The role of two *Pseudomonas aeruginosa* anthranilate synthases in tryptophan and quorum signal production

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Pseudomonas aeruginosa is a Gram-negative, opportunistic pathogen that causes infections in the lungs of individuals with the genetic disease cystic fibrosis. Density-dependent production of toxic factors regulated by the Pseudomonas quinolone signal (2-heptyl-3-hydroxy-4-quinolone; PQS) have been proposed to be involved in *P. aeruginosa* virulence. PQS biosynthesis requires conversion of the central metabolite chorismate to anthranilate by anthranilate synthase. This reaction is also the first step in tryptophan biosynthesis. *P. aeruginosa* possesses two functional anthranilate synthases, TrpEG and PhnAB, and these enzymes are not functionally redundant, as *trpEG* mutants are tryptophan auxotrophs but produce PQS while mutants in *phnAB* are tryptophan prototrophs but do not produce PQS in minimal media. The goal of the work described in this paper was to determine the mechanism for this lack of functional complementation of TrpEG and PhnAB. Our results reveal that overexpression of either enzyme compensates for tryptophan auxotrophy and PQS production in the *trpEG* and *phnAB* mutants respectively, leading to the hypothesis that differential regulation of these genes is responsible for the lack of functional complementation. In support of this hypothesis, *trpEG* was shown to be expressed primarily during low-density growth while *phnAB* was expressed primarily at high density. Furthermore, dysregulation of *phnAB* expression eliminated tryptophan auxotrophy in the *P. aeruginosa trpEG* mutant. Based on these data, we propose a model for anthranilate sequestration by differential transcriptional regulation of the two *P. aeruginosa* anthranilate synthase enzymes.

INTRODUCTION

The Gram-negative, opportunistic pathogen *Pseudomonas aeruginosa* is a primary constituent of chronic, polymicrobial infections in the lungs of individuals with the genetic disease cystic fibrosis (CF) (Delhaes *et al.*, 2012; Harrison, 2007). Because it is intrinsically resistant to many conventional antimicrobial therapies, *P. aeruginosa* is generally the most challenging pathogen to eradicate from these infections, and is the leading cause of morbidity and mortality for individuals with CF (Emerson *et al.*, 2002; Hoiby *et al.*, 1977). *P. aeruginosa* utilizes numerous virulence factors to colonize and persist in the CF lung, and many of those virulence factors are regulated in a density-dependent manner through a process called quorum sensing (QS) (Déziel *et al.*, 2005; Rumbaugh *et al.*, 1999; Schuster & Greenberg, 2006; Whiteley *et al.*, 1999). In a canonical Gram-negative QS system, a small molecule called an autoinducer is synthesized constitutively, and the concentration of autoinducer increases as

cell density increases. Upon reaching a threshold concentration, autoinducers interact with transcriptional regulators to alter gene expression. *P. aeruginosa* has three known QS systems: two require the production of acylhomoserine lactone autoinducers and one utilizes quinolone signals (Pesci *et al.*, 1999; Schuster & Greenberg, 2006). The most potent quinolone signal is the Pseudomonas quinolone signal (2-heptyl-3-hydroxoy-4-quinlone; PQS), which regulates several genes involved in virulence (Déziel *et al.*, 2005; Gallagher & Manoil, 2001). PQS signalling is likely to be relevant in the CF lung, as *P. aeruginosa* produces five times more PQS when grown in CF lung fluids (sputum) compared with when it is grown in a standard laboratory medium (Palmer *et al.*, 2005). Using a defined synthetic CF sputum medium that mimics the nutritional contents of sputum, research in our laboratory has determined that phenylalanine and tyrosine are responsible for enhanced PQS production in CF sputum (Palmer *et al.*, 2007). Subsequent work has demonstrated that aromatic amino acid induction of PQS production is not due to co-regulation of phenylalanine/tyrosine catabolism and PQS biosynthesis (Palmer *et al.*, 2010).

Our favoured model for enhanced PQS production in the presence of phenylalanine and tyrosine involves flux of the central metabolite chorismate, a shared metabolic precursor for aromatic amino acid and PQS biosynthesis (Palmer et al., 2010, 2007). Chorismate is converted to anthranilate by an anthranilate synthase in the first step of PQS biosynthesis, however, degradation of tryptophan via the products of the kynABU operon has also been reported to be a significant source of anthranilate (Farrow & Pesci, 2007). Our measurements of CF sputum have indicated extremely low tryptophan levels (<10 µM) in the CF lung, thus chorismate-mediated synthesis of anthranilate for PQS production is likely to be the relevant biosynthetic scheme in the CF lung (Palmer et al., 2007).

It was discovered in 1990 that P. aeruginosa possesses two functional anthranilate synthases, each comprised of a large and small subunit encoded by the products of the trpE and trpG or phnA and phnB genes, respectively (Essar et al., 1990a, b). Interestingly, early evidence indicated that these enzymes are not functionally redundant, as growth experiments using a marked trpE mutant demonstrated tryptophan auxotrophy while a similar phnA mutant did not (Essar et al., 1990a, b). The authors also observed that the phnA mutant was deficient in production of the virulence factor pyocyanin while the trpE mutant was not (Essar et al., 1990a, b). At the time it was believed that PhnAB generated anthranilate for pyocyanin production, but later work determined that anthranilate is instead a precursor for PQS production, which is required to induce

genes for pyocyanin biosynthesis (Calfee et al., 2001; Gallagher et al., 2002; Mavrodi et al., 2001). These observations led to the hypothesis that TrpEG generates anthranilate for tryptophan biosynthesis while PhnAB generates anthranilate for PQS biosynthesis; however, a mechanism explaining the lack of crosstalk between these pathways has not been determined.

