Identification of mutants with altered phenazine production in *Pseudomonas aeruginosa*

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*Pseudomonas aeruginosa* is an opportunistic human pathogen that causes serious and chronic infections. Many secondary metabolites are secreted throughout its growth, among which phenazine is a known virulence factor and signalling molecule. Phenazine is coordinately controlled by the global regulatory quorum-sensing (QS) systems. Despite the detailed understanding of phenazine biosynthesis pathways in *P. aeruginosa*, the regulatory networks are still not fully clear. In the present study, the regulation of the phzA1B1C1D1E1F1G1 operon (phzA1) has been investigated. Screening of 5000 transposon mutants revealed 14 interrupted genes with altered phzA1 expression, including PA2593 (QteE), which has been identified as a novel regulator of the QS system. Overexpression of qteE in *P. aeruginosa* significantly reduced the accumulation of homoserine lactone signals and affected the QS-controlled phenotypes such as the production of pyocyanin, rhamnolipids and LasA protease and swarming motility. Indeed, overexpression of qteE in *P. aeruginosa* attenuated its pathogenicity in the potato and fruit fly infection models. These findings suggest that qteE plays an important role in *P. aeruginosa* pathogenicity and is part of the regulatory networks controlling phenazine production.

**INTRODUCTION**

*Pseudomonas aeruginosa* is a ubiquitous, Gram-negative, opportunist pathogen that causes serious infections in immunocompromised hosts, including severely burned patients, HIV-infected individuals and cancer patients undergoing chemotherapy (Akiyama et al., 2000; Dénervaud et al., 2004). Many *P. aeruginosa* factors such as alginate, pili and lipopolysaccharide play important roles in *P. aeruginosa* virulence. Known virulence factors in *P. aeruginosa* include toxins (exotoxin A and exoenzyme S), proteases (elastase, LasA protease and alkaline protease), haemolysins and phenazines, which have been found to contribute to *P. aeruginosa* pathogenicity in animal models (Howe & Iglewski, 1984) or clinical studies (Blackwood et al., 1983).

Pyocyanin is the major phenazine compound produced by *P. aeruginosa*, which functions as both an important virulence factor (Lau et al., 2004; Laursen & Nielsen, 2004) and intriguingly a signal molecule (Dietrich et al., 2006). Pyocyanin produced by *P. aeruginosa* in cystic fibrosis lungs has been shown to inhibit the ciliary function of respiratory epithelial cells *in vitro* (Wilson et al., 1987), and can alter the host immune and inflammatory response (Allen et al., 2005; Denning et al., 1998). As bioactive ‘secondary’ molecules, the role of phenazine compounds as signal molecules has recently been explored (Dietrich et al., 2006; Price-Whelan et al., 2006). It has been shown that pyocyanin acts as the final signal in the quorum-sensing (QS) system cascade, and regulates at least 22 genes in *P. aeruginosa* PA14 (Dietrich et al., 2006).

Two homologous operons are involved in the synthesis of phenazine compounds in *P. aeruginosa*, i.e. phzA1B1C1D1G1 (phzA1) and phzA2B2C2D2G2 (phzA2) (Mavrodi et al., 2001). The expression of phzA1 accounts for most of the phenazine production (Chugani et al., 2001; Whiteley et al., 1999). In addition, the gene products of phzM, phzH and phzS are required to convert the intermediate phenazine-1-carboxylic acid to other final products including phenazine-1-carboxamide, 1-hydroxphenazine and pyocyanin (Mavrodi et al., 2001).

In *P. aeruginosa*, there are three intertwined QS systems: the two acyl homoserine lactone (AHL)-mediated las and rhl systems, and the 2-alkyl-4(1H)-quinolone (AHQ) signal-based system. The las and rhl systems consist of the transcriptional activators LasR and RhlR and a signal synthase, LasI and RhlI, respectively. The major AHQ signals include 2-heptyl-3-hydroxy-4-quinolone (the *Pseudomonas* quinolone signal, PQS) and 2-heptyl-4-quinolone. Expression

Abbreviations: AHL, acyl homoserine lactone; PQS, *Pseudomonas* quinolone signal; QS, quorum sensing.
of numerous cellular and secreted virulence factors, such as protease and pyocyanin, is regulated by QS in *P. aeruginosa*.

There is a complex regulatory network that controls the QS systems at both the transcriptional and post-transcriptional level. At least 16 QS regulators have been identified and only seven with known targets in the QS network (Venturi, 2006; Williams *et al.*, 2007). Recently, Siehnel *et al.* (2010) have demonstrated that PA2593 (*qteE*) is a unique negative regulator that blocks QS gene expression and decreases the half-life of the LasR protein without affecting lasR transcription or translation.

