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

Haihua Liang,¹ Jiali Duan,¹ Christopher D. Sibley,² Michael G. Surette² and Kangmin Duan^{1,2}

Correspondence

Kangmin Duan

kduan@nwu.edu.cn or

kduan@ucalgary.ca

¹Key Laboratory of Resources Biology and Biotechnology in Western China, Ministry of Education, Faculty of Life Sciences, Northwest University, Xi'an, Shaanxi 710069, PR China

²Department of Microbiology and Infectious Diseases, Faculty of Medicine, University of Calgary, 3330 Hospital Drive, Calgary, AB T2N 4N1, Canada

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.

Received 17 May 2010

Accepted 10 August 2010

INTRODUCTION

Pseudomonas aeruginosa is a ubiquitous, Gram-negative, opportunistic 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-hydroxyphenazine 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-quinoline (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

Bacterial strain/plasmid	Genotype or phenotype	Reference/source
Strains		
<i>E. coli</i>		
DH5α	F ⁻ <i>mcrA</i> Δ(<i>mrr-hsdRMS-mcrBC</i>)80 <i>dlacZ</i> Δ <i>M15</i> Δ <i>lacX74</i> <i>deoR</i> <i>recA1</i> <i>endA1</i> <i>araD139</i> Δ(<i>ara leu</i>)7697 <i>galU</i> <i>galK</i> λ ⁻ <i>rpsL</i> <i>nupG</i>	Invitrogen
SM10	Mobilizing strain, RP4 integrated in the chromosome, Kn ^r	Simon <i>et al.</i> (1983)
<i>P. aeruginosa</i>		
PAO1	Wild-type, laboratory strain	This laboratory
B36	PA2593 transposon mutant of PAO1, Gm ^r	This study
PAO(Δ2593)	PA2593 replacement mutant of PAO1, PA2593::Gm ^r	This study
PAO1(pUCP26)	Wild-type PAO1 containing pUCP26 plasmid	This study
PAO1(<i>p-qteE</i>)	Wild-type PAO1 containing <i>p-qteE</i> plasmid	This study
JP2	<i>lasI</i> - <i>rhlI</i> double mutant of PAO1	Pearson <i>et al.</i> (1997)
Plasmids		
pMS402	Expression reporter plasmid carrying the promoterless <i>luxCDABE</i> gene	Duan <i>et al.</i> (2003)
PUCP26	<i>P. aeruginosa</i> expression vector	West <i>et al.</i> (1994)
<i>p-qteE</i>	<i>lacp-qteE</i> on pUCP26	This study
pBT20	Mini-TnM delivery vector; Ap ^r , Gm ^r	Kulasekara <i>et al.</i> (2005)
pEX18Ap	<i>oriT</i> ⁺ <i>sacB</i> ⁺ gene replacement vector with multiple-cloning site from pUC18; Ap ^r	Hoang <i>et al.</i> (1998)
pZ1918-lacZGm	Source plasmid of Gm ^r cassette	Schweizer (1992)
pRK2013	Broad-host-range helper vector; Tra ⁺ , Kn ^r	Ditta <i>et al.</i> (1980)
Mini-CTX1	Integration plasmid; Tet ^r	Hoang <i>et al.</i> (2000)
pKD-2593K	PA2593 knockout plasmid pEX18Ap with 465 bp upstream region, Gm ^r - <i>lacZ</i> fragment from pZ1918Gm-lacZ and 442 bp downstream of PA2593 cloned between <i>HindIII</i> and <i>BamHI</i> ; Ap ^r (Cb ^r), Gm ^r	This study
Mini-CTX- <i>phzA1</i>	Mini-CTX1 containing <i>phzA1</i> promoter region; Tc ^r	This study
pKD- <i>pqsH</i>	pMS402 containing <i>pqsH</i> promoter region	Liang <i>et al.</i> (2008)
pKD- <i>pqsR</i>	pMS402 containing <i>pqsR</i> promoter region	Liang <i>et al.</i> (2008)
pKD- <i>rhlI</i>	pMS402 containing <i>rhlI</i> promoter region	Duan & Surette (2007)
pKD- <i>rhlR</i>	pMS402 containing <i>rhlR</i> promoter region	Duan & Surette (2007)
pKD- <i>lasI</i>	pMS402 containing <i>lasI</i> promoter region	Duan & Surette (2007)
pKD- <i>lasR</i>	pMS402 containing <i>lasR</i> promoter region	Duan & Surette (2007)
pKD- <i>rhlA</i>	pMS402 containing <i>rhlA</i> promoter region	Duan <i>et al.</i> (2003)
pKD- <i>lasB</i>	pMS402 containing <i>lasB</i> promoter region	Duan <i>et al.</i> (2003)