Here, we investigated the two P. aeruginosa anthranilate synthases, TrpEG and PhnAB, in order to better characterize their roles in physiology and pathogenesis and determine why anthranilate does not appear to be shared between the tryptophan and PQS biosynthetic pathways. We provide evidence that differential expression of trpEG and phnAB is the mechanism for their lack of functional redundancy.

METHODS

Bacterial strains and media. Strains and plasmids used in this work are listed in Table 1. Luria–Bertani (LB) broth (Fisher) was used for growth of Escherichia coli and P. aeruginosa for molecular cloning and genetic manipulations. For growth and physiology experiments, P. aeruginosa cultures were grown in a MOPS minimal medium, a MOPS-buffered salts base (50 mM MOPS, pH 7.2, 93 mM NH$_4$Cl, 43 mM NaCl, 3.7 mM KH$_2$PO$_4$, 1 mM MgSO$_4$, 3.5 µM FeSO$_4$ heptahydrate) supplemented with 20 mM succinate as a sole source of carbon and energy. When necessary for growth of tryptophan auxotrophic strains (see Table 1), MOPS minimal medium was additionally supplemented with 200 µM tryptophan unless otherwise stated. MOPS buffer was used to wash and starve cells and comprises

Table 1. Strains and plasmids used in this study

All mutant strains and plasmids were generated in this study unless otherwise referenced.

Strain or plasmid	Description
Strains	
E. coli	
DH5α	Wild-type strain for molecular cloning (Sambrook & Russell, 2001)
SM10	Conjugation strain for deletion mutant generation (de Lorenzo & Timmis, 1994)
P. aeruginosa	
PA14	Wild-type Pseudomonas aeruginosa strain (Liberati et al., 2006)
ΔtrpE	trpE clean deletion in PA14 background
ΔkynA	kynA clean deletion in PA14 background
ΔphnAB	phnAB clean deletion in PA14 background
ΔtrpE ΔkynA	trpE and kynA double clean deletions in PA14 background
ΔtrpE ΔphnAB	trpE and phnAB double clean deletions in PA14 background
ΔtrpE ΔpqsA	trpE and pqsA double clean deletions in PA14 background
Plasmids	
pEX18Tc	Gene replacement vector, Tc^R (Hoang et al., 1998)
pJN105	araC-pBAD expression vector, Gm^R (Newman & Fuqua, 1999)
pGPKO-trpE	pEX18-derived trpE deletion vector, Tc^R
pGPKO-kynA	pEX18-derived kynA deletion vector, Tc^R
pGPKO-phnAB	pEX18-derived phnAB deletion vector, Tc^R
pGPKO-pqsA	pEX18-derived pqsA deletion vector, Tc^R
pGP-trpEG	pJN105-derived trpEG overexpression vector, Gm^R
pKP-phnAB	pJN105-derived phnAB overexpression vector, Gm^R

Table 2. Primers used in this study

Underlined sequences represent recognition sites for restriction endonucleases.

Description	Sequence
Deletion vector construction	
trpE flank 1 for	CTGAATTCGTCAACGTCAAGAACATCCGTGAG
trpE flank 1 rev	GTCCAGCCGAGCACAAAAAAGGCATCGGAGGAGAGAACGGTCAAC
trpE flank 2 for	GTTGACCGTTCTCTCCTCCGATGCCTTTTTTGTGCTCGGCTGGAC
trpE flank 2 rev	CTTCTAGAGCTGTACAAGGAAGTGGAGATGTC
kynA flank 1 for	CTGGTACCCGACTTTTTCCGTTCCCTGC
kynA flank 1 rev	GTACTACAGGTTGGAACGGAGCGTGAATCTCCTGAATTCGGC
kynA flank 2 for	GCCGAATTCAGGAGATTCACGCTCCGTTCCAACCTGTAGTAC
kynA flank 2 rev	CTTCTAGAGACATGACCGACGACATCGAC
phnAB flank 1 for	CTGAATTCGGTCAGCAACCTGGAAATCG
phnAB flank 1 rev	CGATGATGAACATGCCGTTGCCATCCCGAGTCGATTCTCAC
phnAB flank 2 for	GTGAGAATCGACTCGGGATGGCAACGGCATGTTCATCATCG
phnAB flank 2 rev	CTTCTAGACGTAAACCTGAGGAGGTGAAC
pqsA flank 1 for	CTGAATTCCCAGAATTGCCACCAAGACTC
pqsA flank 1 rev	GTCCACATTGGCCAACCTGACCCCTTTATCACGACAACCTTC
pqsA flank 2 for	GAAGGTTGTCGTGATAAAGGGGTCAGGTTGGCCAATGTGGAC
pqsA flank 2 rev	CTTCTAGACTATGGCAAGGTGCAACAATGG
Deletion confirmation	
trpE for	GGTACCCTCGACAAGTTGC
trpE rev	CATTGGTGCTGGAACCGCTG
kynA for	CGTACTGCGTTGGTGATGG
kynA rev	CCTCCATCGCATTACTCAGG
phnAB for	CGTGAACATGTTCCTCCAGG
phnAB rev	GGATCGTCTGGGCAACATG
pqsA for	CCAGGCTGAACTCGTTCTCG
pqsA rev	GGTTTCCAAACGCAGCAACC
Expression vectors	
phnAB for	GACTAGTGCGCGCTAGCGTCGCGCAGG
phnAB rev	GCTCTAGACCTGGCAACCGAGCATCGTCG
trpE for	GCCTGCAGCGTTTGCACCCTGTTGACC
trpE rev	GCAGAGCGTCGAGTAAGACGGAAATCAAGAGGTTACAGCC
trpG for	GGCTGTAACCTCTTGATTTCCGTCTTACTCGACGCTCTGC
trpG rev	GCTCTAGAGGTTGACGATGCGATTGAGG
RT-PCR	
phnA for	CGTTGAACGCCAATGGACG
phnA rev	CGGTACGATCTGGAACACG
trpE for	CGTAGTGGTATTCGACAACC
trpE rev	CGATCAGCATCAGGTGCTCG
rplU for	CGCAGTGATTGTTACCGGTG
rplU rev	AGGCCTGAATGCCGGTGATC

MOPS minimal medium without a carbon source. Tetracycline concentrations for *E. coli* and *P. aeruginosa* were 10 and 50 μg ml^{-1} for selection and 5 and 25 μg ml^{-1} for maintenance, respectively. Gentamicin concentrations for *P. aeruginosa* containing pJN105-derived plasmids were 50 μg ml^{-1} for selection and 25 μg ml^{-1} for maintenance. Growth conditions were 37 °C with shaking at 250 r.p.m.

