The MexGHI-OpmD multidrug efflux pump controls growth, antibiotic susceptibility and virulence in Pseudomonas aeruginosa via 4-quinolone-dependent cell-to-cell communication

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INTRODUCTION

Pseudomonas aeruginosa is an ubiquitous Gram-negative γ-proteobacterium capable of causing disease in humans, animals and plants (Cao et al., 2001a; Lyczak et al., 2002). P. aeruginosa produces a vast array of extracellular virulence factors, the majority of which are regulated both in a cell population density-dependent manner, via cell-to-cell communication or ‘quorum sensing’ (Swift et al., 2001; Withers et al., 2001; Bassler, 2002; Smith & Iglewski, 2003), and in a growth phase-dependent manner (Diggle et al., 2002, 2003). P. aeruginosa possesses two N-acylhomoserine lactone (AHL)- dependent quorum sensing systems (reviewed by Winzer & Williams, 2001; Câmara et al., 2002). The las system comprises the N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) synthase, LasI and its cognate response regulator LasR, whilst the rhl system involves the N-butanoyl-L-homoserine lactone (C4-HSL) synthase and the response regulator RhlR (Winzer & Williams, 2001; Câmara et al., 2002). The las system regulates the production of extracellular virulence determinants such as elastase, the LasA protease, alkaline protease and exotoxin A (Winzer & Williams, 2001; Câmara et al., 2002). It also plays a role in controlling the expression of the xcpP and xcpR genes involved in the regulation of the
Type II general secretion pathway (Chapon-Hervé et al., 1997) and has been implicated in the maturation of P. aeruginosa biofilms (Davies et al., 1998). The rhl system controls the production of rhamnolipids, elastase, LasA protease, hydrogen cyanide, pyocyanin, siderophores and the cytotoxic lectins PA-II and PA-III (Diggle et al., 2002; Latifi et al., 1995, 1996; Winson et al., 1995). The las and the rhl systems are hierarchically organized such that the las system exerts transcriptional control over the rhl system (Latifi et al., 1996). However, the rhl system can also function independently of the las system (Diggle et al., 2003). Three independent transcriptome analyses have revealed that the las and the rhl systems influence the expression of more than 5% of the P. aeruginosa PAO1 genome (Schuster et al., 2003; Wagner et al., 2003; Hentzer et al., 2003).

Recently, an additional LuxR homologue, VqsR (virulence and quorum sensing regulation) has been shown to control the expression of a subset of quorum sensing regulated genes and to be needed for the full expression of siderophore biosynthesis and uptake genes (Juhas et al., 2004; Cornelis & Aendekerk, 2004).

P. aeruginosa also releases into the extracellular milieu 2-heptyl-3-hydroxy-4(1H)-quinolone, the pseudomonas quinolone signal (PQS), a molecule which closely resembles the 4-quinolone family of synthetic antimicrobials. The synthesis and action of PQS are modulated by the las and rhl systems respectively (Pesci et al., 1999). LasA regulates the production of PQS, and the addition of exogenous PQS to P. aeruginosa cultures enhances the expression of the elastase gene lasB, rhlII and rhlR as well as the alternative sigma factor, RpoS (Diggle et al., 2003; Pesci et al., 1999; McKnight et al., 2000). These findings suggest that PQS functions as a regulatory link between the las and rhl AHL-dependent quorum sensing systems and the stationary phase (via RpoS). The overlap between the quorum sensing and the RpoS regulons has been confirmed by a recent transcriptome analysis of the genes controlled by RpoS in P. aeruginosa (Schuster et al., 2004). Although the rhl system is active in the absence of PQS, production of certain rhl-dependent phenotypes such as PA-IL lectin and pyocyanin strictly depends on the presence of PQS at the onset of stationary phase (Diggle et al., 2003).

PQS is derived from anthranilate (Calfee et al., 2001), and the structural genes required for PQS biosynthesis have recently been identified (pqsABCD, pqsH plus the anthranilate synthase genes phnAB) together with the transcriptional regulator MvfR (PqsR) and a proposed effector, PqsE (Gallagher et al., 2002; Dèziel et al., 2004). The transcription of pqsH is regulated by the las quorum sensing system, linking AHL-dependent quorum sensing with PQS regulation (Gallagher et al., 2002). Evidence that PQS is produced during human infections has been obtained by the direct detection of PQS in P. aeruginosa strains from cystic fibrosis (CF) patients (Collier et al., 2002) and by data indicating that PQS synthesis is increased in P. aeruginosa isolates from CF airways (Guina et al., 2003).

We have previously described a genetic locus (mexGHI-opmD), conferring resistance to vanadium in P. aeruginosa (Aendekerk et al., 2002), of which the gene products MexH, MexI and OpmD are highly conserved in relation to other components of P. aeruginosa antibiotic efflux pumps (Poole, 2002). Recently, it was shown that a plasmid expressing mexH, mexI and opmD conferred elevated resistance to norfloxacin, ethidium bromide, acriflavine and rhodamine 6G, confirming that MexHI-OpmD functions as a multidrug efflux pump (Sekiya et al., 2003). We knew from our previous work that mutants in the non-coding region, upstream of mexGHI-opmD, in mexI and opmD were severely affected in the production of AHL-regulated exoproducts including elastase, rhamnolipids and pyocyanin (Aendekerk et al., 2002). In addition, the expression of lecA, which encodes the cytotoxic, galactophilic lectin PA-IL of P. aeruginosa, was markedly reduced by a Tn5 insertion into the mexGHI-opmD operon (Diggle et al., 2002). Here we demonstrate that the MexI efflux protein and the OpmD porin play a key role in controlling P. aeruginosa growth, antibiotic susceptibility and virulence via 4-quinolone-dependent cell-to-cell communication. We also show that loss of the pump by mutation may result in the accumulation of a toxic PQS precursor which could be responsible for the severely attenuated phenotype observed.