Transposon mutagenesis. The transposon mutagenesis library was constructed as previously described (Kulasekara *et al.*, 2005; Liang *et al.*, 2008). The donor strain (SM10- λ pir) 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 OD₆₀₀ 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 *Pst*I-digested Gm^r-*lacZ* cassette from pZ1918Gm (Schweizer, 1993) between two PCR fragments of PA2593 cloned in pEX18Ap. The 465 bp upstream fragment was amplified using primer NH1 (5'-CGTAAGCTTATGGCGACCCTCTTCCCG-3') containing a *Hind*III restriction site and NH2 (5'-TCGCTGCAGACTTGCGGCAGGCCACGT-3') containing a *Pst*I site (underlined); the 442 bp downstream region was generated using primers NH3 (5'-CGTCTGCAGCCGGCTGCAAGGCGGTGA-3') containing a *Pst*I site and NH4 (5'-CGCGGATCCACCGGCGGCCAGCGTAGC-3') containing a *Bam*HI 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 *lasI*-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, C₄-HSL, was measured using an *rhlA* promoter-based *P. aeruginosa* strain, pDO100 (pKD-*rhlA*). This detection system was developed by fusing the C₄-HSL-responsive *rhlA* promoter upstream of *luxCDABE* and introducing the construct into pDO100, a *rhlI* 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 rhlI* 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 diluted 1:300 in LB medium, and 90 µl was added to the wells of a 96-well microtitre plate. A 10 µl portion of the samples or medium control was added to the wells. The luminescence value was measured every half hour for a total of 24 h in a Victor³ 1420 multilabel plate reader, and relative levels of AHL were calculated from the maximal values (counts s⁻¹) minus the medium control.

Cloning and overexpression of the PA2593 gene. In order to generate an expression plasmid for PA2593 (*qteE*), a 573 bp DNA fragment, which began at the (PA2593) start codon (ATG) and ended 50 bp downstream from the stop codon, was amplified with the forward primer 5'-CGGCGGAATTCATGCTTATCCATTTCTGC-3' and reverse primer 5'-TCTACTGCAGCTTCAGGCCAGCCCATAG-3' using PAO1 genomic DNA as a template. The forward primer and reverse primer contain an *Eco*RI and a *Pst*I site (underlined), respectively. Vector plasmid pUCP26 (West *et al.*, 1994), which contains a *lac* promoter (*lacp*) to control gene expression, was digested with *Eco*RI and *Pst*I. The prepared plasmid DNA was ligated with the PA2593-containing PCR fragment, which had also been digested with *Eco*RI and *Pst*I, to produce the *qteE* expression plasmid

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

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 A₅₂₀ was measured. Concentrations, expressed as µg pyocyanin produced (ml culture supernatant)⁻¹, were determined by multiplying the A₅₂₀ 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 16 000 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 10 000 g. The resulting pellet was resuspended in 10 mM Na₂HPO₄ (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).

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 × 10⁹ 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

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*, *rhII*, *rhIA*, *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.