DNA manipulations. Standard methods were utilized for handling DNA fragments and plasmids (Ausubel, 2002). Restriction endonucleases and other DNA-modifying enzymes were purchased from New England Biolabs. Genomic DNA preparations were performed using a DNeasy Tissue kit (Qiagen). Plasmid DNA preparations were performed using a GeneJet Plasmid Miniprep kit (Fermentas), and

purification of DNA fragments was done using a GeneJet PCR Purification kit (Fermentas). PCR was performed using the Expand Long Template PCR System (Roche). DNA sequencing to confirm newly generated constructs was performed at the University of Texas at Austin Institute for Cell and Molecular Biology DNA core facility.

Anthranilate synthase phylogenetic analysis. The amino acid sequences for *P. aeruginosa* PA14 TrpE and PhnA were obtained from http://www.pseudomonas.com and used to probe the database of non-redundant protein sequences at http://blast.ncbi.nlm.nih.gov with BLAST. Sequences for the top 30 hits from unique species were aligned using the MEGA 5 muscle alignment feature and phylogenetic trees were generated using the MEGA 5 maximum-likelihood tree generator with 100 bootstrap replicates (Tamura *et al.*, 2011).
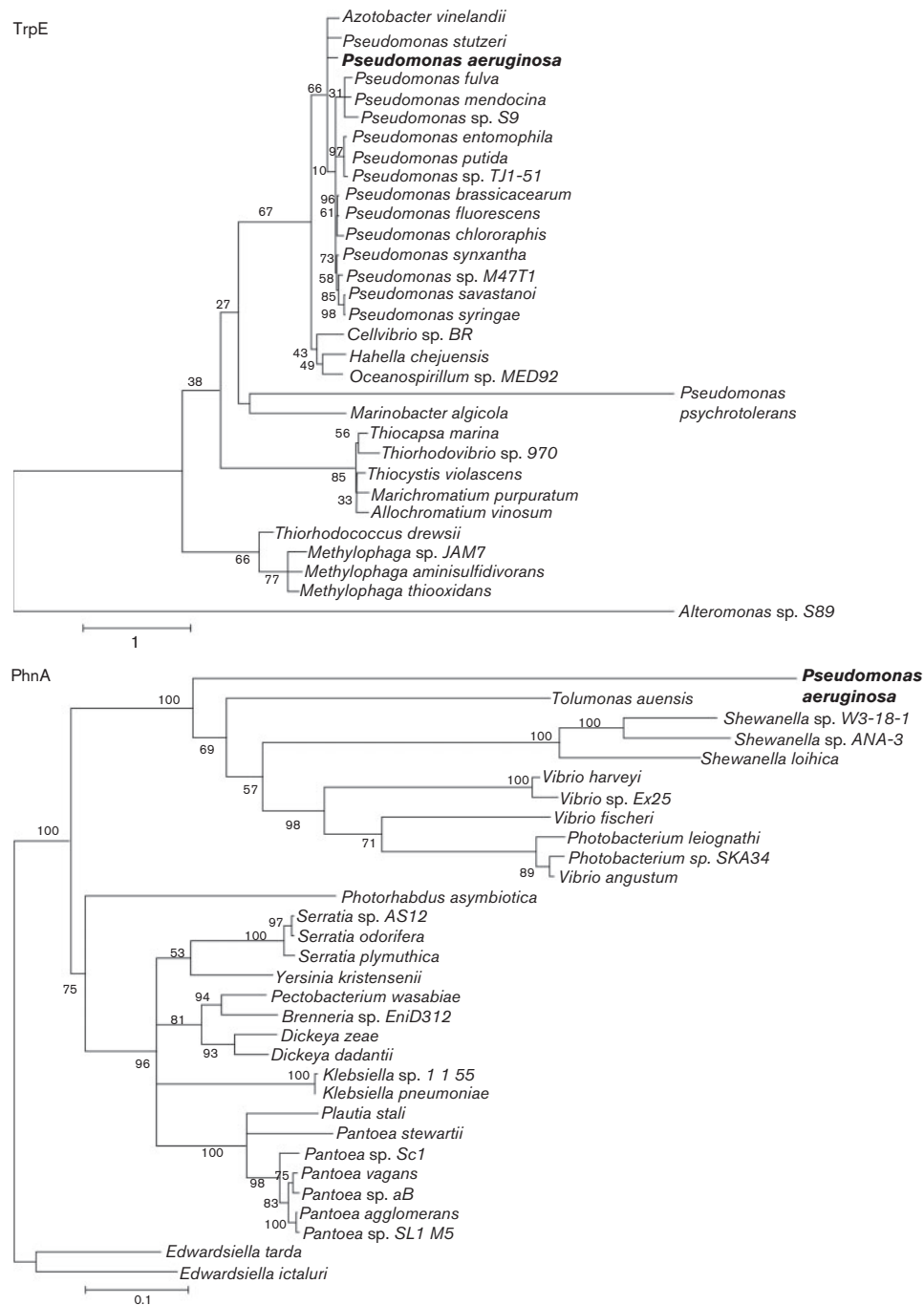
Fig. 1. Anthranilate synthase phylogenetic trees. The top 30 homologues identified by BLAST analysis using the *P. aeruginosa* TrpE and PhnA sequences reveal the evolutionary relationships of each anthranilate synthase enzyme to those of other species. TrpEG are most closely related to anthranilate synthases from other members of the fluorescent pseudomonad family, while PhnAB are most closely related to anthranilate synthases from more distantly related organisms. The absence of a *phnAB*-like operon in other pseudomonads is evidence that PhnAB acquisition occurred after the family's diversification. Bootstrap values at tree nodes indicate likelihood the node represents a genuine phylogenetic relationship.