Although the pathophysiological effects of *P. aeruginosa* phenazines have been well studied (Mavrodi *et al.*, 2001), the regulatory networks of phenazine production are still not fully understood. To better understand the regulatory pathway of phenazine biosynthesis, we screened a transposon mutagenesis library, and identified and characterized several mutants with altered *phzA1* expression. Among them is the PA2593 (*QteE*) mutant, in which *phzA1* expression is completely abolished. Our data showed that the induction of *qteE* in *P. aeruginosa* reduced the accumulation of QS signals and affected QS-controlled virulence factors. Furthermore, overexpression of *qteE* attenuated the pathogenicity of *P. aeruginosa*.

### METHODS

**Bacterial strains and media.** The bacterial strains and plasmids used in this study are listed in Table 1. *P. aeruginosa* wild-type strain and derivatives were routinely grown at 37°C on LB agar plates or in broth [LB broth or M9 minimal medium (BD) supplemented with 0.1% Casamino acids and 0.5% glucose] with shaking at 200 r.p.m. *Agrobacterium tumefaciens* was grown in LB medium at 30°C. Antibiotics were used at the following concentrations where appropriate: for *Escherichia coli* in all media, kanamycin (Kn) at 50 μg ml⁻¹, tetracycline (Tet) at 15 μg ml⁻¹ and ampicillin (Ap) at 100 μg ml⁻¹; for *P. aeruginosa*, gentamicin (Gm) at 75 μg ml⁻¹ in LB or 150 μg ml⁻¹ in Pseudomonas Isolation Agar (PIA; BD), Tet at 75 μg ml⁻¹ in LB or 200 μg ml⁻¹ in PIA, carbenicillin (Cb) at 500 μg ml⁻¹ in PIA and trimethoprim at 300 μg ml⁻¹ in all media.

### Table 1. Strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype or phenotype</th>
<th>Reference/source</th>
</tr>
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<tbody>
<tr>
<td>E. coli</td>
<td>F⁻ merA Δ(mrr-hsdRMS-mcrBC)80dlacZ ΔM15 ΔlacX74 deoR recA1 endA1 araD139 Δ ara leu)7697 galU galK2⁻ rpsL supG</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>SM10</td>
<td>Mobilizing strain, RP4 integrated in the chromosome, Kn²</td>
<td>Simon <em>et al.</em> (1983)</td>
</tr>
<tr>
<td>PAO1</td>
<td>Wild-type, laboratory strain</td>
<td>This laboratory</td>
</tr>
<tr>
<td>PAO(Δ2593)</td>
<td>PA2593 replacement mutant of PAO1, Gm²</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1(pUCP26)</td>
<td>Wild-type PAO1 containing pUCP26 plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>PAO1(p-qteE)</td>
<td>Wild-type PAO1 containing p-qteE plasmid</td>
<td>This study</td>
</tr>
<tr>
<td>JP2</td>
<td>las–rhl double mutant of PAO1</td>
<td>Pearson <em>et al.</em> (1997)</td>
</tr>
<tr>
<td>pMS402</td>
<td>Expression reporter plasmid carrying the promoterless lacCDABE gene</td>
<td>Duan <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>PUCP26</td>
<td><em>P. aeruginosa</em> expression vector</td>
<td>West <em>et al.</em> (1994)</td>
</tr>
<tr>
<td>p-qteE</td>
<td>lac-qteE on pUCP26</td>
<td>This study</td>
</tr>
<tr>
<td>pBT20</td>
<td>Mini-TnM delivery vector; Ap⁵, Gm²</td>
<td>Kulasekara <em>et al.</em> (2005)</td>
</tr>
<tr>
<td>pZ1918-lacZGm</td>
<td>Source plasmid of Gm² cassette</td>
<td>Schweizer (1992)</td>
</tr>
<tr>
<td>pRK2013</td>
<td>Broad-host-range helper vector; Tra⁺, Kn¹</td>
<td>Ditta <em>et al.</em> (1980)</td>
</tr>
<tr>
<td>Mini-CTX1</td>
<td>Integration plasmid; Tet²</td>
<td>Hoang <em>et al.</em> (2000)</td>
</tr>
<tr>
<td>pKD-2593K</td>
<td>PA2593 knockout plasmid pEX18Ap with 465 bp upstream region, Gm²–lacZ fragment from pZ1918Gm-lacZ and 442 bp downstream of PA2593 cloned between HindIII and BamHI; Ap² (Cb²), Gm²</td>
<td>This study</td>
</tr>
<tr>
<td>Mini-CTX-phzA1</td>
<td>Mini-CTX1 containing phzA1 promoter region; Tc²</td>
<td>This study</td>
</tr>
<tr>
<td>pKD-pqsH</td>
<td>pMS402 containing pqsH promoter region</td>
<td>Liang <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>pKD-pqsR</td>
<td>pMS402 containing pqsR promoter region</td>
<td>Liang <em>et al.</em> (2008)</td>
</tr>
<tr>
<td>pKD-rhlI</td>
<td>pMS402 containing rhlI promoter region</td>
<td>Duan &amp; Surette (2007)</td>
</tr>
<tr>
<td>pKD-rhlR</td>
<td>pMS402 containing rhlR promoter region</td>
<td>Duan &amp; Surette (2007)</td>
</tr>
<tr>
<td>pKD-lasI</td>
<td>pMS402 containing lasI promoter region</td>
<td>Duan &amp; Surette (2007)</td>
</tr>
<tr>
<td>pKD-lasR</td>
<td>pMS402 containing lasR promoter region</td>
<td>Duan <em>et al.</em> (2003)</td>
</tr>
<tr>
<td>pKD-lasB</td>
<td>pMS402 containing lasB promoter region</td>
<td>Duan <em>et al.</em> (2003)</td>
</tr>
</tbody>
</table>
Transposon mutagenesis. The transposon mutagenesis library was constructed as previously described (Kulasekara et al., 2005; Liang et al., 2008). The donor strain (SM10-λpir) containing pBT20 and the recipient PAO containing the phzA1-lux reporter fusions (Mini-CTX-phzA1) were scraped from overnight incubated plates and resuspended separately in 0.2 ml LB broth. The bacterial suspensions were adjusted to OD600 40 for the donors and OD 600 20 for the recipient. Twenty-five microlitres of each donor and recipient were mixed together and spotted on an LB agar plate and incubated for 8 h at 37°C. The mating mixtures were scraped and resuspended in 3 ml M9 medium; 100 µl of this suspension was plated on PIA agar plates containing Gm at 150 µg ml⁻¹. A transposon mutant library was constructed by picking 5000 colonies grown on these selective plates.