1.5 ml of solidified 5% sucrose agar in the well of a 24-well plate (Falcon Cat. no. 351147). The plates were placed at 37 °C for 30 min. Male Canton-S flies (3–5 days old) were starved for 3 h before 10–14 flies were added to each well of the 24-well plate. Carbon dioxide was used for anaesthetizing flies throughout the sorting and transferring process. Infection plates were stored at 26 °C in a humidity-controlled environment. The number of live flies was documented at the start of the experiment and continued at 24 h intervals. For each infection group, a minimum of six wells (greater than 60 flies) were used.

RESULTS

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.

To identify the genes disrupted by transposon insertion, the insertion sites were determined by arbitrary primed PCR and subsequent DNA sequencing. The mutated genes identified are listed in Table 2. Some of the genes identified have been determined before; others are novel. Transposon insertion in five different genes caused increased expression of *phzA1*, seven resulted in decreased expression, and two abolished *phzA1* expression. Of the 14 genes identified, PA5191 belongs to the class of hypothetical protein. Two of them, *rsaL* and *vqsM*, have been identified previously as belonging to 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 *phzA1* 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 *phzA1* expression, indicating that this gene is directly connected to *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*

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

Strain	Insertion site	Gene name or number*	Description	Max fold†
Increased expression				
A85	4714837	<i>phzC1</i>	Phenazine biosynthesis protein PhzC	2.5
A3	1558963	<i>rsaL</i>	Regulatory protein RsaL	4.2
B27	4713768	<i>phzM</i> -P‡	Probable phenazine biosynthesis protein	35
A52	1347875	PA1243-P‡	Probable sensor/response regulator hybrid	3.2
A19	5844013	PA5191-P‡	Hypothetical protein	3.5
Decreased expression				
B11	2198301	<i>hmgA</i>	Homogentisate 1,2-dioxygenase	−8.0
A324	649621	<i>apaH</i>	Bis(5′-nucleosyl)-tetraphosphatase	−5.0
B25	2983995	<i>nuoD</i>	NADH dehydrogenase I chain C,D	−2.8
B71	1082025	<i>pqsD</i>	3-Oxoacyl-[acyl-carrier-protein] synthase III	−12
B32	1602178	<i>ccmA</i> -P‡	Haem exporter protein CcmA	−8.5
B38	2965076	<i>cspD</i> -P‡	Cold-shock protein CspD	−7.8
B5	5275134	<i>ilvI</i>	Acetolactate synthase large subunit	−6.0
No expression				
B36	2935823	PA2593-P‡	Hypothetical protein	NE
A280	2448826	<i>vqsM</i>	Probable transcriptional regulator	NE

NE, Expression of the *phzA1* operon in these mutants was very low so an accurate ratio could not be determined.

*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 *rhIA* 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 $\pm$ SD of triplicate cultures. (e) Expression of QS-controlled genes *rhIA* 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.

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

Fig. 3. Expression of QS genes in PAO1 (Δ) and B36 ($\circ$). The assay was independently repeated at least three times and the data shown are representatives of comparable results.

(Heurlier *et al.*, 2004). Measurement of *rhIA* and *lasB* expression in the B36 strain indicated that both *rhIA* 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 (Dziel *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(Δ 2593) and the wild-type PAO1 (data not shown).

Fig. 4. AHL production in PAO1 and PAO1(*p-qteE*). AHL plate bioassay was carried out using *P. aeruginosa* (pDO100-*rhIA*) (a) and *A. tumefaciens* reporter strain (b). On the left, 2 μ l test bacterial cultures ($OD_{600}=1.0$) was inoculated and the plates were incubated at 37 °C for 24 h. The halo zone around bacterial colonies indicates AHL activity. On the right are measurements of the relative amount of AHL. The means of three independent measurements and their SD are represented.

Fig. 5. Expression of PQS genes in PAO1 (Δ) and strain B36 ($\circ$). Light production of *P. aeruginosa* PAO1 containing *pqsR* (a), *pqsH* (b) or *pqsA* (c) *luxCDABE* fusion plasmids was measured.

