signals *N*-butanoyl- and *N*-hexanoyl-homoserine lactone (C4-HSL and C6-HSL) and a repression of 2-heptyl-3-hydroxy-4-quinolone (PQS) synthesis at later growth phases. The effects on C4- and C6-HSL depended upon the expression of *relA*, encoding the (p)pGpp alarmone synthase, which was increased in the *lptA* mutant. Together, the findings support the concept that alterations in membrane properties can act as a trigger for stress-related gene expression.

INTRODUCTION

*Pseudomonas aeruginosa* is a versatile Gram-negative bacterium known to be an opportunistic pathogen of humans, but which can also be found in many diverse environments, such as soils, lakes, mineral water and in association with plants (Goldberg, 2000). Its ubiquitous occurrence makes *P. aeruginosa* a model organism for studying mechanisms of prokaryotic stress adaptation.

When bacterial cultures reach a sufficient cell density, phenotypes are modulated to favour cell survival and to permit the synthesis of secondary metabolites, enzymes and virulence factors, allowing the bacteria to colonize specific ecological niches (Rumbaugh et al., 2000; Winzer & Williams, 2001). This adaptive process relies on the bacterial production of autoinducer molecules that diffuse into the surrounding medium. In *P. aeruginosa*, there are two *N*-acyl homoserine lactone (N-AHL)-based quorum-sensing (QS) systems, the *las* and the *rhl* systems, behaving in a hierarchical manner (reviewed by Câmara et al., 2002). The main
N-AHL signalling molecules of *P. aeruginosa* are N-(3-oxododecanoyl)-l-homoserine lactone (3-oxo-C12-HSL), a product of LasI, and N-butanoyl-l-homoserine lactone (C4-HSL), synthesized by RhlI. 2-Heptyl-3-hydroxy-4-quinolone (PQS) is a third signal molecule that was originally described as a regulator of the production of the LasB protease (Pesci *et al.*, 1999). PQS is derived from anthranilate (Calfee *et al.*, 2001), and the genes responsible for the biosynthesis of PQS have now been identified (Gallagher *et al.*, 2002; D’Argenio *et al.*, 2002; Déziel *et al.*, 2004). Production of PQS is regulated by lasR, and PQS enhances the expression of *rhl*, *rhlR* and the sigma factor *rpoS*. Certain *rhl*-dependent phenotypes, such as pyocyanin synthesis, are dependent on the presence of PQS at the onset of the stationary phase (Diggle *et al.*, 2003).

It has been suggested that under extreme environmental conditions, the QS circuit may help *P. aeruginosa* to adapt to nutritional deficiencies, independently of cell density (Van Delden *et al.*, 2001). During the colonization of a biological niche, *P. aeruginosa* could be exposed to nutritional stress before a critical cell density has been reached. In this situation, premature activation of QS-regulated enzymes could provide the bacteria with the ability to utilize new nutrient sources. It has been demonstrated that both lasR and rhlR gene expression and autoinducer synthesis are prematurely activated during the stringent response induced by overexpression of *Escherichia coli* relA in *P. aeruginosa* PA01. RelA catalyses the synthesis of the alamine guanosine-3',5'-bispyrophosphate (ppGpp) in response to amino acid starvation or energy-source depletion (Van Delden *et al.*, 2001). More recently, Erickson *et al.* (2004) reported that, in conditions of low magnesium, the relA gene of *P. aeruginosa* acts to enhance the production of 3-oxo-C12-HSL and to reduce the production of PQS, when starvation is induced by serine hydroxamate. In contrast to the previous results of Van Delden *et al.* (2001), the effect of relA on the production of N-AHLS was not pronounced under normal magnesium availability, and no effect on C4-HSL production was detected. These data suggest that the connection between the stringent response mediated via relA and the QS system is complex and dependent on the environmental conditions.

The production of ppGpp is correlated not only with nutrient starvation but also with many kinds of growth perturbation (Cashel *et al.*, 1996). When external conditions become unfavourable, bacteria rapidly induce counteractive responses in order to maximize survival. In Gram-negative bacteria, the cell envelope comes into direct contact with the environment, and consequently it is potentially primarily involved in sensing environmental changes and triggering adaptive responses. The impact of variations in the membrane fluidity in the activation of stress responses has been reported for several bacteria (reviewed by Los & Murata, 2004). In cyanobacteria, it is thought that HikK3, a multiple sensor kinase that perceives cold stress, osmotic stress and nutrient stress (Van Waasbergen *et al.*, 2002), is activated via alterations of the cell membrane fluidity (Mikami & Murata, 2003). In *Bacillus subtilis*, DesK/DesR is a two-component system that regulates expression of the *des* gene encoding the Δ5-acyl lipid desaturase. It is believed that a decrease in membrane fluidity activates the DesK/DesR signal transduction cascade, resulting in desaturation of membrane lipids (Albanesi *et al.*, 2004). In *E. coli*, the expression of many regulatory genes is affected by an unbalanced membrane phospholipid composition, an effect that has been called ‘phospholipid specific stress signal’ (Inoue *et al.*, 1997). The class of major phospholipids includes the acidic phosphatidylglycerol (PG) and cardiolipin (CL), and the zwitterionic phosphatidylethanolamine (PE) (White & Tucker, 1969). The deficiency in one of the two acidic phospholipids, induced by genetic manipulation, has been shown to affect not only various membrane functions but also central cellular events, such as transcription of the flagellar operon and the superoxide dismutase genes (Inoue *et al.*, 1997).