**METHODS**

**Growth conditions and strains.** All the bacterial strains and plasmids used in this study are listed in Table 1. The P. aeruginosa strain PA14 mexl and opmD mutants and the PAO1 strain mexl phnA and mexl pqsA double mutants were constructed by allelic exchange as described for the corresponding single mexl PAO1 mutants by Aendekerk et al. (2002). P. aeruginosa strains were grown in Luria broth (LB), Casamino acids medium (CAA) or ox broth (Statens Serum Institut, Copenhagen, Denmark) at 37 °C. When required, synthetic PQS was added to a final concentration of 60 μM, the synthetic AHLS 3-oxo-C12-HSL and C4-HSL at concentrations of 10 or 100 μM, and anthranilate at concentrations of 100 nM to 100 μM. Growth was continuously monitored in the Bioscreen apparatus (Life Technologies), using the following parameters: culture volume 300 μl, temperature 37 °C, shaking for 10 s every 3 min, and reading every 20 min. As inoculum, an overnight culture of PAO1 in CAA was diluted in order to achieve a final OD600 of 0.001. Each culture was inoculated in triplicate and each experiment was repeated three times.

**Synthesis of AHLS and PQS analogues.** C4-HSL and 3-oxo-C12-HSL were synthesized as described previously (Chhabra et al., 1993, 2003), and PQS was synthesized as described by Diggle et al. (2003) using the procedures of Pesci et al. (1999).

**TLC assays for C4-HSL and 3-oxo-C12-HSL detection.** Acidified supernatants from 18 h cultures (OD600 2) in LB at 37 °C were extracted with dichloromethane as described previously (Diggle et al., 2002; Yates et al., 2002) and analysed by TLC. Ten-microlitre samples of synthetic AHLs 3-oxo-C12-HSL and 3-oxo-C12-HSL at concentrations of 10, 100 μM and anthranilate at concentrations of 100 nM to 100 μM. Growth was continuously monitored in the Bioscreen apparatus (Life Technologies), using the following parameters: culture volume 300 μl, temperature 37 °C, shaking for 10 s every 3 min, and reading every 20 min. As inoculum, an overnight culture of PAO1 in CAA was diluted in order to achieve a final OD600 of 0.001. Each culture was inoculated in triplicate and each experiment was repeated three times.
Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or phenotype</th>
<th>Source or reference</th>
</tr>
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<tbody>
<tr>
<td><strong>P. aeruginosa strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type</td>
<td>ATCC 15692</td>
</tr>
<tr>
<td>PAO1 mexl</td>
<td>Mutant with a Gm cassette inserted in the gene mexl</td>
<td>Aendekerk et al. (2002)</td>
</tr>
<tr>
<td>PAO1 opmD</td>
<td>Mutant with a Gm cassette inserted in the gene opmD</td>
<td>Aendekerk et al. (2002)</td>
</tr>
<tr>
<td>PAO1 phnA</td>
<td>Mutant in the phnA gene</td>
<td>University of Washington Genome Center</td>
</tr>
<tr>
<td>PAO1 pqsA</td>
<td>Mutant in the pqsA gene</td>
<td>This work</td>
</tr>
<tr>
<td>PAO1 mexl phnA</td>
<td>Derivative of the phnA mutant with the mexl mutation</td>
<td>This work</td>
</tr>
<tr>
<td>PAO1 mexl pqsA</td>
<td>Derivative of the pqsA mutant with the mexl mutation</td>
<td>This work</td>
</tr>
<tr>
<td>PAO1 lecA':::lux</td>
<td>lecA':::luxCDABE chromosomal reporter fusion in PAO1</td>
<td>Winzer et al. (2000)</td>
</tr>
<tr>
<td>PAO1 lecA':::lux mexl</td>
<td>Derivative of the PAO1 lecA':::lux mutant with the mexl mutation</td>
<td>This work</td>
</tr>
<tr>
<td>PAO1 lecA':::lux opmD</td>
<td>Derivative of the PAO1 lecA':::lux mutant with the opmD mutation</td>
<td>This work</td>
</tr>
<tr>
<td>PA14</td>
<td>Wild-type able to infect different host</td>
<td>Tan et al. (1999)</td>
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<td>Mutant with a Gm cassette inserted in the mexl gene</td>
<td>This work</td>
</tr>
<tr>
<td>PA14 opmD</td>
<td>Mutant with a Gm cassette inserted in the opmD gene</td>
<td>This work</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
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<td>pSB536</td>
<td>AHL biosensor ahyR ahy'::luxCDABE in pAHP13 (AmpR) used for the detection of C4-HSL</td>
<td>Swift et al. (1997)</td>
</tr>
<tr>
<td>pSB1075</td>
<td>AHL biosensor lasR lasF::luxCDABE in pAHP13 (AmpR) used for the detection of 3-oxo-C12-HSL</td>
<td>Winson et al. (1998)</td>
</tr>
</tbody>
</table>

(Winson et al., 1998). For both biosensors, AHLs were visualized as bright spots on a dark background when viewed with a Luminograph LB980 (Berthold) photon video camera.