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

Fig. 6. Effect of *qteE* gene overexpression on *P. aeruginosa* pathogenicity. (a) Potato infection. The photographs were taken 3 days after inoculation. (b) Kaplan–Meier survival curves of *P. aeruginosa* infections. ○, Survival curve of flies feeding on 5% sucrose; □, survival of flies infected with PAO1; △, survival of flies infected with JP2; ▲, survival of flies infected with B36 with induced PA2593 expression. Log-rank analysis (Mantel–Cox) was used to compare infections with the B36 and the PAO1 control ($P < 0.0001$).

(Gallagher *et al.*, 2002). 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 *P_{tac}* 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

model in *P. aeruginosa*. Our data showed that expression of *pqsR* and *pqsH* was drastically decreased in the B36 strain (Fig. 5a, b) while the expression of *pqsA* in the B36 strain showed no changes throughout the growth phase (Fig. 5c), which is intriguing considering that PqsR–PQS directly activates the *pqsABCDE* operon (Wade *et al.*, 2005). Furthermore, it was shown that *qteE* blocks RhlR accumulation independent of the action of *qteE* on LasR. The data in this study suggest that *qteE* might regulate the *rhl* system through PqsR. Previous work showed that PQS positively regulates the *rhl* system (Latifi *et al.*, 1995) while the *rhl* system negatively regulates the PQS system (Diggle *et al.*, 2003). Because the expression of both *pqsR* and *rhlI/rhlR* dramatically decreased in the B36 strain (Figs 3 and 5), it is unlikely that the decreased expression of *pqsR* was due to a decreased *rhl* system. In such a case, *pqsR* expression would increase instead. Adding exogenous C₄-HSL to B36 culture did not restore the expression of *rhlA* to the wild-type level (data not shown). However, it is possible that the induction of *qteE* resulted in decreased expression of *pqsR*, which in turn decreased the *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).

REFERENCES

Akiyama, M., Oishi, K., Tao, M., Matsumoto, K. & Pollack, M. (2000). Antibacterial properties of *Pseudomonas aeruginosa* immunotype 1 lipopolysaccharide-specific monoclonal antibody (MAb) in a murine thigh infection model: combined effects of MAb and ceftazidime. *Microbiol Immunol* **44**, 629–635.

Allen, L., Dockrell, D. H., Pattery, T., Lee, D. G., Cornelis, P., Hellewell, P. G. & Whyte, M. K. (2005). Pyocyanin production by *Pseudomonas aeruginosa* induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. *J Immunol* **174**, 3643–3649.

Baert, B., Baysse, C., Matthijs, S. & Cornelis, P. (2008). Multiple phenotypic alterations caused by a *c*-type cytochrome maturation *ccmC* gene mutation in *Pseudomonas aeruginosa*. *Microbiology* **154**, 127–138.

Blackwood, L. L., Stone, R. M., Iglewski, B. H. & Pennington, J. E. (1983). Evaluation of *Pseudomonas aeruginosa* exotoxin A and elastase as virulence factors in acute lung infection. *Infect Immun* **39**, 198–201.

Chambers, C. E., Visser, M. B., Schwab, U. & Sokol, P. A. (2005). Identification of N-acylhomoserine lactones in mucopurulent respiratory secretions from cystic fibrosis patients. *FEMS Microbiol Lett* **244**, 297–304.

Chugani, S. A., Whiteley, M., Lee, K. M., D’Argenio, D., Manoil, C. & Greenberg, E. P. (2001). QscR, a modulator of quorum-sensing signal synthesis and virulence in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **98**, 2752–2757.

de Kievit, T., Seed, P. C., Nezezon, J., Passador, L. & Iglewski, B. H. (1999). RsaL, a novel repressor of virulence gene expression in *Pseudomonas aeruginosa*. *J Bacteriol* **181**, 2175–2184.

Dénervaud, V., TuQuoc, P., Blanc, D., Favre-Bonté, S., Krishnapillai, V., Reimann, C., Haas, D. & van Delden, C. (2004). Characterization of cell-to-cell signaling-deficient *Pseudomonas aeruginosa* strains colonizing intubated patients. *J Clin Microbiol* **42**, 554–562.