We have examined the potential linkage between the changes in membrane properties, RelA expression and the N-AHLS-based QS system using *P. aeruginosa* as a model micro-organism. *De novo* synthesis of phospholipid in bacteria uses glycerol-3-phosphate (G3P) and fatty acids as the main substrates (Cronan & Rock, 1996). Two acyltransferase reactions sequentially transfer acyl groups from acyl-acyl carrier protein (acyl-ACP) or acyl-coenzyme A (acyl-CoA) to G3P. The second enzyme in the pathway of membrane phospholipid biosynthesis in *E. coli* is a lysophosphatic acid acyltransferase (EC 2.3.1.51), encoded by *pslC*. This enzyme transfers the acyl chain to lysophosphatic acid (LPA). The product, phosphatic acid, is subsequently converted into the major membrane phospholipids PE, PG and cardiolipin (Athenstaedt & Daum, 1999). Certain bacteria, such as *Neisseria meningitidis*, have been proven to possess two functional PslC homologues. Due to differences in substrate specificity, inactivation of one of these two LPA acyltransferases alters the fatty acid profile of phospholipids (Shih *et al.*, 1999). In a parallel manner, we decided to alter the phospholipid composition of *P. aeruginosa* by identifying and disrupting the gene encoding the main PslC homologue.

We have identified and inactivated the *lptA* gene encoding a LPA acyltransferase, characterized the resulting modifications in phospholipid composition and investigated the effects on the production of signalling molecules. We have demonstrated that inactivation of *lptA* affects the fatty acid composition of the membrane phospholipids and that these modifications are sufficient to activate the premature expression of a number of genes, including *relA* and those involved in the QS system, resulting in a premature production of N-AHL signalling compounds. These effects, which occurred independently of cell density, external stress exposure and nutrient starvation, provide further support for the concept that changes in the membrane structure can modulate stress-related gene expression in *P. aeruginosa*. 

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*References*


METHODS

Bacterial strains and growth conditions. All strains of P. aeruginosa PAO1 wild-type and derived mutants were maintained on Casamino acids (CAA) medium (Cornelis et al., 1992). P. aeruginosa PAO1 strains other than our laboratory strain were used and have been described previously (Beatson et al., 2002; Stover et al., 2000; Jacobs et al., 2003). The PAO1 relA mutant (ID 433) and pheA mutant (ID 7254) were obtained from the University of Washington Genome Centre (http://www.genome.washington.edu/UWGC/pseudomonas/). The P. aeruginosa PAO1 ppgR (MP551) mutant was constructed by Gallagher & Manoil (2001). Unless otherwise indicated, 50 ml cultures were inoculated from an overnight preculture and incubated at 37 °C for 200 r.p.m. Antibiotics were added to P. aeruginosa strains at the following concentrations: kanamycin (Km) 200 μg ml⁻¹, chloramphenicol (Cm) 300 μg ml⁻¹, tetracycline (Tc) 100 μg ml⁻¹, gentamicin (Gm) 100 μg ml⁻¹, spectinomycin (Sp) 600 μg ml⁻¹ and streptomycin (Sm) 600 μg ml⁻¹. E. coli strains were grown at 37 °C in LB with the appropriate antibiotics: 100 μg Km ml⁻¹, 100 μg Ap ml⁻¹, 25 μg Cm ml⁻¹ and 15 μg Tc ml⁻¹.

Construction and complementation of the P. aeruginosa PAO1 lptA mutant. The Gm cassette was amplified by PCR from the pBBR1MCS-5 vector (Kovach et al., 1995) with Taq polymerase (Life Technologies) using the following primers: gent1, 5’-tccgccggacaccgaggaaacgg-3’ and gent2, 5’-ttccgcggagatctggtctggcag-3’. The 800 bp fragment was digested with SacI and incubated at 37 °C for 2 h. Antisense and Cm sensitivity. The gene replacements were confirmed by resistance and Cm sensitivity. The gene replacements were confirmed by PCR using the following primers: Hdi1, 5’-agaggagaatcatctgctgagcactgcagc-3’ and Hdi2, 5’-ccctctcagagaacaccgac-3’.

The plasmid pPALPtA, containing the PAO006 and lptA genes from PAO1, together with the original promoter, cloned in the expression vector pBBR1-MCS (Kovach et al., 1995), was introduced into the appropriate strains by triparental mating using the helper strain carrying PRK2913. Transconjugants were selected on CAA containing chloramphenicol.

Motility assays. Swimming was analysed on tryptone swim plates (1 %, w/v, tryptone, 0.5 %, w/v, NaCl, 0.3 %, w/v, agar), which were inoculated using a sterile toothpick with bacteria from colonies grown overnight on LB agar at 37 °C. The plates were incubated at 37 °C and motility-assessed qualitatively by examining the circular zone of motile bacteria emerging away from the inoculation point.

Swarming was analysed on agar plates containing 0-5 % agar or agarose and 8 g l⁻¹ Difco nutrient broth supplemented with 5 g glucose l⁻¹. Bacteria from an overnight culture were inoculated onto plates using a sterile toothpick, and the plates were incubated overnight at 37 °C. A positive result was recorded as a branching growth over the agar surface away from the inoculation point.

Analysis of cellular fatty acid composition. The fatty acid composition of whole cells was assessed through fatty acid methyl ester (FAME) analysis by Accugenix, Newark, USA and Microcheck, Northfield, USA.