**TLC analysis of PQS production.** For the detection of extracellular PQS, cell-free spent supernatants were prepared from 18 h cultures (10 ml at OD600 2) and extracted with 10 ml acidified ethyl acetate. The organic phase was dried and resuspended in 50 μl methanol. Intracellular PQS was detected after disruption by sonication of a cell pellet prepared from a 40 ml overnight culture resuspended in 20 ml LB. PQS was then extracted from the supernatant of the cell lysate using equal volumes of acidified ethyl acetate. The solvent was evaporated and resuspended in 100 μl methanol. Ten-microlitre samples of each extracellular and intracellular extract were spotted onto normal-phase silica 60F254 (Merck) TLC plates, pretreated by soaking in 5% K2HPO4 for 30 min and activated at 100 °C for 1 h. Extracts were separated using a dichloromethane/methanol (95:5, v/v) system until the solvent front reached the top of the plate. PQS was visualized under UV light and identified by comparison with a synthetic standard (5 μl of a 10 mM stock).

**Antibiotic resistance test.** Antibiotic resistance was measured by the filter-disk assay method using commercially available disks (Fluka). The following antibiotics were tested: spectinomycin (15 μg per disk), tetracycline (30 μg), nalidixic acid (30 μg), chloramphenicol (30 μg), kanamycin (30 μg), rifampicin (15 μg) and carbencillin (30 μg). Three millilitres of a cell suspension (5 x 106 c.f.u. ml−1) was added to a LB plate and left for 30 min, after which the cell suspension was removed and the plate dried under laminar flow. The disks were applied to the plates (four per plate) and the plates incubated for 18 h at 37 °C. The diameter of the inhibition ring was then measured. Each experiment was done in triplicate.

**Quantification of pyocyanin.** For pyocyanin analysis bacteria were spread onto PAB agar (Difco) in numbers that enabled confluent growth and incubated at 37 °C for 48 h. Pyocyanin was extracted from the agar medium and quantified according to Mavrodj et al. (2001).

**Measurement of bioluminescence.** Bioluminescence was measured as a function of cell density using a combined automated luminometer–spectrophotometer (LUCYI, Anthos Labtech). Overnight cultures of the P. aeruginosa lecA':::luxCDABE reporter gene fusion were diluted to OD600 0-01 in LB and 200 μl of this dilution was added to each well of a LUCY plate. Where required, PQS (60 μM) or C4-HSL (100 μM) or both were added. Bioluminescence and optical density were determined. Luminescence is given, in relative light units (RLU) divided by OD600, indicating the approximate light output per cell.

**RT-PCR.** RNA was extracted from P. aeruginosa PAO1, mexl and opmD grown in LB using the High Pure RNA Isolation Kit (Roche Diagnostics). cDNA was synthesized using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia). For PQS biosynthesis gene expression, RT-PCR was done using two sets of primers: pqsA1 and pqsA2 or phnA1 and phnA2 (Table 2). The primers used for lasI and rhlI transcript detection are also shown in Table 2. As a control, RT-PCR was done using primers oprL-1 and oprL-2 for the amplification of the housekeeping gene oprL (Lim et al., 1997). The complete list of primers used in this study is shown in Table 2.

**Construction of the PAO1 pqsA, mexl pqsA and mexl phnA mutants.** A pqsA chromosomal deletion mutant in PAO1 lacking 1172 internal nucleotides was constructed as follows. Using PAO1 DNA as a template, a 2600 bp fragment containing the intact pqsA gene (1554 bp) was amplified using the primer pair pqsAUF containing a Sall restriction site (5'-TAGGTGTGCACTTGCGCA-GGCTCGGC-3') and pqsAUR containing a HindIII restriction site (5'-GGCTAAAGCTTGGGCAATCCAGGT-3'). The resulting PCR product was digested with Sall and HindIII and cloned into similarly digested pUC18, resulting in the plasmid pUCpqsA. To introduce a deletion of the recombinant pqsA gene, the primer pair DpqsAUP (5'-TCGGGAGCTTACGACCCGAAAG-3') and DpqsAUR (5'-TTCCCCATGACGGATTTGTAATTACA-3') was used in conjunction with inverse PCR using pUCpqsA DNA as a template. The resulting blunt-ended PCR product containing a 1172 bp deletion in pqsA was self-ligated, resulting in the plasmid...
pUCpqsA. The PCR product was excised from the vector using SalI and XbaI, and cloned into the suicide vector pDM4 (Milton et al., 1996) digested with SalI and Xbal, in the resulting plasmid pDM4pqsA. Allelic exchange using pDM4pqsA contained in E. coli S17-1 Δpir with PA01 resulted in a P. aeruginosa strain (PA01 pqsA) containing an in-frame deletion of the pqsA gene. This deletion was confirmed by PCR analysis (data not shown). The phnA deletion mutant is a transposon mutant from PAO1 obtained from the University of Washington Genome Center. The mexI pqsA or mexI phnA double mutants were made by inserting a GmR cassette in the mex gene of the pqsA and phnA mutants as previously described (Aendekerk et al., 2002).

**Virulence assays in plants and animals**

**Animals.** Lung infection in rats was performed as previously reported (Song et al., 1998). PAO1 and mexI strains were first cultured on agar plates, and then one colony of each strain was inoculated into ox broth and cultured at 37°C. Cultured on agar plates, and then one colony of each strain was cultured to the stationary phase at 37°C. Mutants were grown overnight in LB medium or until they reached a density of 10^8 c.f.u. ml^{-1}. Challenge concentrations for each rat (female, 7-week-old Lewis rats with a body weight of between 150 and 200 g). The challenge concentration for bronchus for each rat was determined from a series of pilot studies. The mortality rate was followed up to 7 days post-challenge, after which all animals were sacrificed. Lung pathology was expressed as lung index of macroscopic pathology (LIMP) and pathological scoring. LIMP represents the lung area pathology was expressed as lung index of macroscopic pathology (LIMP) and pathological scoring. LIMP represents the lung area.