Denning, G. M., Wollenweber, L. A., Railsback, M. A., Cox, C. D., Stoll, L. L. & Britigan, B. E. (1998). *Pseudomonas* pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infect Immun* **66**, 5777–5784.

Déziel, E., Lépine, F., Milot, S., He, J., Mindrinos, M. N., Tompkins, R. G. & Rahme, L. G. (2004). Analysis of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines (HAQs) reveals a role for 4-hydroxy-2-heptylquinoline in cell-to-cell communication. *Proc Natl Acad Sci U S A* **101**, 1339–1344.

Dietrich, L. E., Price-Whelan, A., Petersen, A., Whiteley, M. & Newman, D. K. (2006). The phenazine pyocyanin is a terminal signalling factor in the quorum sensing network of *Pseudomonas aeruginosa*. *Mol Microbiol* **61**, 1308–1321.

Diggle, S. P., Winzer, K., Chhabra, S. R., Worrall, K. E., Camara, M. & Williams, P. (2003). The *Pseudomonas aeruginosa* quinolone signal molecule overcomes the cell density-dependency of the quorum sensing hierarchy, regulates *rhl*-dependent genes at the onset of stationary phase and can be produced in the absence of LasR. *Mol Microbiol* **50**, 29–43.

Ditta, G., Stanfield, S., Corbin, D. & Helinski, D. R. (1980). Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. *Proc Natl Acad Sci U S A* **77**, 7347–7351.

Dong, Y. H., Zhang, X. F., Xu, J. L., Tan, A. T. & Zhang, L. H. (2005). VqsM, a novel AraC-type global regulator of quorum-sensing signalling and virulence in *Pseudomonas aeruginosa*. *Mol Microbiol* **58**, 552–564.

Duan, K. & Surette, M. G. (2007). Environmental regulation of *Pseudomonas aeruginosa* PAO1 Las and Rhl quorum-sensing systems. *J Bacteriol* **189**, 4827–4836.

Duan, K., Dammel, C., Stein, J., Rabin, H. & Surette, M. G. (2003). Modulation of *Pseudomonas aeruginosa* gene expression by host microflora through interspecies communication. *Mol Microbiol* **50**, 1477–1491.

Essar, D. W., Eberly, L., Hadero, A. & Crawford, I. P. (1990). Identification and characterization of genes for a second anthranilate synthase in *Pseudomonas aeruginosa*: interchangeability of the two anthranilate synthases and evolutionary implications. *J Bacteriol* **172**, 884–900.