The phospholipid composition of P. aeruginosa WT and lptA mutant, and the fatty acid content of each individual phospholipid were determined by Mynefield Lipid Analysis, Dundee, Scotland. Briefly, the samples were extracted from cell pellets, obtained from cultures of P. aeruginosa strains grown in LB at 37 °C to OD₆₀₀ 0-5, using the Folch method (2:1:0.8 chloroform/methanol/KCl by vol.; Folch et al., 1957). Extracts were first spotted onto an aluminium-backed Silica Gel 60 TLC plate (Merck) and separated in two directions (first direction solvent mix, 65:25:4 chloroform/methanol/water by vol.; second direction solvent mix, 40:20:30:10:1:8 g chloroform/methanol/petroleum ether/acetic acid/boric acid by vol./v/v). The lipid spots were identified qualitatively, against standards, under a UV lamp after spraying a primuline solution (0.01 mg in 100 ml of aceton/water, 60:40, v/v). Weighed extracts, with C17:0 phosphatidylcholine added as internal standard, were spotted onto glass-backed Silica Gel 60 TLC plates (Merck) and separated into phospholipid species by one-dimensional TLC using the solvent system 80:12:15:4 chloroform/methanol/acetic acid/water. Each species band was scraped from the glass plate, and C21:0 methyl ester was added as a second internal standard and transsterified before being analysed by gas chromatography using flame ionization detection (FID) (Ichihara et al., 1996).

Cytoplasmic membrane fluidity assay (membrane polarization). Bacterial cells grown in LB at 37 °C to exponential phase were harvested by centrifugation and washed twice in sterile 15 mM Tris/HCl buffer (pH 7.0), and resuspended in the same buffer to OD₆₀₀ 0-4. The fluorescent membrane probe 1,6-diphenyl-1,3,5-hexatriene (DPH, Molecular Probes) (12 mM stock solution in tetrahydrofuran) was added to 3 ml of each sample (to a final concentration of 4 μM) in a quartz cuvette. The mixtures were mixed for 10 min in the dark at room temperature to allow probe incorporation into the cytoplasmic membrane. Fluorescence polarization was measured using a spectrofluorometer (Photon Technology International Inc., London, ON, Canada) equipped with a temperature-controlled cuvette holder and a magnetic cuvette stirrer. Excitation and emission wavelengths were set at 358 and 428 nm, respectively. The experiment was performed at 37 °C using the conditions described by Vincent et al. (2004). Data were recorded using FELIX software (Version 1.4, Photon Technology International). The correlation factor G was calculated as the ratio of parallel and perpendicular emitted light when the excitation light was horizontal. The degree of polarization (P) was calculated from the emission fluorescence intensities measured parallel (I1) and perpendicular (I2) to the plane of excitation light according to the following equation:

\[ P = 1 - \frac{I2}{I1} \]

Detection of N-AHLS and PQS by TCL. For N-AHL detection, acidified cell-free supernatants from LB cultures at 37 °C were extracted with dichloromethane (Diggle et al., 2002). PQS was extracted from the cultures (LB, 37 °C) with acidified ethyl acetate, as described by Diggle et al. (2003). TCL was used to characterize N-AHL (Shaw et al., 1997) and PQS (Diggle et al., 2003) production in P. aeruginosa PAO1 and lptA mutants. N-AHLS were detected on the TCL plate by overlay with the reporter strains Chromobacterium violaceum CV026, capable of sensing shorter-chain N-AHL molecules through the production of a violet pigment (Ravn et al., 2001), and Agrobacterium tumefaciens NTI, which carries a lacZ transcriptional fusion under the control of a N-AHL-inducible promoter for longer-chain N-AHLS (Shaw et al., 1997). PQS and anthranilate standards, as well as pggR (PQS-) and pheA (reduced anthranilate production) mutants were used as controls. It has been shown that the PQS⁻ mutant accumulates and excretes anthranilate into the growth medium (Déziel et al., 2004). Anthranilate is not easily distinguished from PQS under the conditions used, since both migrate at a close distance and display the same blue fluorescence (S. Diggle, personal communication). However, we have noticed that the spots of anthranilate, but not of PQS, turn yellow when the TCL plates are left for 24 h at room temperature, which allows an accurate analysis.

Comparative RT-PCR analysis. Total RNAs were purified from bacteria grown in LB medium at 37 °C to OD₆₀₀ 0-3, 0-5 and 0-8.
Two to four millilitres of culture were mixed with 8 ml RNA protect Bacteria Reagent (Qiagen) and, after centrifugation, pellets were stored at \(-80^\circ\)C for 24 h. Pellets were resuspended in Tris/EDTA containing lysozyme (1 mg ml\(^{-1}\)) for 10 min at 37 \(^\circ\)C, and RNA was then purified using the RNAeasy Midi columns (Qiagen). Contaminating DNA was removed by treatment with RQ1 RNase-free DNase I (Promega) at 0-025 U \(\mu l\)^{-1} for 1 h at 37 \(^\circ\)C. DNase was eliminated by RNA clean-up on an RNAeasy Midi column (Qiagen). cDNA synthesis was performed using RT SuperScript II (Invitrogen) with 10 \(\mu g\) RNA and 400 U reverse transcriptase. The reaction was carried out in a final volume of 20 \(\mu l\) for 1 h at 37 \(^\circ\)C. After RNase treatment for 1 h at 37 \(^\circ\)C (RNase I, 0-2 U \(\mu l\)^{-1}, Roche), cDNAs were purified using the RNAeasy mini kit from Qiagen. PCRs were carried out with the PCR master mix from Promega using the primers described in Table 1 and 50 ng cDNA template for 20 to 25 cycles. The proC gene, which is constitutively expressed (Savl et al., 2003), was used as the internal control to verify the absence of significant variation in cDNA level for housekeeping genes in all samples.

**Quantification of \(\beta\)-galactosidase activity from relA–lacZ translational fusion.** The translation of relA was evaluated using a strain carrying a lacZ translational fusion in the relA gene (mutant ID 433; Jacobs et al., 2003) and a lpta derivative of this strain isolated as described above. Both strains were grown in LB medium at 37 \(^\circ\)C, and \(\beta\)-galactosidase assays were performed as described by Miller (1972). Data are the mean of three independent experiments with duplicated samples. Data are presented as the mean \(\pm\) SD.

**Measurement of rhoI, lasI and pqsA transcription by lacZ translational fusions.** The level of pqsA transcription in *P. aeruginosa* WT and the lpta mutant was monitored using the vector pLPO996 (pqsA–IacZ translational fusion; McGrath et al., 2004).