**Plants.** P. aeruginosa strain PA14 and the isogenic mexI and opmD mutants were grown overnight in LB medium or until they reached the stationary phase at 37°C, and washed in 10 mM MgSO_4. Cells were then diluted to 10^7 and 10^8 c.f.u. ml^{-1}. Ten microlitres of diluted cells was inoculated with a micropipette into the stems of Romaine lettuce plants. The stems were washed with 0.1% bleach and placed on 15 cm diameter Petri dishes containing a Whatman filter impregnated with 10 mM MgSO_4. The midrib of each lettuce leaf was inoculated with each of the three P. aeruginosa strains to be tested. For some experiments, PQS (60 μM) was either co-injected with the bacteria or injected on its own. The plates were kept in a growth chamber at 28°C and symptoms monitored daily for 5 days.

### RESULTS

**MexI and OpmD are required for AHL and PQS production**

We have previously shown that mutations in the P. aeruginosa genes mexI and opmD from the MexGHI-OpmD efflux system result in the inability to produce detectable levels of C6-HSL and C4-HSL using the AHL biosensor strain Chromobacterium violaceum CV026 (Aendekerk et al., 2002). However, CV026 has a relatively low sensitivity for C4-HSL (McClean et al., 1997) and is not activated by 3-oxo-C12-HSL. To gain better insights into the production of AHLs by the mexI and opmD mutants, two alternative and more sensitive AHL biosensor strains were used in conjunction with TLC (Diggle et al., 2002; Yates et al., 2002). These were the lux-based E. coli(pSB536) for the detection of C4-HSL and E. coli(pSB1075) for 3-oxo-C12-HSL (Swift et al., 1997; Winson et al., 1998). We also investigated the effect of the mexI and opmD mutations on PQS production using the TLC-based method described by Calfee et al. (2001). Fig. 1 shows that the mexI and opmD mutants produced no detectable 3-oxo-C12-HSL or PQS and markedly reduced levels of C4-HSL in spent-culture supernatants. However, the addition of 60 μM synthetic PQS to cultures of mexI and opmD mutants resulted in the restoration of both 3-oxo-C12-HSL (Fig. 1a) and C4-HSL production (Fig. 1b), although in the mexI mutant, 3-oxo-C12-HSL levels did not return to wild-type (Fig. 1a, lane 5). In contrast, exogenous provision of 3-oxo-C12-HSL did not restore PQS synthesis in these mutants (data not shown), demonstrating that the PQS-negative phenotype was not simply due to a non-functioning las system. This is believed to be the first time that 3-oxo-C12-HSL synthesis has been shown to be PQS-dependent. Furthermore, no intracellular PQS (Fig. 1c) or AHLs (data not shown) could be detected in either the mexI or opmD mutants, indicating that the loss of the AHLs was not a consequence of the inability to export 3-oxo-C12-HSL or PQS.

**Table 2. Primers used for RT-PCR**

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer designation</th>
<th>Primer sequence</th>
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<tbody>
<tr>
<td>oprL</td>
<td>oprL-1</td>
<td>5’-ATGGAATGTGAATTCGACC-3’</td>
</tr>
<tr>
<td></td>
<td>oprL-2</td>
<td>5’-CTCTTTAGCAGGGAGGGACG-3’</td>
</tr>
<tr>
<td>lasI</td>
<td>lasI-1</td>
<td>5’-TCAAAAAGGTCGCGCGGCAA-3’</td>
</tr>
<tr>
<td></td>
<td>lasI-2</td>
<td>5’-TAAACGGGATCTGGGTCTTT-3’</td>
</tr>
<tr>
<td>rhII</td>
<td>rhII-1</td>
<td>5’-AGTACCGAATTGCTCTGGA-3’</td>
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<td>rhII-2</td>
<td>5’-TCACCCGACCGACCAGCGGTA-3’</td>
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<tr>
<td>pqsA</td>
<td>pqsA-1</td>
<td>5’-AAATGGCCGACCTCACATTCT-3’</td>
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<td>pqsA-2</td>
<td>5’-TCCTCGATAGTGTGTCCT-3’</td>
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<td>5’-ACTGGAAAGAAGTCTGGA-3’</td>
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<tr>
<td></td>
<td>phnA-2</td>
<td>5’-TGGGTGTCGAGCGCACA-3’</td>
</tr>
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</table>
Expression of quorum sensing regulated virulence determinants in the mexI and opmD mutants can be restored by exogenously supplied PQS

The fact that mutations in mexI and opmD abolish or drastically reduce the production of quorum sensing signal molecules suggests that this may also result in a decreased production of quorum sensing regulated virulence determinants in these mutants. If this is the case, since PQS has a central role in quorum sensing-mediated gene expression (Diggle et al., 2003) and can restore AHL production in the unstabilized mexI and opmD mutants, addition of PQS to these mutants should restore the production of virulence determinants. In our previous work, we showed that the production of elastase and pyocyanin was drastically reduced in the mexI and mexGHI-opmD mutant (Fig. 2). In contrast, the provision of exogenous PQS to the mexI mutant restored lecA expression to wild-type levels (Fig. 2). Similar results were obtained with the opmD mutant in the lecA::lux expression (data not shown). This is consistent with our recent work (Diggle et al., 2003), in which we showed that inhibition of PQS biosynthesis, in the presence of a functional rhl system, abolished PA-IL production. Addition of a combination of PQS and C4-HSL to these mutants further enhanced lecA::lux expression (data not shown). In contrast, 3-oxo-C12-HSL had no effect on lecA transcriptional levels (data not shown), as shown previously (Winzer et al., 2000).