Generation of deletion mutants. Unmarked deletion mutants were constructed by allelic exchange as described previously with some modifications (Hoang *et al.*, 1998). To generate deletion constructs, ~1000 bp regions upstream and downstream of the desired locus to be deleted were PCR amplified using *P. aeruginosa* PA14 DNA as template and primers listed in Table 2. Amplicons were concatenated by overlap extension PCR, digested with *Eco*RI and *Xba*I or *Kpn*I and *Xba*I (see restriction sites in Table 2) and

ligated into pEX18Tc to generate the deletion vectors listed in Table 1. Deletion constructs were conjugated into PA14, or a previously generated mutant strain for double mutants, using the conjugation-competent *E. coli* strain SM10. Mutants were selected by growth on LB plates containing 50 μg tetracycline ml^{-1} and 25 μg nalidixic acid ml^{-1} for merodiploid transconjugants. Subsequent growth on LB plates containing 10 % sucrose was used to select for excision of the integrated plasmid with the desired deletion locus. Deletions were confirmed by PCR using confirmation primers outside the amplified flanking regions (Table 2) as well as phenotypic characterization for tryptophan auxotrophy (Δ*trpE*, Δ*trpE* Δ*phnAB*, Δ*trpE* Δ*pqsA*), PQS production (Δ*phnAB*, Δ*trpE* Δ*phnAB*, Δ*trpE* Δ*pqsA*) and ability to grow on tryptophan (Δ*kynA*, Δ*trpE* Δ*kynA*).

Growth curves. To determine growth characteristics of PA14, Δ*phnAB* and Δ*trpE* strains, cultures of each were grown overnight in MOPS minimal medium supplemented with 200 μM tryptophan. Overnight cultures were subcultured into fresh MOPS minimal medium supplemented with 200 μM tryptophan and grown to mid-exponential phase (OD$_{600}$ 0.05) at which point cells were washed twice, concentrated and starved for 2 h in MOPS buffer. Washed, starved, exponential-phase cells were used to inoculate 25 ml MOPS minimal medium with no tryptophan to OD$_{600}$ 0.01, and growth was measured by OD$_{600}$ readings every 30 min upon the initiation of exponential growth. To demonstrate tryptophan-dependent growth of Δ*trpE*, tryptophan was added to a final concentration of 200 μM three hours after inoculation. Growth was monitored for 8 h after inoculation, and the generation times for each culture were calculated. The growth curve was repeated three times.

PQS extractions. Overnight cultures of indicated strains grown in MOPS minimal medium supplemented with 200 μM tryptophan when necessary were subcultured into fresh MOPS minimal medium to OD$_{600}$ 0.05 and grown to mid-exponential phase (OD$_{600}$ 0.5). Mid-exponential-phase cells were washed twice, concentrated and starved for 2 h in MOPS buffer. Washed, starved, exponential-phase cells were used to inoculate 12.5 ml MOPS minimal medium containing 20 mM succinate supplemented with 5 mM of the stated amino acid or anthranilate. Cultures were grown for 24 h (final OD$_{600}$ values were consistently within a range of 1.5–2.0) and 10 ml were removed and extracted twice with an equal volume of ethyl acetate (Fisher) acidified with 150 μl glacial acetic acid l^{-1} (Fisher). Ethyl acetate extracts were dried completely under a constant stream of N$_2$ gas and resuspended in methanol (Fisher). PQS in extracts was resolved by separation on thin layer chromatography plates (EMD) as described previously (Palmer *et al.*, 2007, 2010, 2011). PQS spots were imaged with excitation by long-wavelength UV light, and spots were quantified by densitometry using GeneTools software compared with a standard curve of synthetic PQS standards spotted on the same plate. For TLC images, 250 ng synthetic PQS or anthranilate were spotted on the TLC plate as migration standards.

Overexpression of TrpEG and PhnAB. Overexpression constructs were generated by PCR amplification of the *trpE* and *trpG* genes and the *phnAB* operon using PA14 genomic DNA as template and primers listed in Table 2. Because the *trpE* and *trpG* genes are not adjacent on the *P. aeruginosa* chromosome (Essar *et al.*, 1990b), overlap extension PCR was used to generate a fused construct. This was not necessary for *phnAB*, which are present in an operon (Cao *et al.*, 2001; Essar *et al.*, 1990a). The *trpEG* and *phnAB* PCR products were digested with *Pst*I and *Xba*I or *Spe*I and *Xba*I (see restriction sites in Table 2), respectively, and ligated into the arabinose-inducible vector pJN105 to generate pGP-*trpEG* and pKP-*phnAB* (Table 1). Expression constructs and empty pJN105 were introduced into relevant *P.*

aeruginosa strains by MgCl$_2$ transformation as described previously (Whiteley *et al.*, 2000).

To determine whether overexpression of PhnAB could restore growth to PA14 Δ*trpE*, washed, starved, exponential-phase Δ*trpE* cells containing pKP-*phnAB* or empty pJN105 were prepared as described above and used to inoculate 3 ml MOPS minimal medium with 0.5 % arabinose. After overnight growth (~16 h), a photograph was taken to demonstrate final growth yield. To determine whether overexpression of TrpEG restores PQS production in a minimal medium, washed, starved, exponential-phase PA14 and Δ*phnAB* cells containing either pGP-*trpEG* or empty pJN105 were prepared as described above and used to inoculate 12.5 ml MOPS minimal medium with 0.5 % arabinose. PQS was extracted and quantified as described above.