- Gallagher, L. A., McKnight, S. L., Kuznetsova, M. S., Pesci, E. C. & Manoil, C. (2002). Functions required for extracellular quinolone signaling by *Pseudomonas aeruginosa*. *J Bacteriol* **184**, 6472–6480.
- Guerra-Santos, L. H., Käppeli, O. & Fiechter, A. (1986). Dependence of *Pseudomonas aeruginosa* continuous culture biosurfactant production on nutritional and environmental factors. *Appl Microbiol Biotechnol* **24**, 443–448.
- Heurlier, K., Williams, F., Heeb, S., Dormond, C., Pessi, G., Singer, D., Camara, M., Williams, P. & Haas, D. (2004). Positive control of swarming, rhamnolipid synthesis, and lipase production by the posttranscriptional RsmA/RsmZ system in *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **186**, 2936–2945.
- Hoang, T. T., Karkhoff-Schweizer, R. R., Kutchma, A. J. & Schweizer, H. P. (1998). A broad-host-range Flp-FRT recombination system for site-specific excision of chromosomally-located DNA sequences: application for isolation of unmarked *Pseudomonas aeruginosa* mutants. *Gene* **212**, 77–86.
- Hoang, T. T., Kutchma, A. J., Becher, A. & Schweizer, H. P. (2000). Integration-proficient plasmids for *Pseudomonas aeruginosa*: site-specific integration and use for engineering of reporter and expression strains. *Plasmid* **43**, 59–72.
- Howe, T. R. & Iglewski, B. H. (1984). Isolation and characterization of alkaline protease-deficient mutants of *Pseudomonas aeruginosa* in vitro and in a mouse eye model. *Infect Immun* **43**, 1058–1063.
- Kessler, E., Safrin, M., Olson, J. C. & Ohman, D. E. (1993). Secreted LasA of *Pseudomonas aeruginosa* is a staphylytic protease. *J Biol Chem* **268**, 7503–7508.
- Koch, A. K., Kappeli, O., Fiechter, A. & Reiser, J. (1991). Hydrocarbon assimilation and biosurfactant production in *Pseudomonas aeruginosa* mutants. *J Bacteriol* **173**, 4212–4219.
- Köhler, T., Curty, L. K., Barja, F., van Delden, C. & Pechère, J.-C. (2000). Swarming of *Pseudomonas aeruginosa* is dependent on cell-to-cell signaling and requires flagella and pili. *J Bacteriol* **182**, 5990–5996.
- Kulasekara, H. D., Ventre, I., Kulasekara, B. R., Lazdunski, A., Filloux, A. & Lory, S. (2005). A novel two-component system controls the expression of *Pseudomonas aeruginosa* fimbrial cup genes. *Mol Microbiol* **55**, 368–380.
- Kurachi, M. (1958). Studies on the biosynthesis of pyocyanine. Isolation and determination of pyocyanine. *Bull Inst Chem Res Kyoto Univ* **36**, 163–173.
- Latifi, A., Winson, M. K., Foglino, M., Bycroft, B. W., Stewart, G. S., Lazdunski, A. & Williams, P. (1995). Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* **17**, 333–343.
- Lau, G. W., Hassett, D. J., Ran, H. & Kong, F. (2004). The role of pyocyanin in *Pseudomonas aeruginosa* infection. *Trends Mol Med* **10**, 599–606.
- Laursen, J. B. & Nielsen, J. (2004). Phenazine natural products: biosynthesis, synthetic analogues, and biological activity. *Chem Rev* **104**, 1663–1686.
- Liang, H., Li, L., Dong, Z., Surette, M. G. & Duan, K. (2008). The YebC family protein PA0964 negatively regulates the *Pseudomonas aeruginosa* quinolone signal system and pyocyanin production. *J Bacteriol* **190**, 6217–6227.
- Mavrodi, D. V., Ksenzenko, V. N., Bonsall, R. F., Cook, R. J., Boronin, A. M. & Thomashow, L. S. (1998). A seven-gene locus for synthesis of phenazine-1-carboxylic acid by *Pseudomonas fluorescens* 2-79. *J Bacteriol* **180**, 2541–2548.
- Mavrodi, D. V., Bonsall, R. F., Delaney, S. M., Soule, M. J., Phillips, G. & Thomashow, L. S. (2001). Functional analysis of genes for biosynthesis of pyocyanin and phenazine-1-carboxamide from *Pseudomonas aeruginosa* PAO1. *J Bacteriol* **183**, 6454–6465.
- McGrath, S., Wade, D. S. & Pesci, E. C. (2004). Dueling quorum sensing systems in *Pseudomonas aeruginosa* control the production of the *Pseudomonas* quinolone signal (PQS). *FEMS Microbiol Lett* **230**, 27–34.