mexI and opmD mutants show enhanced antibiotic resistance which can be reversed upon addition of exogenous PQS

We have previously described the role of the mexGHI-opmD locus in resistance to vanadium in P. aeruginosa (Aendekerk et al., 2002). Also, a mutant (ncr, with an insertion in the non-coding upstream region) unable to express mexGHI-opmD was shown to be more resistant to tetracycline and to the combination of ticarcillin and clavulanic acid (Aendekerk et al., 2002). To investigate whether mutations in mexI and opmD confer altered susceptibility to other antibiotics, we tested a range of these against the two mutants. Confirming and extending our previous results, we observed that the mexI and opmD mutants became
resistant to kanamycin and spectinomycin, while they displayed a decreased sensitivity to carbenicillin, nalidixic acid, tetracycline, chloramphenicol and rifampicin (Table 3). Furthermore, the sensitivity towards all antibiotics was partially or totally restored upon provision of exogenous PQS to the mutants, in a way similar to complementation by partially or totally restored upon provision of exogenous PQS (Aendekerk et al., 2002).

To investigate whether mutations in mexI and opmD affected the growth of P. aeruginosa, which could also account for the increase in antibiotic resistance observed, the growth of both mutants was monitored. As shown in Fig. 4, in LB liquid medium at 37 °C, an extended lag phase was observed for both mutants (20–40 h) compared with the wild-type (8 h), although the growth rate was

### Table 3. Antibiotic sensitivity of wild-type, mexI and opmD mutants in the absence and presence of PQS

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>PAO1</th>
<th>PAO1 + PQS</th>
<th>mexI</th>
<th>mexI + PQS</th>
<th>opmD</th>
<th>opmD + PQS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kanamycin</td>
<td>1·35 ± 0·05</td>
<td>2·0 ± 0·01</td>
<td>0</td>
<td>1·55 ± 0·05</td>
<td>0</td>
<td>1·65 ± 0·05</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>2·9 ± 0·1</td>
<td>4·1 ± 0·1</td>
<td>0</td>
<td>1·25 ± 0·05</td>
<td>0</td>
<td>1·3 ± 0·1</td>
</tr>
<tr>
<td>Carbenicillin</td>
<td>3·6 ± 0·1</td>
<td>4·9 ± 0·1</td>
<td>1·15 ± 0·05</td>
<td>4·25 ± 0·05</td>
<td>1·15 ± 0·05</td>
<td>4·4 ± 0·1</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>2·55 ± 0·05</td>
<td>2·65 ± 0·05</td>
<td>1·45 ± 0·05</td>
<td>2·1 ± 0·1</td>
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<td>Rifampicin</td>
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<td>3·35 ± 0·05</td>
<td>1·75 ± 0·05</td>
<td>3·0 ± 0·01</td>
<td>1·6 ± 0·1</td>
<td>3·2 ± 0·1</td>
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<td>Tetracycline</td>
<td>2·85 ± 0·05</td>
<td>5·0 ± 0·01</td>
<td>2·15 ± 0·05</td>
<td>4·75 ± 0·05</td>
<td>2·2 ± 0·1</td>
<td>4·6 ± 0·1</td>
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<tr>
<td>Chloramphenicol</td>
<td>3·55 ± 0·05</td>
<td>4·8 ± 0·1</td>
<td>3·0 ± 0·01</td>
<td>4·35 ± 0·05</td>
<td>3·05 ± 0·05</td>
<td>4·25 ± 0·05</td>
</tr>
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</table>

**Mutation of mexI and opmD results in growth deficiency which is restored upon addition of exogenous PQS**

To understand the mechanism leading to the reduction in the production of virulence determinants in the MexGHI-OpmD mutants, and as the mexI and opmD mutations exert a profound effect on cell-to-cell communication, we investigated whether this was due to a reduction in the expression of lasI, rhlI (encoding the 3-oxo-C12-HSL and the C4-HSL synthases, respectively) or pqsA and phnA, which are two key genes in the biosynthesis of PQS (Gallagher et al., 2002; Déziel et al., 2004). Detection of transcripts for these genes was carried out using RT-PCR. After 18 h growth (OD$_{600}$ 2), the cDNAs corresponding to all four genes were present in the parent PAO1 strain while in both the mexI and opmD mutants, only the lasI and rhlI transcripts were present (Fig. 3a). A more detailed analysis of pqsA and phnA expression as a function of growth revealed that the corresponding transcripts could be detected at OD$_{600}$ 0·6 and 1·2, but not at OD$_{600}$ 1·6. We conclude that, the absence of PQS production in the mexI and opmD mutants is a result of the inhibition of the expression of PQS biosynthetic genes during growth. The fact that the absence of 3-oxo-C12-HSL did not correlate with the inhibition of lasI transcription suggested the presence of an alternative, post-transcriptional mechanism preventing the biosynthesis of this quorum sensing signal molecule.