Tryptophan molar growth yield and semiquantitative RT-PCR. To determine the tryptophan molar growth yield for Δ*trpE*, washed, starved, exponential-phase Δ*trpE*, Δ*trpE* Δ*phnAB* or Δ*trpE* Δ*pqsA* cells were prepared as described above and used to inoculate 100 μl MOPS minimal medium supplemented with increasing concentrations of tryptophan from 0 to 200 μM in the wells of a Nunc 96-well plate. Each well contained a borosilicate glass bead (Fisher) to improve aeration. Plates were grown overnight (~16 h) after which glass beads were removed and the volume in each well was increased to 200 μl by addition of MOPS buffer. Final growth yield for each well was determined by measuring OD$_{600}$ using a BioTek SynergyMx 96-well plate reader with Gen5 software. Growth yields were averaged from five wells for each tryptophan concentration, and each plate set containing the entire range of tryptophan concentrations was repeated three times with unique biological replicates each time. For experiments with exogenous PQS, the same protocol and media were used with the addition of 20 μM PQS throughout growth and starvation. Reported OD$_{600}$ readings are corrected for dilution in MOPS buffer and spectrophotometric path length.

To determine whether *phnAB* expression correlates with abolishment of Δ*trpE* tryptophan auxotrophy, washed, starved, exponential-phase Δ*trpE* cells were inoculated into 96-well plates as described above
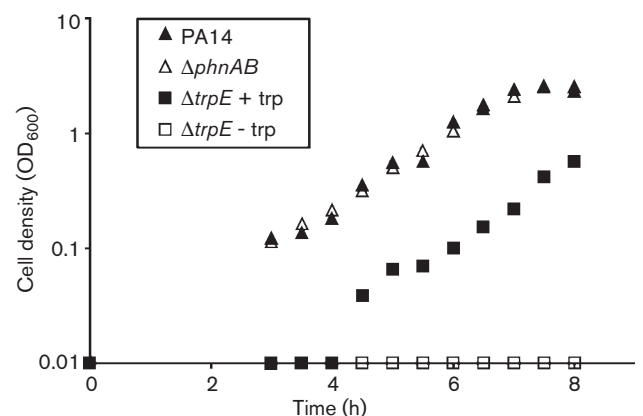
Fig. 2. Determination of Δ*trpE* tryptophan auxotrophy. Washed, starved, exponential-phase PA14, Δ*phnAB* and Δ*trpE* strains were grown in MOPS minimal medium with no tryptophan. Wild-type growth was observed for Δ*phnAB*; however, Δ*trpE* did not grow until the addition of tryptophan to a final concentration of 200 μM after three hours of non-growth. Δ*trpE* did not grow in the absence of tryptophan.

except that all wells contained MOPS minimal medium supplemented with 200 μM tryptophan. The RNA-stabilizing agent RNALater (Ambion) was added 1 : 1 (v/v) to wells containing cultures grown to OD$_{600}$ 0.07, 0.2 and 1.0. To determine *trpE* expression levels, washed, starved, exponential-phase PA14 cells were inoculated into 25 ml MOPS minimal medium and RNALater was added 1 : 1 (v/v) to cultures grown to OD$_{600}$ 0.15, 0.30 and 1.8. For all RT-PCR experiments, total RNA was extracted using RNA bee (Tel-test) as per the manufacturer's instructions. DNA contaminants were removed by DNase treatment (Promega) and confirmed by PCR amplification of the *rplU* gene using primers listed in Table 2 as previously described (Palmer *et al.*, 2010, 2007; Schuster *et al.*, 2003). RNA integrity was confirmed by gel electrophoresis, and cDNA was synthesized using a SuperScript II First-Strand cDNA synthesis kit (Invitrogen) with random primers as described previously (Palmer *et al.*, 2010, 2007; Schuster *et al.*, 2003). cDNA was purified and used as the template for semiquantitative RT-PCR using *phnA* or *trpE* primers listed in Table 2 to PCR amplify *phnA* from 25 ng or *trpE* from 20 ng cDNA, and *rplU* primers listed in Table 2 to PCR amplify *rplU* from 5 ng cDNA as a constitutively expressed loading control. RNA alone and genomic DNA served as negative and positive controls, respectively.

RESULTS AND DISCUSSION

Evidence that *phnAB* was acquired by horizontal gene transfer

P. aeruginosa appears to be rare in that it possesses two functional anthranilate synthases, and it is probably not a coincidence that this bacterium also produces over 50 unique quinolone compounds that require anthranilate for biosynthesis (Essar *et al.*, 1990a, b; Lépine *et al.*, 2004). A complete quinolone biosynthesis operon has been identified in *Burkholderia pseudomallei*, *Burkholderia thailandensis* and *Burkholderia ambifaria*, though these organisms produce fewer quinolones than *P. aeruginosa* (Diggle *et al.*, 2006; Lépine *et al.*, 2004; Vial *et al.*, 2008). *Pseudomonas putida* also contains quinolone biosynthesis genes throughout its genome (Diggle *et al.*, 2006). Among the quinolones reportedly produced by these organisms is the immediate precursor to PQS, 2-heptyl-4-quinoline (HHQ) (Diggle *et al.*, 2006); however, a BLAST analysis using the *P. aeruginosa* PA14 TrpEG and PhnAB sequences performed for this work indicated that these organisms only possess homologues of TrpEG. As a first step toward determining how *P. aeruginosa* came to possess two anthranilate synthases, we used BLAST analysis of the TrpE and PhnA protein sequences to identify the most closely related anthranilate synthase homologues. Identified sequences were aligned and used to generate phylogenetic trees. Our results demonstrate that TrpE shares significant homology with the tryptophan biosynthesis enzymes of closely related species including many pseudomonads (Fig. 1). By contrast, the protein sequence of PhnA was most closely related to multiple divergent species (Fig. 1). The GC content of *phnA* (68 %) is similar to that of the rest of the *P. aeruginosa* genome (66 %). By including many new genomes which have been sequenced since 2004, these results extend the findings of a study by Xie and colleagues

indicating that *P. aeruginosa* acquired the *phnAB* operon from the tryptophan biosynthetic machinery of another bacterium via horizontal gene transfer and that this event probably occurred after the diversification of fluorescent pseudomonads, as no close relatives of *P. aeruginosa* possess a *phnAB*-like operon (Merino *et al.*, 2008; Xie *et al.*, 2004).