Construction of the PA2593 knockout mutant. For gene replacement, the previously described sacB-based strategy was employed (Hoang et al., 1998). The suicide plasmid pKD2593K for gene replacement was constructed by inserting the PstI-digested Gm⁻-lacZ cassette from pZV1918Gm (Schweizer, 1993) between two PCR fragments of PA2593 cloned in pEX18Ap. The 465 bp upstream fragment was amplified using primer NH1 (5'-CGTATCAGCTTCTTTGGCAGC3') containing a HindIII restriction site and NH2 (5'-TCGCTGAGCAGCAGCCGAGCGACG3') containing a PstI site (underlined); the 442 bp downstream region was generated using primers NH3 (5'-GCTGCTGAGCAGCAGCCGAGCGACG3') containing a PstI site and NH4 (5'-CGCAGTACGCGGCAGCGACG3') containing a BamHI site (underlined). The fragments were ligated with pEX18Ap, yielding pKD2593K. The PA2593 mutant PAO1(Δ2593) was generated by a triparental mating procedure employing the helper vector PRK2013 as previously described (Ditta et al., 1980).

Bioassay of AHL activity. The last-dependent 3-oxo-C₁₂-HSL produced by P. aeruginosa was estimated using a modified A. tumefaciens system (Chambers et al., 2005). The autoinducer of the rhl system, C₁₂-HSL, was measured using an rhlA promoter-based P. aeruginosa strain, pDO100 (pKD-rhlA). This detection system was developed by fusing the C₁₂-HSL-responsive rhlA promoter upstream of luxCDABE and introducing the construct into pDO100, a rhl mutant strain (Liang et al., 2008). Two microlitres of test bacterial culture (OD600 = 1.0) of wild-type PAO1, PAO1(pUCP26), PAO1 (p-qteE) and PAO1 lasI double mutant (JP2) was inoculated onto the seeded bioassay plate, respectively. The plates were incubated at 37°C for 24 h. The dark halo zone around bacterial colonies indicates AHL activity. The assays were also carried out in LB broth using the reporter fusions. Briefly, an overnight culture of the reporter strain was added to 900 µl of LB medium and, after gentle tip poking, the inoculated slices were prepared by centrifugation of overnight culture at 5000 rpm and removing the supernatant. Swarm agar plates were incubated for 24 h at 37°C and then incubated for an additional 24 h at room temperature for a total of 48 h. Photographs were taken with the LAS-3000 imaging system (Fuji Life Sciences).