To examine the transcription of rhoI and lasI, the vectors pMP01c and pMP02c were constructed as follows: a 910 bp fragment upstream from the start codon of rhoI was amplified with the high-fidelity pfx turbo taq polymerase (Invitrogen), using the primers rhoI A, 5'-ccgaattccgggagggctgc-3' and rhoIB, 5'-aactgcgtcgcgactccgtg-3'. The 370 bp fragment upstream from the start codon of lasI was amplified in the same way with the primers lasIA, 5'-cggaattcgcgcctgtcgtc-3' and lasIB, 5'-aactgcgtcgcgactccgtc-3'. The PCR products were digested with EcoRI and PstI, and inserted into the corresponding sites of the promoterless vector pMP220 (Spanik et al., 1987). The resulting pMP01c and pMP02c vectors were transferred into *P. aeruginosa* PAO1 WT and the lpta mutant by electroporation, and colonies were selected on 60 \(\mu g\) tetracycline ml\(^{-1}\). The inserts were verified by nucleotide sequencing (Lark Technology, UK). \(\beta\)-Galactosidase activity in *P. aeruginosa* WT and the lpta mutant, carrying pLPO996, pMP01c or pMP02c, was quantified as described by Miller (1972). Overnight cultures were washed and inoculated to a starting OD\(_{660}\) of 0-08. Cultures were grown for 24 h and \(\beta\)-galactosidase was assayed in duplicate and repeated at least three times. Data are presented as the mean \(\pm\) SD.

**RESULTS**

**Identification of lpta, a plsC homologue in *P. aeruginosa* PAO1**

The enzyme LPA acyltransferase is encoded by plsC in *E. coli* and shows strong sequence conservation across species. This facilitated a bioinformatics approach to identify the putative *P. aeruginosa* homologue of this enzyme. Using the BLAST tool of the PAO1 Genome Sequence website (www.pseudomonas.com), a gene (PA0005) encoding a protein that displayed homology to *E. coli* PlsC was identified. The protein encoded by PA0005 displays 28 % identity with PlsC of *E. coli* and 36 % identity with NlaA from *Neisseria meningitidis*, which has also been identified as a functional LPA acyltransferase (Shih et al., 1999). Like *E. coli* PlsC, the PA0005 protein product possesses consensus sequences specific for phospholipid and glycerol acyltransferases (blocks IPB00213A and IPB213B, DEBG, GenomeNet).

Two approaches were taken to test whether PA0005 encodes a functional LPA acyltransferase. First, the PA0005 gene was cloned into the vector pBBR1MCS and introduced into an *E. coli* plsC temperature-sensitive (ts) mutant (Coleman, 1990). Heterologous expression of PA0005 allowed the *E. coli* plsC ts mutant to grow at 42 \(^\circ\)C, proving that the PA0005 gene product can carry out the LPA acyltransferase reaction (data not shown). Second, a mutant strain deleted in the PA0005 gene was constructed by allele replacement, and the effect on membrane fatty acids was determined. FAME profiling on whole *P. aeruginosa* PAO1 WT and mutant cells showed that absence of a functional PA0005 was correlated with an increase in saturated and unsaturated C18 fatty acids and a parallel decrease in saturated and unsaturated C16 fatty acids (Table 2). The fatty acid profile of the mutant was also characterized by the absence of 3-hydroxy dodecanoic acid, tetradecanoic acid and cyclo-heptadecanoic acid. Complementation of the mutant with the *pta* gene in trans restored a normal fatty acid ratio (Table 2). This phenotype mirrored that of mutation of the *N. meningitidis* LPA acyl-transferase, NlaA. (Shih et al., 1999). Together, the conserved structural features, the functional complementation of the *E. coli* plsC mutant and the effect of mutation of PA0005 on the fatty acid profile of *P. aeruginosa* indicate that PA0005 encodes an LPA acyltransferase, justifying its designation as *lpta* (Lyso\_Phosphatidic acid acylTransferase A).

<table>
<thead>
<tr>
<th>Table 1. Primers used in comparative RT-PCR studies</th>
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<tbody>
<tr>
<td>Target gene</td>
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<tr>
<td>---</td>
</tr>
<tr>
<td>rhoI</td>
</tr>
<tr>
<td>pqsC</td>
</tr>
<tr>
<td>pqsA</td>
</tr>
<tr>
<td>lasB</td>
</tr>
<tr>
<td>lasI</td>
</tr>
<tr>
<td>proC</td>
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</table>
Table 2. Fatty acid profile of bacterial cells as determined by fatty acid methyl ester (FAME) analysis