TrpE is required for growth without tryptophan

It has previously been reported that a mutation in *trpE* results in tryptophan auxotrophy in *P. aeruginosa*, while *phnA* and *phnB* mutants retain the ability to grow in the
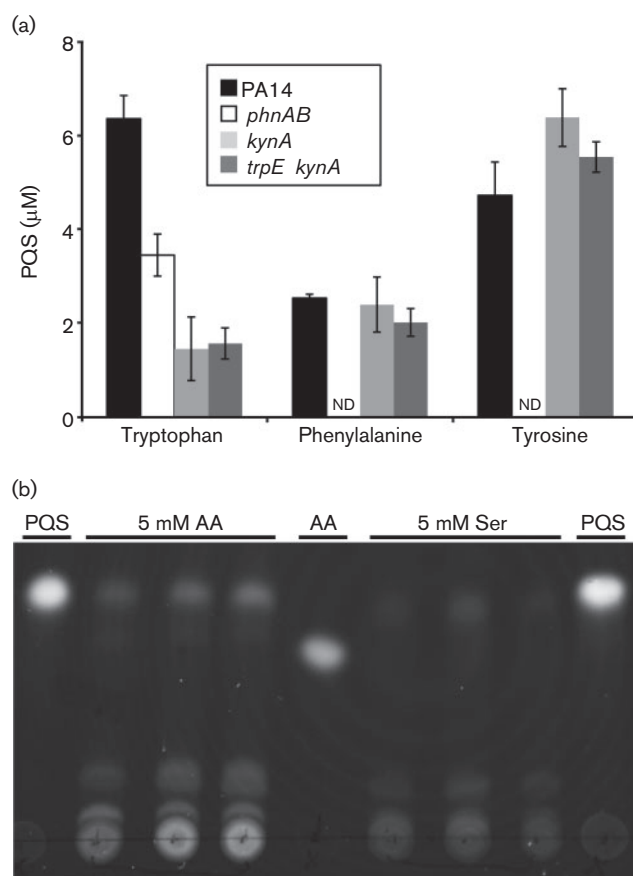
Fig. 3. PQS production in the presence of aromatic amino acids and anthranilate. (a) PQS was extracted and quantified from whole cultures of PA14, Δ*phnAB*, Δ*kynA* and Δ*trpE* Δ*kynA* grown for 24 h in MOPS minimal medium supplemented with 5 mM of the indicated aromatic amino acids. ND indicates no detectable levels of PQS and error bars represent SEM. (b) PQS was extracted from whole cultures of Δ*phnAB* grown for 24 h in MOPS minimal medium supplemented with 5 mM anthranilate or serine. Extracts were separated by TLC and imaged under long-wave UV light. Extractions from three independently grown cultures are shown for anthranilate and serine addition. 250 ng synthetic PQS (PQS) or anthranilate (AA) were included as TLC migration standards.

absence of tryptophan (Essar *et al.*, 1990a, b). To determine whether unmarked deletion mutants also displayed these phenotypes, Δ*trpE* and Δ*phnAB* mutants were generated. *P. aeruginosa* PA14, Δ*trpE* and Δ*phnAB* were grown to exponential phase, washed, starved and inoculated into MOPS minimal medium with no tryptophan, and growth was monitored over 8 h. *P. aeruginosa* Δ*phnAB* grew at the same rate without tryptophan, however, only upon addition of tryptophan to a final concentration of 200 μM after 3 h of non-growth was Δ*trpE* able to grow at the same rate as wild-type *P. aeruginosa* (Fig. 2). These results demonstrated that Δ*trpE* is a tryptophan auxotroph and that endogenous *phnAB* is unable to complement tryptophan auxotrophy in Δ*trpE*.

## PhnAB supplies anthranilate for PQS production

P. aeruginosa enhances production of PQS in the presence of aromatic amino acids (Farrow & Pesci, 2007; Palmer *et al.*, 2007, 2005). For tryptophan, catabolism via the products of the *kynABU* genes generates anthranilate for PQS production (Farrow & Pesci, 2007); however the role of PhnAB during growth with tryptophan was not known. Examination of PQS production in the presence of tryptophan revealed that wild-type *P. aeruginosa* and Δ*phnAB* produced ~6 and ~3.5 μM PQS, respectively (Fig. 3a). The decreased levels of PQS in Δ*phnAB* suggest that PhnAB generates anthranilate for PQS biosynthesis even in the presence of high levels of tryptophan, indicating that degradation of tryptophan via KynABU is not the only source of anthranilate when the amino acid is in excess (Farrow & Pesci, 2007). This was confirmed as Δ*kynA*, which is unable to degrade tryptophan and Δ*trpE* Δ*kynA* produced low levels (~1 μM) of PQS in the presence of 5 mM tryptophan (Fig. 3a). These experiments were performed using fresh tryptophan stock solutions to minimize possible confounding effects of spontaneous tryptophan degradation in solution. Because PhnAB is the only known source of anthranilate in Δ*trpE* Δ*kynA*, it is likely that PhnAB is a source of anthranilate for quinolone biosynthesis in the presence of tryptophan; however, construction of a *trpE kynA phnAB* quadruple mutant would be necessary to confirm this.