Potato inoculation. P. aeruginosa cells were prepared by centrifugation of overnight culture at 5000 g for 10 min and diluted to different densities in phosphate buffer (0.05 M, pH 8.0). Mature potato tubers were surface-sterilized with 70% ethanol and sliced into 3 mm thickness, and placed on Petri dishes with wet filter paper to keep them moist. To each slice, 10 µl P. aeruginosa dilutions (2 x 10⁶ c.f.u. ml⁻¹) were inoculated after gentle tip poking. The inoculated slices were incubated at 28°C for 48 h, unless otherwise indicated, before photographs were taken and the extent of maceration was determined.

Fly infections. Infections using a fly model were adapted from the fly feeding assay developed by Chugani et al. (2001). Broth cultures were adjusted to an OD₆₀₀ of 2.0 using the medium that the strain was grown in. Then, 1.5 ml of the culture was collected by centrifugation, the supernatant was removed and the pellet was resuspended in 100 µl 5% sucrose. The resuspended cells were spotted onto a sterile filter (Whatman GF/A 21 mm) that was placed on the surface of

p-qteE. The constructed plasmid p-qteE was then transformed into P. aeruginosa by electroperoration.

Measurement of pyocyanin production. Pyocyanin was extracted from culture supernatants and measured using previously reported methods (Essar et al., 1990; Kurachi, 1958). Briefly, 3 ml chloroform was added to 5 ml culture supernatant. After extraction, the chloroform layer was transferred to a fresh tube and mixed with 1 ml 0.2 M HCl. After centrifugation, the top layer (0.2 M HCl) was removed and its Abs₅₂₀ was measured. Concentrations, expressed as µg pyocyanin produced (ml culture supernatant)⁻¹, were determined by multiplying the Abs₅₂₀ by 17.072 (Kurachi, 1958).

Rhamnolipid assay. Rhamnolipid in P. aeruginosa culture fluids was detected as previously described (Pearson et al., 1997). Cells from mid-exponential-phase cultures grown in Peptone Tryptone Soya Broth (5% Peptone; 0.25% Tryptone Soya Broth, pH 7.0) were washed and resuspended in modified GS medium (Medium Group C) (Guerra-Santos et al., 1986) to an OD₆₀₀ of 0.2 and incubated at 37°C with shaking. Cultures were incubated for a total of 80 h and then centrifuged at 16000 g for 5 min. The supernatants were sterilized by passing through 0.22 µm pore-size filters. Filtrates were extracted twice with 2 vols diethyl ether. The pooled ether extracts were extracted once with 20 mM HCl, and the ether phase was evaporated to dryness. The residue was dissolved in water. Rhamnose content in each sample was determined by comparison to rhamnose standards using duplicate orcinol assays (Koch et al., 1991). Rhamnolipid was determined by the relation that 1.0 mg rhamnose corresponds to 2.5 mg rhamnolipid.

Staphylolytic activity assay. LasA protease activity was determined by measuring the ability of P. aeruginosa culture supernatants to lyse boiled Staphylococcus aureus cells (Kessler et al., 1993). A 30 ml overnight culture of S. aureus was boiled for 10 min and then centrifuged for 10 min at 10000 g. The resulting pellet was resuspended in 10 mM NaHPO₄ (pH 7.5) and adjusted to an OD₆₀₀ of 0.9. A 100 µl aliquot of bacterial supernatant was then added to 900 µl S. aureus suspension, and the OD₆₀₀ was determined after 2, 6, 10, 14, 18, 22, 26, 30, 45, 60 and 90 min.