**PQS biosynthetic gene expression is switched off during the growth of the mexI and opmD mutants**

Fig. 3. RT-PCR analysis of transcripts. (a) Transcripts for oprL (house-keeping control gene), lasI, rhlI, pqsA and phnA, in wild-type, mexI mutant and opmD mutant strains (left to right). The cultures were grown in LB medium until they reached an OD$_{600}$ of 2. (b) Transcripts for pqsA in wild-type, mexI and opmD mutants as a function of the OD$_{600}$ in LB medium (indicated on the right). Identical results were obtained for the phnA transcripts (results not shown).
similar between the mutants and the wild-type. Interestingly, whilst addition of 10 μM 3-oxo-C12 to the medium had no effect, the presence of 60 μM PQS considerably shortened the lag phase (Fig. 4). This suggests that only exogenously supplied PQS can overcome the growth defect in the P. aeruginosa pump mutants. Although these experiments were done in the Bioscreen apparatus, where the cultures are under lower oxygen tension, similar results (extended lag phases for mexI and opmD mutants and growth stimulation by addition of PQS) were obtained when the cultures were incubated in flasks and shaken at 150 r.p.m. (results data not shown).

The extended lag phase of mexI and opmD mutants is probably due to the accumulation of a toxic PQS precursor

It has recently been shown that anthranilate, the product of the anthranilate synthase PhnAB, is a biosynthetic precursor of PQS, thought to be modified by PqsA during the initial steps in the biosynthesis of this 4-quinolone signal molecule (Essar et al., 1990; Gallagher et al., 2002; Déziel et al., 2004). In addition, it has been demonstrated that a pqsA mutant accumulates and excretes anthranilate into the growth medium (Déziel et al., 2004), suggesting the existence of an efflux mechanism for this PQS precursor. Hence the inhibition of pqsA transcription in the pump mutants may result in the accumulation of anthranilate or a metabolic derivative which may exert a toxic effect on the cells and hence affect growth. This would suggest that mexGHI-opmD is involved in the excretion of these toxic metabolites. To investigate this hypothesis we first constructed a P. aeruginosa mexI phnA double mutant (which does not produce anthranilate and does not produce PQS) was characterized by a very long lag phase and a diminished growth rate (Fig. 5), in contrast to the single phnA or pqsA mutants, which did not present any
growth alterations (Fig. 5). Interestingly, although exogenous PQS reduced the lag phase of the \textit{mexI} mutant (Fig. 4) it had no effect on the \textit{mexI phnA} or \textit{mexI pqsA} double mutants (data not shown). Taken together, these data indicate that the delayed growth observed in the pump mutants is caused by the accumulation of anthranilate or a metabolic derivative and that the \textit{mexGHI-opmD} pump might be involved in the detoxification of these compounds.

**Mutation of \textit{mexI} and \textit{opmD} severely compromises virulence in \textit{P. aeruginosa}**

The fact that a mutation in the MexGHI-OpmD pump affected the production of some quorum sensing regulated virulence determinants suggested that it could also have significant implications in the ability of this organism to cause disease. Consequently, we tested the effect that mutations in \textit{mexI} and \textit{opmD} had on the virulence of \textit{P. aeruginosa} using both an animal and a plant model of infection. As the animal model we used a rat lung infection model (Song \textit{et al}, 1998) in which rats were infected intratracheally in the left lung with either the wild-type PAO1 (40 animals) or the \textit{mexI} mutant (20 animals). Whilst 32 (80\%) animals infected with the wild-type died, only 1 rat infected with the \textit{mexI} did (Fig. 6a). Analysis of bacterial numbers present in the lung at the end of the experiments revealed large differences between the \textit{mexI} mutant \((4 \times 10^6\) c.f.u. per lung) and the wild-type \((2 \times 10^8\) c.f.u. per lung) even though 15 times more cells from the \textit{mexI} mutant were used for the infection. Pathological examination of the lungs from the infected rats (Fig. 6a) showed the presence of multiple abscesses in the animals infected with the wild-type PAO1 while only a single abscess was detected in the lungs from the rats infected with the \textit{mexI} mutant.

The \textit{mexI} and \textit{opmD} mutations were then transferred by allelic exchange to the highly plant- and animal-virulent \textit{P. aeruginosa} PA14 strain, and the virulence of the mutants was tested in a lettuce infection assay where PA14, in contrast to PAO1, is known to induce necrotic lesions (Rahme \textit{et al}, 1995). Fig. 6(b) shows that the \textit{mexI} and \textit{opmD} mutants did not cause any necrosis of the leaves whilst typical disease signs (necrosis and maceration) were clearly observed for the wild-type. The fact that PQS can restore the quorum sensing regulated phenotypes tested suggests that one of the reasons for the reduced virulence of the \textit{P. aeruginosa mexI} and \textit{opmD} mutants is the absence of this signal molecule. To investigate whether this is the case we tested whether co-inoculation of the PA14 \textit{mexI} and \textit{opmD} mutants with PQS could restore their ability to cause necrotic lesions in the lettuce infection assay. Fig. 6(b) shows that the addition of PQS indeed restored virulence in both of the mutants. However for the \textit{mexI} mutant this restoration was only partial. Inoculation of PQS alone did not cause any necrosis (results not shown).

**DISCUSSION**

In this work, we have demonstrated a key role for the MexI and OpmD proteins, two components of a newly discovered efflux system in \textit{P. aeruginosa} (Aendekerk \textit{et al}, 2002; Sekiya \textit{et al}, 2003), in the production of 4-quinolone and AHL quorum sensing signal molecules.

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**Fig. 6.** (a) Lungs from rats infected with the wild-type \textit{P. aeruginosa} PAO1 (right) and the \textit{mexI} mutant (left). Multiple abscesses are present in the wild-type-infected lung while only one is visible for the \textit{mexI}-infected lung (shown by arrows). The table shows a summary of the results from these virulence studies. (b) Plant (lettuce) infection assay with \textit{P. aeruginosa} PA14 wild-type and its corresponding \textit{mexI} and \textit{opmD} mutants (1), wild-type PA14, \textit{mexI} and \textit{mexI} co-inoculated with 60 \textmu M PQS (2) and wild-type PA14, \textit{opmD} and \textit{opmD} co-inoculated with PQS (3).
in this organism, and in promoting cell growth and in virulence. Furthermore, we have shown that loss by mutation of the pump enhances rather than reduces resistance to several different classes of antibiotics, a process which can be reversed by the provision of the PQS signal molecule. We believe that this is the first example of mutants in an efflux system displaying such strong phenotypes, including obvious growth defects.