Our group has previously reported that in addition to tryptophan, phenylalanine and tyrosine also enhance PQS production (Palmer *et al.*, 2007, 2005). However unlike tryptophan, this induction is not due to catabolism of these amino acids to anthranilate, but instead increased flux of shared precursors of anthranilate biosynthesis to PQS biosynthesis. Thus, we hypothesized that deletion of *phnAB* would completely eliminate PQS production in the presence of phenylalanine and tyrosine unless *trpEG* could complement this deletion. To test this, PQS was extracted and quantified from washed, starved, exponential-phase cells inoculated into MOPS minimal medium supplemented with 5 mM phenylalanine or tyrosine. *P. aeruginosa*

PA14, Δ*trpE* Δ*kynA* and Δ*kynA* produced ~2 μM PQS in the presence of 5 mM phenylalanine and ~5–6 μM PQS in the presence of 5 mM tyrosine (Fig. 3a). No detectable PQS was produced by Δ*phnAB*, indicating that PhnAB is the anthranilate synthase responsible for PQS production in the presence of phenylalanine and tyrosine (Fig. 3a). Consistent with Farrow and Pesci's observations, Δ*phnAB* PQS production is restored upon growth with anthranilate (Fig. 3b) (Palmer *et al.*, 2010; Farrow & Pesci, 2007; Palmer *et al.*, 2007). Taken together, these experiments indicate that TrpEG-generated anthranilate is used for tryptophan biosynthesis while PhnAB-generated anthranilate is used for quinolone biosynthesis.
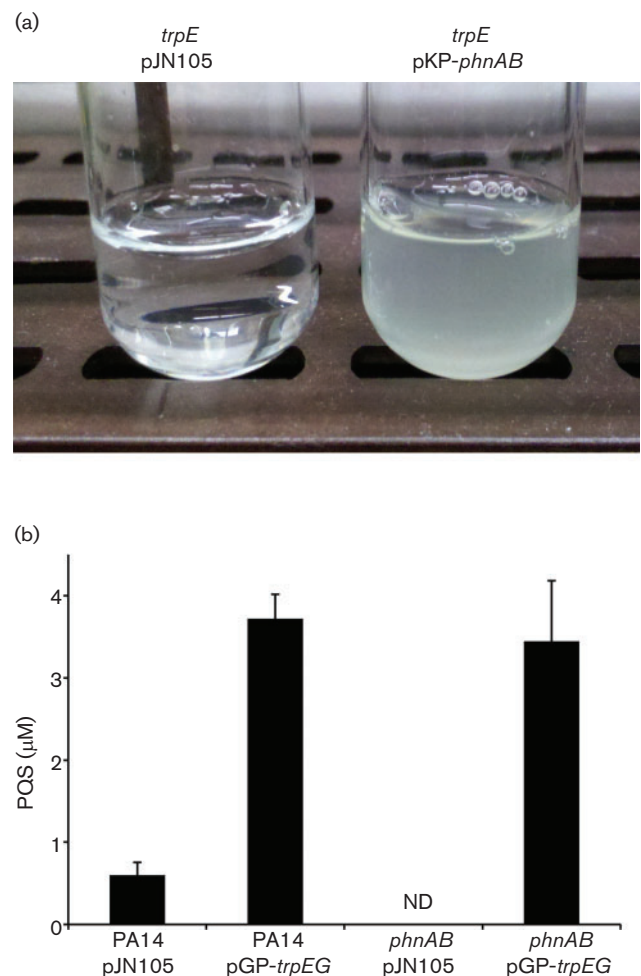
Fig. 4. Overexpression of anthranilate synthase genes results in pathway crosstalk. (a) Overexpression of *phnAB* in Δ*trpE* restored growth in MOPS minimal medium without tryptophan, while empty vector did not. (b) Overexpression of fused *trpEG* restored the ability of Δ*phnAB* to generate PQS in a minimal medium, while empty vector did not. ND indicates no detectable levels of PQS and error bars represent SEM.
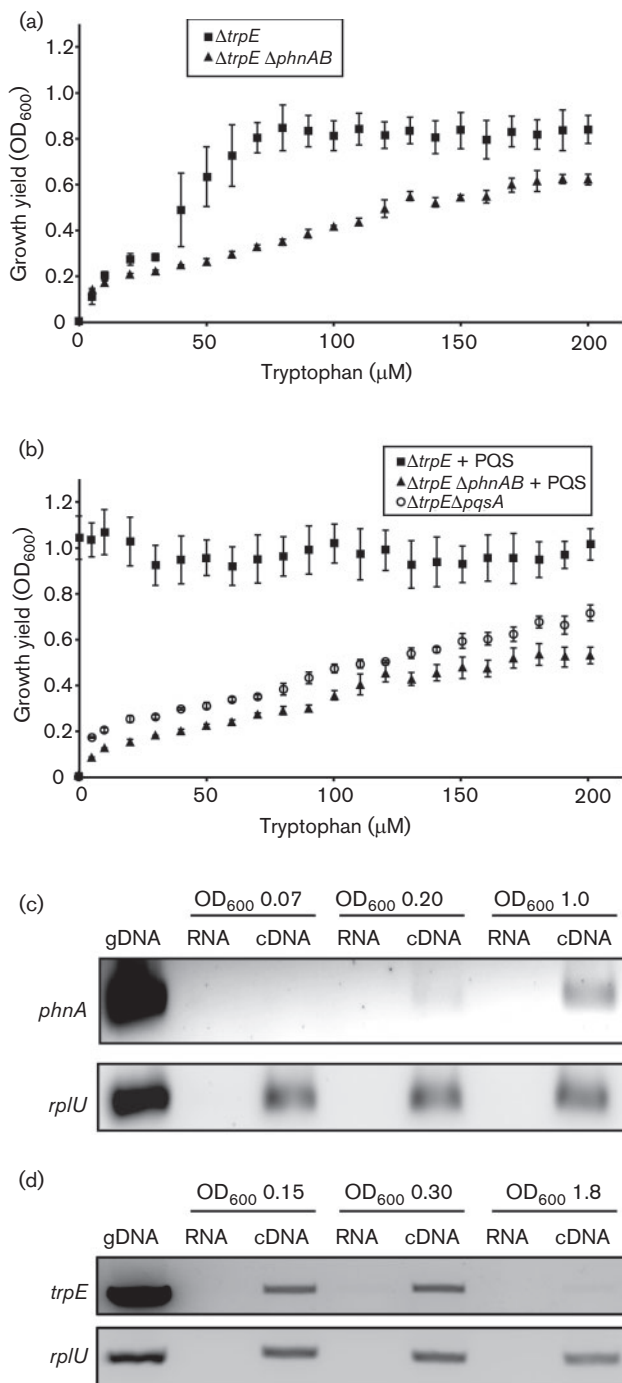
Fig. 5. $\Delta trpE$ tryptophan auxotrophy is dependent upon *phnAB* expression. (a) The final growth yields of $\Delta trpE$ and $\Delta trpE$ $\Delta phnAB$ on increasing concentrations of tryptophan were determined from OD_{600}. $\Delta trpE$ growth became tryptophan-independent upon reaching $OD_{600} \sim 0.5$. Growth of $\Delta trpE$ $\Delta phnAB$ was tryptophan-dependent. (b) Exogenous addition of 20 μM PQS rescued $\Delta trpE$ tryptophan auxotrophy, while $\Delta trpE$ $\Delta phnAB$ remained auxotrophic for tryptophan in the presence of PQS. Growth yields were also tryptophan-dependent in $\Delta trpE$ $\Delta pqsA$, which is unable to generate PQS. (c) RT-PCR confirms that *phnA* expression levels correspond to the loss of $\Delta trpE$ tryptophan auxotrophy. Expression of *phnA* was analysed at three stages of growth (OD_{600} 0.07, 0.2 and 1.0) by PCR amplification from 25 ng cDNA, and levels were compared with constitutive expression of *rplU*, amplified from 5 ng cDNA. Genomic DNA (gDNA) and RNA served as positive and negative controls, respectively. Gel images are inverted for clarity. (d) RT-PCR confirms that *trpE* expression levels decrease late in growth when *phnAB* expression levels are increasing. Expression of *trpE* was analysed at OD_{600} 0.15, 0.3 and 1.8 by PCR amplification from 20 ng cDNA, and levels are compared with constitutive expression of *rplU*, amplified from 5 ng cDNA. gDNA and RNA serve as positive and negative controls, respectively. Gel images are inverted for clarity.