Swarming motility assay. Swarm medium was based on M8 minimal medium (Köhler et al., 2000), supplemented with MgSO₄ (1 mM), glucose (0.2 %) and Casamino acids (0.5 %), and solidified with 0.5 % agar. Bacteria were spot inoculated onto swarm agar plates as 2.5 µl aliquots taken directly from overnight LB cultures. Swarm agar plates were incubated for 24 h at 37°C and then incubated for an additional 24 h at room temperature for a total of 48 h. Photographs were taken with the LAS-3000 imaging system (Fuji Life Sciences).
Fig. 1. Phenotypes of *P. aeruginosa* cultured on LB agar (a) or in LB broth (b) overnight. (c) Expression of *phzA1* in the B36 mutant strain (○) and wild-type PAO1 (△) containing a Mini-CTX-*phzA1* fusion. The assay was independently repeated at least three times and the data shown are representatives of comparable results. (d) Pyocyanin production in PAO1, PAO1(pUCP26), PAO1(p-*qteE*) and JP2. Values represent the mean of three independent experiments ± SD. (e) Alignment of the NNCT-(N)12-AGNN elements of *lasI*, *hcnA*, *rsaL*, *rhlI*, *rhlA*, *qscR* and PA2593. Nucleotides with a grey background represent bases present in all *las*-box elements. Note: PA2593 contains two *las*-box fragments. (f) RT-PCR analysis of *qteE* expression in B36, PAO1(p-*qteE*) and PAO1. The amount of RNA used was 1 μg. The 16S rRNA gene of *P. aeruginosa* was used as the internal control to verify the absence of significant variation at the cDNA level in the samples. This experiment was repeated twice and similar results were obtained.
The loss of phenazine production alters the balance and changes the colony appearance. To identify the genes disrupted by transposon insertion, the insertions were screened for altered colony morphology. The distinctive colour of this mutant is indicative of altered phenazine compound production. The transposon inserted in five different genes caused increased expression of the phzA1 operon, seven resulted in decreased expression, and two abolished it. Of the 14 genes identified, PA5191 belongs to the class of hypothetical protein. Two of them, rsaL and vqsM, have been identified previously as being involved in the las and rhl QS regulon (de Kievit et al., 1999; Dong et al., 2005). Five genes (phzC1, phzM, hmgA, pqsD and ccmA) have been reported to be required for the synthesis of phenazine (Rodríguez-Rojas et al., 2009; Baert et al., 2008; Gallagher et al., 2002; Mavrodi et al., 1998). Interestingly, one mutant strain (B36) exhibited abolished expression (Fig. 1c). Sequence analysis showed that the transposon inserted 28 bp upstream of the PA2593 gene, which encodes a hypothetical protein of 190 amino acids residues (Stover et al., 2000). Recently, Siehnel et al. (2010) have shown that PA2593 (named QteE) is a unique regulator, blocks QS gene expression and decreases the half-life of the LasR protein without affecting lasR transcription or translation. The distinctive colour of this mutant is indicative of altered phenazine compound production (Fig. 1a). Alteration of qteE abolished expression, indicating that this gene is directly connected to the phzA1 expression (Fig. 1c). This result is consistent with the decreased pyocyanin in strain PAO1(p-qteE) compared with the wild-type PAO1 (Fig. 1d). Analysis of the P. aeruginosa

### Isolation and identification of P. aeruginosa mutants with altered phzA1 expression

We identified transposon insertion mutants of P. aeruginosa strain PAO1 with decreased or increased production of the phenazine pigment pyocyanin by visual screening. In addition, mutants with changed luminescence value, i.e. changed phzA1 expression, were collected. In several of the mutants, the changes in the expression of the phzA1 operon resulted in colony colour changes in agreement with the fact that phenazine compounds are the major contributors to the colour of P. aeruginosa cultures. Examples of these mutants producing distinct pigments are shown in Fig. 1(a, b). P. aeruginosa produces several pigmented compounds in addition to phenazine. The final greenish colour of P. aeruginosa cultures is the combination of these pigments. The loss of phenazine production alters the balance and therefore changes the colony appearance.