Previous studies have suggested a role for *P. aeruginosa* efflux pumps in the transport of quorum sensing signal molecules to the extracellular milieu. The MexAB-OprM efflux system of *P. aeruginosa* has been proposed to be involved in the efflux of 3-oxo-C12-HSL while C4-HSL appears to diffuse freely out of the cells (Pearson et al., 1999). In addition this efflux pump has been shown to play an important role in the invasiveness of *P. aeruginosa* and it has been suggested to be involved in the export of virulence determinants (Hirakata et al., 2002). However the mechanism involved was not elucidated. Köhler et al. (2001) have also proposed that the inducible MexEF-OprN system might be responsible for the efflux of PQS. These authors found that overexpression of this pump, achieved via the nfxC mutation, restored the activity of the MexT activator and resulted in the decreased transcription of *rhlI* and a reduction of PQS and C4-HSL production (Köhler et al., 1999, 2001). They suggested that either PQS or a precursor might be the substrate of the MexEF-OprN pump. It is remarkable that the phenotypes described by Köhler and co-workers for the nfxC mutant overexpressing *mexEF-oprN* are similar to those we describe here and in our previous work (Aendekerk et al., 2002), including decreased production of virulence factors and signal molecules, and lack of virulence in a model of acute pneumonia in mice (Cosson et al., 2002). Recently, Ramsey & Whiteley (2004) described a genetic locus, *dad6* (dynamic attachment deficient), corresponding to the PA2491 gene and encoding an oxidoreductase; mutants in this gene showed a defect in the establishment of biofilms. This gene is located upstream of the *mexT* regulator gene and the *mexEF-oprN* genes and its inactivation results in increased expression of the *mexEF-oprN* genes and decreased expression of the PQS biosynthesis genes (Ramsey & Whiteley, 2004). In contrast, our data presented here are believed to be the first to conclusively show that the absence of an efflux pump dramatically affects the production of quorum sensing signal molecules via the repression of PQS biosynthesis genes in *P. aeruginosa* while the data of Köhler et al. (2001) and Ramsey & Whiteley (2004) suggest that overproduction of MexEF-OprN results in the same phenotype. Microarray analysis of the expression of different efflux porin genes showed that, from the genes encoding members of the Opm porin family (Hancock & Brinkman, 2002; Jo et al., 2003) only oprM, oprJ, oprA, oprD and oprL are highly transcribed (Jo et al., 2003).

However, that study was done in the PAK strain of *P. aeruginosa*, which does not produce PQS (Lépine et al., 2003). Using *P. aeruginosa* PA01, Murata et al. (2002) detected the production of OpmB and OpmD by Western blot analysis, with OpmD increasing at the onset of the stationary phase, corresponding to the time of PQS appearance.

Our studies also demonstrate that the absence of the main components of the MexGHI-OpmD pump does not affect the response to exogenous PQS as demonstrated by the restoration of pyocyanin production (data not shown) and the expression of a *lecA*:::lux fusion, both traits known to be controlled by PQS and the Rhl system (Diggle et al., 2003; Gallagher et al., 2002; Déziel et al., 2004; D’Argenio et al., 2002).

An unexpected finding was the growth-phase-dependent shut-down of transcription of the PQS biosynthetic genes *pqsA* and *phnA* in the *mexI* and *opmD* mutants. This result may suggest the involvement of the pump in the efflux of a toxic compound (explaining the reduced growth rate) linked to the production of PQS. As already noted, no PQS could be detected inside the cells of the *mexI* and *opmD* mutants. The precursor of PQS is anthranilate (Calfee et al., 2001). The *phnA* and *phnB* genes encode the two components of an anthranilate synthase and were first described as being important for the production of phenazines, including pyocyanin (Essar et al., 1990, Anjaiah et al., 1998). Subsequently it was demonstrated that *phnAB* are necessary for PQS biosynthesis, providing an explanation as to why mutants in these genes failed to produce phenazines (Gallagher et al., 2002).