- Pearson, J. P., Pesci, E. C. & Iglewski, B. H. (1997). Roles of *Pseudomonas aeruginosa* las and rhl quorum-sensing systems in control of elastase and rhamnolipid biosynthesis genes. *J Bacteriol* **179**, 5756–5767.
- Piper, K. R. & Farrand, S. K. (2000). Quorum sensing but not autoinduction of Ti plasmid conjugal transfer requires control by the opine regulon and the antiactivator TraM. *J Bacteriol* **182**, 1080–1088.
- Price-Whelan, A., Dietrich, L. E. & Newman, D. K. (2006). Rethinking ‘secondary’ metabolism: physiological roles for phenazine antibiotics. *Nat Chem Biol* **2**, 71–78.
- Rodríguez-Rojas, A., Mena, A., Martín, S., Borrell, N., Oliver, A. & Blázquez, J. (2009). Inactivation of the *hmgA* gene of *Pseudomonas aeruginosa* leads to pyomelanin hyperproduction, stress resistance and increased persistence in chronic lung infection. *Microbiology* **155**, 1050–1057.
- Schuster, M., Lostroh, C. P., Ogi, T. & Greenberg, E. P. (2003). Identification, timing, and signal specificity of *Pseudomonas aeruginosa* quorum-controlled genes: a transcriptome analysis. *J Bacteriol* **185**, 2066–2079.
- Schweizer, H. P. (1992). Allelic exchange in *Pseudomonas aeruginosa* using novel ColE1-type vectors and a family of cassettes containing a portable *oriT* and the counter-selectable *Bacillus subtilis* *sacB* marker. *Mol Microbiol* **6**, 1195–1204.
- Schweizer, H. P. (1993). Two plasmids, X1918 and Z1918, for easy recovery of the *xylE* and *lacZ* reporter genes. *Gene* **134**, 89–91.
- Sibley, C. D., Duan, K., Fischer, C., Parkins, M. D., Storey, D. G., Rabin, H. R. & Surette, M. G. (2008). Discerning the complexity of community interactions using a *Drosophila* model of polymicrobial infections. *PLoS Pathog* **4**, e1000184.
- Siehnelt, R., Traxler, B., An, D. D., Parsek, M. R., Schaefer, A. L. & Singh, P. K. (2010). A unique regulator controls the activation threshold of quorum-regulated genes in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **107**, 7916–7921.
- Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Nat Biotechnol* **1**, 784–791.
- Stover, C. K., Pham, X. Q., Erwin, A. L., Mizoguchi, S. D., Warrenner, P., Hickey, M. J., Brinkman, F. S., Hufnagle, W. O., Kowalik, D. J. & other authors (2000). Complete genome sequence of *Pseudomonas aeruginosa* PAO1, an opportunistic pathogen. *Nature* **406**, 959–964.
- Venturi, V. (2006). Regulation of quorum sensing in *Pseudomonas*. *FEMS Microbiol Rev* **30**, 274–291.
- Wade, D. S., Calfee, M. W., Rocha, E. R., Ling, E. A., Engstrom, E., Coleman, J. P. & Pesci, E. C. (2005). Regulation of *Pseudomonas* quinolone signal synthesis in *Pseudomonas aeruginosa*. *J Bacteriol* **187**, 4372–4380.
- West, S. E., Schweizer, H. P., Dall, C., Sample, A. K. & Runyen-Janecky, L. J. (1994). Construction of improved *Escherichia-Pseudomonas* shuttle vectors derived from pUC18/19 and sequence of the region required for their replication in *Pseudomonas aeruginosa*. *Gene* **148**, 81–86.
- Whiteley, M., Lee, K. M. & Greenberg, E. P. (1999). Identification of genes controlled by quorum sensing in *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 13904–13909.

Williams, P., Winzer, K., Chan, W. C. & Camara, M. (2007). Look who's talking: communication and quorum sensing in the bacterial world. *Philos Trans R Soc Lond B Biol Sci* **362**, 1119–1134.

Wilson, R., Pitt, T., Taylor, G., Watson, D., MacDermot, J., Sykes, D., Roberts, D. & Cole, P. (1987). Pyocyanin and 1-hydroxyphenazine

produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro. *J Clin Invest* **79**, 221–229.

Zhang, Y., Wang, L., Zhang, S., Yang, H. & Tan, H. (2008). *hmgA*, transcriptionally activated by Hpda, influences the biosynthesis of actinorhodin in *Streptomyces coelicolor*. *FEMS Microbiol Lett* **280**, 219–225.