The numbers represent the percentage of each fatty acid in a total cell extract of *P. aeruginosa* PAO1 WT, lptA mutant and lptA mutant carrying the lptA gene in trans [lptA (pPALptA)]. The data for WT and the lptA mutant represent the mean±SD of two independent analyses. Significant changes in fatty acid ratio are highlighted in bold.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Wild-type</th>
<th>lptA</th>
<th>lptA (pPALptA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10:0 3OH</td>
<td>3.87±0.40</td>
<td>3.17±0.17</td>
<td>3.61±0.40</td>
</tr>
<tr>
<td>12:0</td>
<td>3.61±0.00</td>
<td>3.35±0.04</td>
<td>2.75±0.00</td>
</tr>
<tr>
<td>12:0 2OH</td>
<td>4.45±0.05</td>
<td>3.86±0.10</td>
<td>5.42±0.05</td>
</tr>
<tr>
<td>12:0 3OH</td>
<td>4.37±0.02</td>
<td>4.35±0.34</td>
<td>3.98±0.02</td>
</tr>
<tr>
<td>12:1 3OH</td>
<td>0.42±0.40</td>
<td>–</td>
<td>0.41±0.40</td>
</tr>
<tr>
<td>14:0</td>
<td>0.70±0.00</td>
<td>0.80±0.80</td>
<td>0.56±0.00</td>
</tr>
<tr>
<td>16:1 (n-7)c/15</td>
<td>15.82±1.90</td>
<td>4.56±0.15</td>
<td>15.00±1.90</td>
</tr>
<tr>
<td>iso 2OH</td>
<td>24.09±1.90</td>
<td>17.03±1.46</td>
<td>30.16±1.90</td>
</tr>
<tr>
<td>17:0 cyclo</td>
<td>0.70±0.50</td>
<td>–</td>
<td>1.82±0.00</td>
</tr>
<tr>
<td>18:1 (n-9)c</td>
<td>–</td>
<td>1.04±0.40</td>
<td>–</td>
</tr>
<tr>
<td>18:1 (n-7)c</td>
<td>40.56±1.30</td>
<td>58.07±1.18</td>
<td>34.40±1.30</td>
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<tr>
<td>18:0</td>
<td>0.54±0.01</td>
<td>3.26±0.50</td>
<td>0.47±0.01</td>
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<td>19:0 cyclo (n-8)c</td>
<td>0.87±0.60</td>
<td>0.51±0.32</td>
<td>1.42±0.60</td>
</tr>
</tbody>
</table>

**Effects of the *P. aeruginosa lptA* mutation on membrane properties**

Since the bulk of fatty acids in the cell are found in the membrane, the FAME analysis indicates that the fatty acid composition of the membrane phospholipids is altered in the lptA mutant (Table 2). To determine whether this reflected an alteration in the levels of phospholipids, detailed analysis was carried out by two-dimensional TLC (Fig. 1) and gas chromatography (Table 3). Interestingly, neither the relative quantity of the four phospholipids, nor the total amount of phospholipids is significantly affected by the mutation of lptA, although the lptA mutant displays a slight reduction in phosphatidylcholine (PC) level compared to WT (Table 3). The modifications in the proportions of C16 and C18 fatty acids (Table 2) are conserved in each of the four main phospholipids, PE, PG, cardiolipin and PC, as demonstrated by FAME analysis of individual phospholipids (Table 4). It is notable that the decreased percentage in C16 fatty acids and the increase in the C18 fatty acids was also observed in PC, which is synthesized via the phosphatidylcholine synthase (PCS) pathway, in which choline is condensed directly with CDP-diacylglyceride to form PC (Wilderman et al., 2002). These data confirm that the alteration occurred in a common upstream enzymatic step.

Here we have shown that the effect of the mutation of lptA is to change the fatty acid profile of the membrane phospholipids by increasing the proportion of longer-chain (C18) fatty acids. Such modifications are known to decrease the membrane fluidity, since longer-chain fatty acids promote acyl chain packing by more easily spanning the width of the bilayer, conferring a more gel-like structure to the membrane (Russell, 1989). The higher proportion of total cis monounsaturated fatty acids may reflect the induction of adaptive mechanisms in the lptA mutant to counteract reduced membrane fluidity. This could occur via the introduction of a double bond during fatty acid synthesis (de Mendoza et al., 1983), or via the activity of cis/trans isomerase (Diefenbach et al., 1992). An in vivo assay was used to directly assess the membrane fluidity of WT and mutant *P. aeruginosa* strains. This assay measured the fluorescence anisotropy of a hydrophobic fluorescent probe 1-[4-(trimethylamino)phenyl]-6-phenyl-hexa-1,3,5-triene (DPH) inserted into the cytoplasmic membranes of *P. aeruginosa* WT and lptA mutant grown to exponential phase in LB.
medium at 37 °C. The rotation of the fluorescent probe in the cytoplasmic membrane depends on the membrane viscosity. The relationship between the probe polarization ratio and membrane fluidity is an inverse one (Vincent et al., 2004). The polarization value was 0.191 ± 0.005 (n = 3) for the wild-type and 0.213 ± 0.009 (n = 3) for the lptA mutant. Small differences in polarization can reflect significant changes in membrane fluidity; therefore, these data indicate that there is a measurable decrease in fluidity of the membrane in the lptA mutant. Such an alteration in membrane fluidity might be expected to have a negative effect on bacterial growth, particularly under low-temperature conditions where adaptation would normally occur in the wild-type. To assess this, doubling times of the lptA mutant were compared to wild-type over a range of temperatures (Fig. 2). It was found that growth of P. aeruginosa PA01 in LB (rich) medium at 37 °C was only slightly affected by inactivation of lptA [the doubling time was increased from 40 to 50 min; Fig. 2(a)] and the final OD of both cultures was identical. Interestingly, however, the lptA mutant displayed a cold-sensitive phenotype, with significantly reduced growth compared to the wild-type at 11 °C [the doubling time was increased from 230 min to 750 min; Fig. 2(b)]. One explanation for this is that the higher level of C18 fatty acids in the mutant does not allow a sufficient increase in membrane fluidity for full adaptation to growth at low temperature. Alternatively, rigid membranes could become leaky, allowing loss of intracellular ions from the cell. It has been reported that pcs

Table 3. Comparative phospholipid content of P. aeruginosa PA01 WT and lptA mutant

Analyses were performed twice with duplicate samples, as described in Methods. The results represent the mean and SD of four values.