### RESULTS

#### Table 2. List of mutants in which at least twofold changes in phzA1 expression were observed

<table>
<thead>
<tr>
<th>Strain</th>
<th>Insertion site</th>
<th>Gene name or operon*</th>
<th>Description</th>
<th>Max fold†</th>
</tr>
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<tbody>
<tr>
<td><strong>Increased expression</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A85</td>
<td>4714837</td>
<td>phzCl</td>
<td>Phentazine biosynthesis protein PhzC</td>
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<td>A3</td>
<td>1558963</td>
<td>rsaL</td>
<td>Regulatory protein RsaL</td>
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<tr>
<td>B27</td>
<td>4713768</td>
<td>phzM-Pt</td>
<td>Probable phenazine biosynthesis protein</td>
<td>35</td>
</tr>
<tr>
<td>A52</td>
<td>1347875</td>
<td>PA1243-Pt</td>
<td>Probable sensor/response regulator hybrid</td>
<td>3.2</td>
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<td>A19</td>
<td>5844013</td>
<td>PA5191-Pt</td>
<td>Hypothetical protein</td>
<td>3.5</td>
</tr>
<tr>
<td><strong>Decreased expression</strong></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B11</td>
<td>2198301</td>
<td>hmgA</td>
<td>Homogentisate 1,2-dioxygenase</td>
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</tr>
<tr>
<td>A324</td>
<td>649621</td>
<td>apaH</td>
<td>Bis(3'-nucleosyl)-tetraphosphatase</td>
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<td>B25</td>
<td>2983995</td>
<td>nusD</td>
<td>NADH dehydrogenase 1 chain C,D</td>
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<td>B71</td>
<td>1080205</td>
<td>pqsD</td>
<td>3-Oxoacyl-[acyl-carrier-protein] synthase III</td>
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<tr>
<td>B32</td>
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<td>ccmA-Pt</td>
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<td>ilvI</td>
<td>Acetolactate synthase large subunit</td>
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<td></td>
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<td>B36</td>
<td>2935823</td>
<td>PA2593-Pt</td>
<td>Hypothetical protein</td>
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<tr>
<td>A280</td>
<td>2448826</td>
<td>vqsM</td>
<td>Probable transcriptional regulator</td>
<td>NE</td>
</tr>
</tbody>
</table>

*The gene number or name is from http://www.pseudomonas.com
†Maximal ratio of expression (measured in counts s⁻¹) between the mutant and the wild-type.
§Transposon inserted at the promoter region of the gene/operon.
**Fig. 2.** Phenotypes and expression of *rhlA* and *lasB* in wild-type and B36 strains. (a) Protease activity. PAO1, PAO1(pUCP26), PAO1(p-qteE) and JP2 (*lasI/rhlI* double mutant) were patched on an LB agar plate supplemented with 2% milk. The clear zone surrounding bacterial colonies after incubation at 37 °C for 18 h indicates the enzyme activity. (b) LasA protease activity. Culture supernatants were analysed after 13 h growth and LB medium was used as a control. All of the experiments shown represent a single experiment, although each experiment was repeated at least three times with similar results. (c) Swarming motility of PAO1 and B36. (d) Rhamnolipid production. The values are means ± SD of triplicate cultures. (e) Expression of QS-controlled genes *rhlA* and *lasB* in the B36 strain (◯) and PAO1 (△). These assays were repeated at least three times and the data shown are representatives of comparable results.

QteE expression attenuates virulence in *P. aeruginosa*
PAO1 genome data has revealed that qteE contains two las-box elements (Fig. 1e) while a previous study showed that qteE is controlled by QS systems (Schuster et al., 2003).

To determine whether any of the genes directly regulate phzA1 expression rather than as a consequence of impaired growth, we also examined the growth of each mutant in LB. The mutants had a similar rate of growth in LB when compared with wild-type PAO1 (data not shown).

**Overexpression of qteE in *P. aeruginosa* reduces virulence factor production**

As shown in Table 2, the expression of the phenazine operon (phzA1) was abolished in the B36 mutant. Sequence analysis showed that the Tn–Gm cassette inserted at 28 bp upstream of the translational start codon (ATG) of the PA2593 (qteE) gene. We hypothesized that the phenotypic change was due to enhanced expression of qteE rather than to inactivation of qteE. This seemed a reasonable prediction based on the fact that the mariner transposon used in this study harbours an outward-directed Ptac promoter. To address this possibility that qteE was overexpressed in the mutant, the qteE gene was cloned in pUCP26 under the control of the lac promoter. The resulting plasmid, p-qteE, was transformed into wild-type PAO1, yielding the PAO1(p-qteE) strain. The overexpression of qteE in these *P. aeruginosa* strains was confirmed by reverse transcriptase PCR analysis (Fig. 1f). The colour of *P. aeruginosa* harbouring the fusion plasmid p-qteE (PAOB1) was clearly different from that containing empty vector alone on LB agar plates or in broth (Fig. 1a, b), suggesting that induction of qteE in *P. aeruginosa* decreases phenazine production. This was confirmed by examining pyocyanin production, and overexpression of qteE reduced pyocyanin levels to those seen in a *P. aeruginosa* mutant lacking QS (Fig. 1d).

This observation led us to test the effect of qteE on other virulence factors. As shown in Fig. 2, overexpression of qteE in *P. aeruginosa* also reduced proteolytic activity, LasA protease, rhamnolipid production and swarming motility. Rhamnolipid and protease are encoded by rhlA and lasB, respectively. They are major virulence factors secreted during the growth of *P. aeruginosa*, capable of degrading and inactivating biological and immunological tissues.