In a previous report we described that a *trpC* mutant failed to produce phenazines and excreted anthranilate into the medium (Anjaiah et al., 1998). Addition of increasing amounts of tryptophan to the *trpC* mutant restored the production of phenazines while decreasing the concentration of anthranilate in the spent medium, due to feedback inhibition of the TrpEG anthranilate synthase (Essar et al., 1990). These results suggest that (i) anthranilate synthesized by the TrpEG anthranilate synthase cannot be channelled into the synthesis of PQS, (ii) high anthranilate concentrations inhibit the production of phenazines, eventually by repressing *phnA* transcription and (iii) anthranilate is excreted from the cells. One possibility is that, in the absence of efflux mediated by MexI-OpmD, accumulation of intracellular anthranilate leads to the progressive transcriptional shutdown of the PQS biosynthesis genes. Our physiological studies of the double *mexI phnA* and *mexl pqsA* mutants indicate that the accumulation of anthranilate in the cell may be responsible for the growth defect in the absence of the pump, since the *mexl pqsA* mutant is almost non-viable, in contrast to a *pqsA* mutant which grows in a manner similar to the parent strain. Anthranilate is also a direct precursor of catechol biosynthesis. Catechols have been shown to exert toxic effects in cells such as generation of reactive oxygen species by redox reactions, oxidative DNA damage and protein damage by thiol arylation or oxidation, and they can also interfere with electron transport (Schweigert et al.,...
Catechols can also complex metals, such as iron (Schweigert et al., 2001). Accumulation of catechols inside the cells in the absence of the MexGHI-OpmD pump could also explain the observed increased sensitivity to vanadium in a mutant affected in this pump (Aendekerk et al., 2002), which could be due to the accumulation of catechol–vanadium complexes. The incomplete restoration of growth in the case of the mexl phnA double mutant could be explained by the fact that a second route exists in pseudomonads for the production of anthranilate via the degradation of tryptophan by the enzyme tryptophan 2,3-dioxygenase (TDO, encoded by PA 2579) into N-formylkynurenine, followed by the conversion of this molecule into N-kynurenine by a formamidase (PA2081), and finally the degradation of N-kynurenine into anthranilate by a kynureninase (PA2080) (Kurnasov et al., 2003; Matthys et al., 2004). Supporting this proposition, it has been shown that a transposon insertion in the TDO gene suppresses the autolysis phenotype caused by an overproduction of PQS in P. aeruginosa (D’Argenio et al., 2002). It is also worth noting a recently published report showing the presence of an efflux system in E. coli that acts as a ‘metabolic relief valve’ by pumping out p-hydroxybenzoic acid, which is a precursor of ubiquinones (Van Dyk et al., 2004).

Despite the loss of AHL production in the mexl and opmD mutants, both the lastl and rhlR transcripts were present in both the exponential and the stationary phase. Previously, it has been shown that the absence of MvfR (PqsR), which is a positive regulator of PQS biosynthesis, does not affect the transcription of lasR or rhlR (Cao et al., 2001b). These data suggest that the restoration of AHL production in the pump mutants by exogenous PQS must therefore be occurring at the post-transcriptional level. It is possible that this involves the post-transcriptional regulatory protein RsmA, which influences the expression of both lasl and rhlR (Pessi et al., 2001), since PQS is capable of overcoming the RsmA-dependent repression of lecA, a gene which is known to be positively regulated by both C4-HSL and PQS (Diggle et al., 2003). Clearly, addition of PQS to the opmD mutant results in the complete restoration of production of 3-oxo-C12, while the effect is less pronounced in the case of the mexl mutant. One possibility is that Mexl is the most important component of the pump and that PQS stimulates the transcription of the pump. Indeed, as opmD is the last gene of the operon, mexl should be transcribed in the opmD mutant (Aendekerk et al., 2002).

Analysis of the P. aeruginosa PA01 genome sequence suggests that this opportunistic human pathogen, which exhibits innate resistance to multiple antibiotic classes, possesses at least ten different RND (Resistance-Nodulation-Cell Division) type multi-drug efflux pumps (Stover et al., 2000). For those pumps that have been extensively characterized, genetic disruption generally leads to increased susceptibility to antimicrobial agents. Paradoxically, here we show that disruption of the MexGHI-OpmD efflux pump enhances the resistance of P. aeruginosa to a variety of antibiotics including β-lactams, aminoglycosides and quinolones (Table 3).

Interestingly, Maseda et al. (2004) showed that overproduction of the MexEF-OprN pump results in increased resistance to quinolones, but hypersusceptibility to most β-lactams. The same authors demonstrated that C4-HSL is needed for maximal expression of mexAB-oprM in stationary phase and that this is antagonized by the MexT regulator. For both the mexl and opmD mutants, the provision of exogenous PQS restored susceptibility to for example kanamycin and spectinomycin. These data indicate that PQS-dependent cell-to-cell communication in P. aeruginosa is also involved in controlling susceptibility to antimicrobial agents. This is further highlighted by the increase in susceptibility of the parent PAO1 strain cultured in the presence of PQS. The mechanism involved is not known but may be a consequence of the PQS-dependent repression of other multi-drug efflux pumps. Although there is no published information on the regulation of any of the RND efflux pumps by PQS, both the mexGHI-opmD gene cluster (qsc133; Whiteley et al., 1999) and the mexAB-oprM cluster (Maseda et al., 2004) are positively regulated by AHL-dependent quorum sensing. Alternatively, PQS may directly regulate genes involved in controlling cell envelope permeability. Further work will clearly be required to determine the mechanism by which PQS modulates antibiotic susceptibility.

Mutation of both mexl and opmD rendered P. aeruginosa unable to cause necrosis and macerate plant tissues. Likewise, the mexl mutant was found to be avirulent in an acute rat lung infection model. This is in agreement with a previous study which showed that biosynthetic mutants unable to produce PQS are avirulent in a pathogenicity assay employing the nematode Caenorhabditis elegans (Gallagher & Manoil, 2001). Interestingly, in the plant infection model, the virulence of both mexl and opmD mutants could be restored to different levels by the provision of exogenous PQS. The fact that PQS only partially restored virulence in the mexl mutant could be explained by the inability of PQS to fully restore 3-oxo-C12-HSL production in this mutant. The restoration of virulence confirms that PQS uptake occurs via an alternative pathway to export and that PQS uptake is not dependent on the presence of a functional MexGHI-OpmD pump or the quorum sensing circuitry. These results clearly reveal the central role played by PQS as the primary quorum sensing signal molecule required for the healthy growth and full virulence of P. aeruginosa. As such a 4-quinolone-dependent cell-to-cell communication, as well as the MexGHI-OpmD pump, may attractive targets for the development of novel anti-pseudomonal drugs.

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