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>Normalized percentage content</th>
<th>Percentage content by milligram of lipid extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>IptA</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>PG</td>
<td>15:12</td>
<td>1:99</td>
</tr>
<tr>
<td>PC</td>
<td>4:05</td>
<td>0:17</td>
</tr>
<tr>
<td>PE</td>
<td>68:37</td>
<td>3:72</td>
</tr>
<tr>
<td>Cardiolipin</td>
<td>12:45</td>
<td>1:55</td>
</tr>
<tr>
<td>Total</td>
<td>100:00</td>
<td>7:43</td>
</tr>
</tbody>
</table>

Table 4. Fatty acid profile of phospholipids of P. aeruginosa PA01 WT and lptA mutant

The values represent the percentage of each corresponding fatty acid in the phospholipid and are the mean of four experiments (two biological replicates of two samples). Significant changes in fatty acid ratio between P. aeruginosa WT and the lptA mutant are highlighted in bold.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Cardiolipin</th>
<th>Phospholipid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>IptA</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>14:0</td>
<td>0:88</td>
<td>0:02</td>
</tr>
<tr>
<td>15:0</td>
<td>0:28</td>
<td>0:10</td>
</tr>
<tr>
<td>16:2</td>
<td>0:49</td>
<td>0:25</td>
</tr>
<tr>
<td>17:0</td>
<td>0:34</td>
<td>0:04</td>
</tr>
<tr>
<td>16:3</td>
<td>0:21</td>
<td>0:05</td>
</tr>
<tr>
<td>C17:0cy</td>
<td>2:03</td>
<td>1:67</td>
</tr>
<tr>
<td>18:0</td>
<td>1:43</td>
<td>0:70</td>
</tr>
<tr>
<td>18:1(n-9)</td>
<td>0:66</td>
<td>0:15</td>
</tr>
<tr>
<td>18:1(n-7)</td>
<td>44:30</td>
<td>1:11</td>
</tr>
<tr>
<td>18:2</td>
<td>0:21</td>
<td>0:00</td>
</tr>
<tr>
<td>C19:0cy</td>
<td>3:70</td>
<td>2:91</td>
</tr>
<tr>
<td>20:1</td>
<td>0:00</td>
<td>0:00</td>
</tr>
</tbody>
</table>
mutants of *P. aeruginosa* (PC deficient) are more difficult to recover from stocks stored in 15% glycerol at −70 °C (Wilderman et al., 2002). This phenotype is also observed in the lptA mutant. Therefore, we can not exclude that the cold-sensitive phenotype of the lptA mutant is related to the observed decrease in PC level. Links have also been found between membrane fluidity and motility (Liaw et al., 2004; Lai et al., 2005), therefore we also looked at this phenotype in the lptA mutant. It was found that the lptA mutant was significantly impaired in both swimming (Fig. 3) and swarming motility (not shown), even at 37 °C, and that this phenotype was complemented by the lptA gene in trans. All these data are consistent with the hypothesis that deleting the lptA gene leads to structural changes in the bacterial membrane that also affect membrane properties such as fluidity.

**Production of quorum-sensing signalling molecules is altered in the lptA mutant**

As mentioned previously, emerging data suggest that the quorum-sensing (QS) system of *P. aeruginosa* can, in stressful conditions, be part of a global adaptive response that occurs independently of cell density. This motivated us to investigate the impact that changes in membrane structure may have on the QS system.

No significant difference in the amount of signalling molecules was found in the supernatants from stationary-phase cultures of PAO1 WT versus the membrane-modified lptA mutant. Interestingly however, the absence of LptA in *P. aeruginosa* correlated with premature production of C4-HSL and C6-HSL (Fig. 4a) during the exponential phase of growth (OD$_{600}$ 0·3 and 0·6), indicating that advanced production of these QS molecules is a phenotype of the lptA mutant. Introduction of the lptA gene in trans restored C4-HSL and C6-HSL to WT level. In contrast, no significant difference was observed in the relative levels of 3-oxo-C12-HSL between the lptA mutant and the WT strain under the same conditions at either OD$_{600}$ 0·6 (Fig. 4b) or OD$_{600}$ 0·3 (data not shown).

In contrast to the increased production of some N-AHL molecules, the *P. aeruginosa* lptA mutant displayed reduced production of PQS, monitored at the onset of the stationary phase (Fig. 4c) when PQS production is initiated (Diggle et al., 2003). The mutant did, however, accumulate anthranilate, the precursor of PQS (Gallagher et al., 2002), both in...
early exponential phase and in stationary phase (Fig. 4d).

Introduction of the lptA gene in trans into the lptA mutant restored PQS production and abolished anthranilate accumulation (data not shown). For all signalling molecules analysed, comparable results were seen when comparing P. aeruginosa PAO1 strains from several sources (cited in Methods) with their cognate lptA mutants, ruling out a possible effect of spontaneously acquired mutations in our laboratory strain, which could affect the production of signal molecules (Beatson et al., 2002). Furthermore, similar results were obtained if the bacterial cells were lysed prior to extraction of the signalling compounds (data not shown). This eliminated the possibility that differences in the levels of N-AHL and PQS signal molecules in the medium were due to differences in the diffusion rate through the altered membrane, rather than to differences in the level of synthesis.

The QS system is altered at the transcriptional level in the P. aeruginosa membrane-modified lptA mutant