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**Fig. 3.** Expression of QS genes in PAO1 (△) and B36 (○). The assay was independently repeated at least three times and the data shown are representatives of comparable results.
(Heurlier et al., 2004). Measurement of rhlA and lasB expression in the B36 strain indicated that both rhlA and lasB were drastically suppressed in the B36 strain (Fig. 2e). These results clearly indicate that qteE affects the expression of virulence factors in P. aeruginosa.

Repression of QS systems due to overexpression of qteE

The observation that induction of qteE repressed many QS-controlled phenotypes suggests that qteE is involved in the regulation of QS in P. aeruginosa. Therefore, we compared the expression of QS genes (lasR, lasI, rhlR and rhlI) in B36 and PAO1. The data showed that induction of qteE repressed lasI, rhlR and rhlI expression with no effect on lasR (Fig. 3). Consistent with the changed synthase gene expression in B36 (Fig. 3), the levels of AHLs in PAO1(p-qteE) were decreased compared to those in the wild-type PAO1 (Fig. 4). These observations are in agreement with the work of Siehnel et al. (2010).

It has been shown that PQS positively regulates the rhl system and the rhl system negatively regulates the PQS system, and LasR positively regulates the PQS system (McGrath et al., 2004; Wade et al., 2005). As a negative regulator qteE blocks QS gene expression by decreasing the half-life of the LasR protein (Siehnel et al., 2010). This made us investigate the connection between qteE induction and the PQS system. It has been shown that the product of pqsH is required to convert the PQS precursor 4-hydroxy-2-heptyl quinoline that is synthesized by PqsABCD into PQS (Décézi et al., 2004) and MvfR (PqsR) modulates the expression of multiple QS-regulated virulence factors (Gallagher et al., 2002). The data indicate that the expression of pqsR and pqsH was decreased in the B36 transposon insertion mutant strain compared to the wild-type PAO1 (Fig. 5a, b). However, the expression of pqsA in strain B36 showed an identical level to that of the wild-type (Fig. 5c). No differences in pqsR and pqsH expression were observed between the qteE replacement mutant strain PAO(A2593) and the wild-type PAO1 (data not shown).
Attenuated pathogenicity in the qteE overexpression strain

As the induction of qteE in P. aeruginosa could reduce virulence factor gene expression and repress the QS system, we verified the impact of qteE expression on P. aeruginosa pathogenicity using the potato tuber infection model. P. aeruginosa that expressed qteE caused only minor soft rot disease symptoms in potato (Fig. 6a).

Drosophila melanogaster has been adopted as a model to analyse P. aeruginosa virulence and interactions between this bacterium and innate host defences. Feeding P. aeruginosa to fruit flies demonstrated the contribution of QS, the stringent response and possibly pyocyanin in pathogenesis. We adapted the feeding assay developed by Sibley et al. (2008) in the 24-well plate format to accommodate screening large numbers of assays. Flies were fed with PAO1 and the B36 mutant strain and fly survival was monitored daily. The group of flies fed with 5% sucrose alone was used as a control. The results are presented in Fig. 6(b). The data showed a significant difference in fly survival between the B36 strain and PAO1 (P<0.0001).

These results clearly demonstrate that induction of qteE could attenuate P. aeruginosa pathogenicity in vivo.

DISCUSSION

Phenazine compounds have been shown to be important virulence factors in P. aeruginosa (Lau et al., 2004; Laursen & Nielsen, 2004). Recently, it has been shown that the phenazine pyocyanin is a terminal signalling molecule in the QS network of P. aeruginosa (Dietrich et al., 2006). However, the physiological roles of phenazine compounds in nature are still not fully understood. In the present study, we performed transposon mutagenesis with PAO1 and isolated several mutants with altered phzA1 expression. Some of the mutated genes are known regulators of the QS system. The rsaL gene is known to bind lasI promoter and repress several virulence factors including lasB and phzA1 (de Kievit et al., 1999). vqsM is an AraC-type regulator controlling QS signalling and virulence in PAO1 (Dong et al., 2005). The identification of pqsD confirms the requirement of the PQS system in phzA1 expression.
The hmgA gene encodes a homogentisate 1,2-dioxygenase, which is responsible for the catabolism of tyrosine. The colour of the hmgA mutant is deep-red due to the accumulation of homogentisate through oxidation, polymerization and finally formation of pyomelanin (Zhang et al., 2008). Baert et al. (2008) showed that ccmA is required for phenazine synthesis but it has no effect on QS expression. Four genes identified (ilval, apaH, nuoD and cspD) are also involved in phenazine synthesis, but the detailed mechanism needs to be investigated in future. Another mutant identified, B27, was deep-green in colony colour, indicative of increased phenazine production. Also the expression of the phzA1 operon is upregulated by about 35-fold in this mutant compared to wild-type PAO1 (Table 2). The sequencing analysis indicated that the transposon inserted at 669 bp upstream of the translational start codon of the phzM gene. It seems plausible to assume that the phenotypic change was due to enhanced expression of phzM considering that the mariner transposon used in the study harbours an outward-directed Ptac promoter. This is also consistent with previous work demonstrating that phzM positively regulates phzA1 expression (Mavrodi et al., 2001). Importantly, the data indicate that mutation of the phzC1 gene promoted phzA1 activity, suggesting that phenazine biosynthesis is auto-regulated in P. aeruginosa (unpublished data).