The premature synthesis of the signal molecules in the lptA mutant is consistent with the hypothesis that alterations of the phospholipid composition activate the QS-dependent signal-transduction cascade in P. aeruginosa. To examine this phenomenon in greater molecular detail, we compared the expression of genes associated with the synthesis of QS signal molecules, C4-HSL, 3-oxo-C12 and PQS, in the WT and the lptA mutant. We also extended this analysis to include lasB (encoding elastase B), a gene regulated by the QS system. RT-PCR on RNA from PAO1 WT and the lptA mutant, extracted at three points in the exponential growth phase (OD_{600} 0·3, 0·5 and 0·8), was carried out using specific primers for each gene (Table 1). As shown in
Fig. 5(a), inactivation of lptA correlated with an increased expression of rhlI (encoding C4-HSL synthase) in cultures at OD$_{600}$ 0.3 and 0.5 and 0.8, and of lasI (encoding 3-oxo-C12-HSL synthase) at OD$_{600}$ 0.3 and 0.5. The overexpression of rhlI and lasI in the lptA mutant during the exponential phase of growth was confirmed by monitoring the β-galactosidase activity of rhlI–lacZ and lasI–lacZ transcriptional fusions in both WT and lptA backgrounds, using the plasmids pMP01c and pMP02c, respectively. As shown in Fig. 5(b), the lptA mutant carrying either the pMP01c or the pMP02c plasmid displays a significant increase in β-galactosidase activity compared to the WT in the first stage of growth. The difference in rhlI transcriptional level was significant at mid-exponential phase (OD$_{600}$ 0.5–0.8). Although relatively small, the difference in rhlI expression between WT and the lptA mutant is consistent with the increased synthesis of C4-HSL in the mutant (Fig. 4a). The differences we observed between RT-PCR and lacZ fusion in monitoring the transcript-level changes between WT and the lptA mutant may reflect an impaired stability of the plasmid in the lptA mutant.

Expression of pqsC and pqsA (directing PQS synthesis) was down-regulated in the lptA mutant under these conditions is shown in Fig. 2(a).
Role of relA in alteration of QS signalling molecule production in response to engineered modifications of *P. aeruginosa* membranes

Since the data on the QS molecules indicated that changes in the membrane properties caused by *lptA* inactivation led to a phenotype consistent with a stress response, we decided to investigate the role of the ppGpp synthase RelA in this process. RelA (via production of ppGpp) mediates the stringent response, can integrate many environmental signals and is known to control the QS system (Van Delden et al., 2001; Erickson et al., 2004). To assess the role of RelA, we inactivated *lptA* in a relA background and examined C4-HSL, C6-HSL and PQS production in the *relA* single mutant and in the *relA lptA* double mutant. In contrast to *E. coli*, in which the SpoT enzyme can also catalyse ppGpp formation, a *relA* mutant of *P. aeruginosa* produces no detectable amount of ppGpp (Erickson et al., 2004; Perron et al., 2005). As shown in Fig. 6(a), at OD<sub>600</sub> 0.3, C6-HSL production in the *relA lptA* strain is similar to the WT, whereas the *relA* mutant shows a production of C6-HSL intermediate between those of the WT and the *lptA* mutant. At OD<sub>600</sub> 0.3, the level of C4-HSL produced in the WT supernatant is still below the threshold of sensitivity of our detection assay, as shown also in Fig. 4(a), while the level of C4-HSL from the *lptA* mutant sample is already detectable. Since loss of RelA is sufficient to abolish the premature production of C4-HSL in the *lptA* mutant, the (p)ppGpp alarmone is implicated in the regulatory cascade initiated by the alteration of membrane structure. The *relA lptA* mutant is still repressed for PQS production however (Fig. 6b) and still accumulates anthranilate compared to the WT and the *relA* mutant (Fig. 6c). ppGpp has been proven to repress PQS production in *P. aeruginosa* (Erickson et al., 2004). However, in the *lptA* mutant, it seems that the repression of PQS synthesis is not mediated via RelA and ppGpp alone.

To further investigate the role of RelA in this signal pathway, we monitored expression of *relA* in the *P. aeruginosa* PAO1 WT and the *lptA* background using a chromosomal *relA*–*lacZ* translational fusion (Fig. 7). Although at higher OD<sub>600</sub> values there is no significant change in the level of expression of *relA*, expression is advanced in the *lptA* mutant compared to WT. These data indicate that alteration of...
the membrane leads to upregulation of the expression of relA early in the growth cycle.

**DISCUSSION**

**Links between membrane phospholipid composition and membrane structure**

Our approach to altering the membrane structure of *P. aeruginosa* has been to mutate the lptA gene encoding an orthologue of PlsC, the single LPA acyltransferase of *E. coli* that catalyses a common step in the synthesis of all phospholipids in that organism. Phospholipid biosynthesis is an essential process in bacteria, and since the *P. aeruginosa* lptA mutant is viable, it follows that *P. aeruginosa* must possess alternative enzymes with LPA acyltransferase activity. *In silico* analysis of the PAO1 genome revealed the presence of three other genes whose products share identical consensus sequences specific for acyltransferases (blocks IPB002123B): PA4351, PA3267 and PA2537. Of these three gene products, only PA4351 shows significant similarity with LptA (25% identity), PlsC (26% identity) and NlaA (31% identity). NlaA has been described as a second LPA acyltransferase of *N. meningitidis* (Shih et al., 1999) and possesses, like PlsC homologues, the additional IPB002123A block/domain. Although disruption of PA4351 resulted in a viable mutant, several attempts to construct a double lptA PA4351 mutant were unsuccessful, supporting the hypothesis that the combination of both mutations is lethal. Moreover, inactivation of PA4351 does not affect the fatty acid composition of the membrane (data not shown), suggesting that LptA is the main LPA acyltransferase in *P. aeruginosa* PAO1. The phosphate-starvation-induced PA4351 gene (Lewenza et al., 2005) encodes a protein with 50% similarity to the OlsA acyltransferase involved in ornithine lipid synthesis in *Sinorhizobium meliloti* (Weissenmayer et al., 2002). It has been suggested that the present group of ‘lysophosphatidic acid acyltransferases’ encompasses numerous subgroups with slightly different and overlapping biochemical activities.