Finally, two hypothetical genes (PA2593 and PA5193) were also isolated with altered phzA1 expression. Recently, Siehnel et al. (2010) demonstrated that PA2593 (qteE) is a unique regulator that blocks QS gene expression and decreases the half-life of the LasR protein without affecting lasR transcription or translation. It independently blocks RhlR protein accumulation and signalling by the rhl system. The present study investigated the impact of qteE on both QS and P. aeruginosa pathogenicity. Using the B36 strain and luxCDABE fusions, our results showed that the induction of qteE in P. aeruginosa affected the expression of virulence factors such as phzA1, rhlA, lasB and other QS-controlled phenotypes (Figs 1 and 2). Overexpression of qteE repressed QS activity and decreased signal production (Figs 3, 4 and 5) while the expression of lasR was not affected (Fig. 3a). Finally, our data showed that induction of qteE in P. aeruginosa attenuated pathogenicity in both the potato and Drosophila models (Fig. 6).

Siehnel et al. (2010) demonstrated that bacteria lacking qteE have rapidly rising expression of QS at low cell density and no quorum threshold is apparent. This model is similar to that in A. tumefaciens, where an anti-activator (TraM) inhibits QS in the prequorum period (Piper & Farrand, 2000). However, it is not clear whether other regulators such as pqsR and qscR contribute to the QS

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model in *P. aeruginosa*. Our data showed that expression of \textit{pqsR} and \textit{pqsH} was drastically decreased in the B36 strain (Fig. 5a, b) while the expression of \textit{pqsA} in the B36 strain showed no changes throughout the growth phase (Fig. 5c), which is intriguing considering that \textit{PqsR–PQS} directly activates the \textit{pqsABCDE} operon (Wade \textit{et al.}, 2005). Furthermore, it was shown that \textit{qteE} blocks RhlR accumulation independent of the action of \textit{qteE} on LaR. The data in this study suggest that \textit{qteE} might regulate the \textit{rhl} system through PqsR. Previous work showed that PQS positively regulates the \textit{rhl} system (Latifi \textit{et al.}, 1995) while the \textit{rhl} system negatively regulates the PQS system (Diggel \textit{et al.}, 2003). Because the expression of both \textit{pqsR} and \textit{rhlI/rhlR} dramatically decreased in the B36 strain (Figs 3 and 5), it is unlikely that the decreased expression of \textit{pqsR} was due to a decreased \textit{rhl} system. In such a case, \textit{pqsR} expression would increase instead. Adding exogenous C4-HSL to B36 culture did not restore the expression of \textit{rhlA} to the wild-type level (data not shown). However, it is possible that the induction of \textit{qteE} resulted in decreased expression of \textit{pqsR}, which in turn decreased the \textit{rhl} system. However, we have not yet determined whether QteE directly interacts with PqsR or the PQS system. Analysis of interactions between QteE and the PQS system using an electrophoretic mobility shift assay is under way. Further studies are needed to establish the links and connections between the players in the complicated QS-regulatory networks of *P. aeruginosa*.

For a long time, phenazines and quinolones have been recognized as antimicrobial agents. The discovery that quinolone PQS and the phenazine pyocyanin can act as intercellular signals indicates that these ‘secondary’ metabolites could play important primary roles in natural settings. The connection between phenazine compounds and cell–cell signalling calls for better understanding of phenazine regulation. The identification of the genes involved in phenazine production is a prerequisite for such understanding.

**ACKNOWLEDGEMENTS**

We thank our laboratory members for critically reading the manuscript. This work was supported by a grant from the National Natural Science Foundation of China (No. 30870097) and by NWU Doctorate Dissertation Fund (08YYB05).

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