In the wild-type, the fatty acid composition of each of the four phospholipids PG, PE, cardiolipin and PC, which we report here for the first time, was comparable. Furthermore, a very similar alteration in the fatty acid composition of each phospholipid was seen as a consequence of lptA mutation. This is consistent with the view that these fatty acid chains are added to a common phospholipid precursor and also with the proposed role of LptA in this process. The differing substrate specificity of LptA and of the alternative acyltransferases for fatty acids with respect to saturation and chain length presumably leads to the synthesis of phospholipids with different fatty acid components in the wild-type and the lptA mutant. We have demonstrated that the shift towards increased C18 and reduced C16 fatty acids in the lptA mutant leads to reduced membrane fluidity, as measured using a fluorescent probe. The cold sensitivity and lack of motility of the lptA mutant are phenotypes consistent with a reduction in membrane fluidity (Schujman et al., 2003; Liaw et al., 2004; Lai et al., 2005).

**Altering membrane structure leads to induction of a stress-responsive signalling cascade**

A number of studies have identified membrane sensors and membrane fluidity as being important for the bacterial stress response (Los & Murata, 2004). In this study, we addressed the hypothesis that changing the membrane structure in *P. aeruginosa* would activate downstream signalling pathways that would be important for bacterial adaptation. The data obtained support this hypothesis and indicate that changing membrane structure leads to increased expression of the stringent response regulator RelA. In turn, this activation of RelA results in increased expression of the lasI and rhlI genes, which direct synthesis of N-AHL cell–cell signalling molecules. Since the function of RelA is to produce ppGpp and (p)ppGpp (Erickson et al., 2004; Perron et al., 2005), it is likely that increased expression of the N-AHL-synthase...
genes arises because of elevated levels of ppGpp in the lptA mutant. Indeed, a link between relA and the N-AHLs-based QS system has been reported previously (Van Delden et al., 2001; Erickson et al., 2004). Consistent with the elevated expression of rhlR, we found that there was advanced production of C4-HSL and C6-HSL in the lptA mutant relative to wild-type. It might have been expected that the increased transcription of lasI in the lptA mutant at low cell density would lead to similarly elevated levels of 3-oxo-C12-HSL in the mutant, but this was not observed. The reasons for this are unknown, and the results may reflect an uncoupling between transcript levels, production and/or the active transport of the molecule. While C4-HSL diffuses freely in the medium, 3-oxo-C12-HSL requires the MexAB-oprM efflux system to be exported (Pearson et al., 1999). In addition, since the WT strain already starts to produce 3-oxo-C12-HSL in high amounts at low cell density, we cannot exclude that small differences in the level of production between WT and mutant do in fact occur, and that our biosensor-based detection assay is not sensitive enough to detect these small variations.

Because of the linkage between fatty acid biosynthesis and N-AHL synthesis in P. aeruginosa, it is important to consider other explanations for increased levels of C4-HSL and C6-HSL in the lptA mutant. One hypothesis could be that the inactivation of the main LPA acyltransferase LptA influences the availability of the acyl-ACP required for the synthesis of N-AHLs. Since N-AHLs derive their invariant lactone rings from S-adenosylmethionine and their variable acyl chains from the cellular acyl-ACP pool (Hoang & Schweizer, 1999), this could potentially increase the substrate availability for N-AHL synthases. However, we have shown that the premature production of N-AHLs does not occur in the relA lptA double mutant, although this strain exhibits the same changes in phospholipid fatty acid ratio as the lptA mutant (data not shown). These data seem to exclude a simple effect of substrate availability. It also points to a role for ppGpp in triggering the synthesis of N-AHLs, since in P. aeruginosa this alarmone is only synthesized via RelA, and a relA mutant has been proven to be totally defective in ppGpp synthesis (Erickson et al., 2004).

In addition to effects on AHL production, the lptA mutant also displayed alterations in the synthesis of another cell–cell signalling molecule, PQS. In this case, a decrease in production of PQS along with accumulation of anthranilate, a precursor molecule, was observed. This deficiency is explained by a substantial decrease in pqsA and pqsC transcripts, which encode PQS biosynthetic enzymes. Although, as with the effects on AHL synthesis, this phenotype was reversed on complementation with the wild-type lptA gene, wild-type levels of PQS were not restored in the relA lptA double mutant. This suggests that altering the membrane structure affects expression of genes in the PQS biosynthetic pathway independently of the ppGpp stress-response signal. Initially, that conclusion may appear to be at variance with a study that reported a decrease in PQS production in P. aeruginosa PA01 treated with serine hydroxamate, which triggers ppGpp production (Erickson et al., 2004). Under such conditions, inactivating relA relieved the repression of PQS synthesis. The conclusions of that study and our work are not mutually exclusive, however, and merely serve to demonstrate the complexity of regulation of PQS synthesis.

Conclusions

This study establishes that alterations in membrane composition and structure can be perceived by P. aeruginosa as a stress signal. For a ubiquitous species that can occupy a diversity of ecological niches, the ability to sense and adapt to environmental conditions is an important trait. Our data show that, independent of nutrient starvation or cell density, RelA is activated in response to a genetically induced alteration of membrane structure, and support the hypothesis that environmentally induced changes in membrane structure or composition will lead to a similar stress response. A separate proteomic study reported an increase in the level of RelA protein in P. aeruginosa under magnesium limitation (Guina et al., 2003), and it is known that membrane fluidity can be altered by the presence of ions such as Ca\(^{2+}\), Mg\(^{2+}\) and Sr\(^{2+}\) (Rilfors et al., 1984). It is tempting, therefore, to postulate that magnesium limitation and induced-phospholipid alterations increase the level of RelA via a similar mechanism. Activation of RelA leads to an increase in levels of ppGpp, an increased level of AHL synthases, and a range of well-documented downstream responses that facilitate the bacterium in adapting to new environmental conditions. The key question is what links alterations in the membrane to activation of RelA? This is not yet known, but further work will attempt to establish this mechanistic link and to identify other regulators that may play a role in the bacterial response to alterations in membrane composition and structure. This will shed further light on how ubiquitous bacteria, such as P. aeruginosa, are able to adapt their physiology and life cycle to allow growth in diverse environments.

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