phenotypes was tested (Fig. 4). Overexpression of PhnAB restored growth of $\Delta trpE$ in the absence of tryptophan, while no growth was observed for $\Delta trpE$ cells containing empty vector (Fig. 4a). To determine whether PQS production could be restored in $\Delta phnAB$, PQS was extracted and quantified from $\Delta phnAB$ cells overexpressing TrpEG or containing empty vector. Expression of TrpEG in wild-type *P. aeruginosa* and $\Delta phnAB$ resulted in high levels of PQS production (Fig. 4b). This effect was dependent upon TrpEG, as $\Delta phnAB$ cells containing empty vector did not produce detectable levels of PQS (Fig. 4b). These experiments indicate that it is possible for metabolic crosstalk to occur between the tryptophan and quinolone biosynthesis pathways, however, why this does not occur with the endogenous enzymes remains unclear.

$\Delta trpE$ tryptophan auxotrophy is dependent upon *phnAB* expression

One possible explanation for the lack of crosstalk of TrpEG- and PhnAB-generated anthranilate is that PhnAB is expressed at levels too low to compensate for loss of TrpEG. Expression of *phnAB* is cell-density dependent, showing increased expression at mid- to late-exponential phase (Cao *et al.*, 2001); thus it is possible that sufficient PhnAB levels are not present at low cell densities to complement $\Delta trpE$-mediated tryptophan auxotrophy. However at high cell densities, we hypothesize that tryptophan auxotrophy would be eliminated in $\Delta trpE$ due to increased *phnAB* expression. To test this hypothesis, washed, starved, exponential-phase $\Delta trpE$ cells were grown overnight in MOPS minimal medium supplemented with tryptophan at concentrations ranging from 0 to 200 μM and final growth yields were determined. Results revealed that $\Delta trpE$ final

Overexpression of either anthranilate synthase complements the loss of the other

The inability of these seemingly redundant anthranilate synthases to compensate for each other in genetic experiments is an interesting conundrum. In order to determine whether it is possible for anthranilate to cross pathways, overexpression constructs were generated for TrpEG and PhnAB and their ability to cross complement each of the respective anthranilate synthase mutant

growth yields were dependent upon tryptophan concentrations until mid-exponential phase, at which point Δ*trpE* reached maximal growth yields regardless of the concentration of tryptophan in the medium (Fig. 5a). When *phnAB* was deleted in Δ*trpE*, growth yields remained dependent on the tryptophan concentration in the medium (Fig. 5a).

The previous results suggest that PhnAB is a source of anthranilate for tryptophan biosynthesis at high cell densities. If this is the case, we hypothesized that addition of PQS to low-density cultures would eliminate the Δ*trpE* auxotrophy since *phnAB* is induced by PQS. In support of this hypothesis, induction of *phnAB* expression with a physiologically relevant concentration of PQS (20 μM) resulted in complete rescue of tryptophan auxotrophy in Δ*trpE*, as Δ*trpE* reached maximal growth yields regardless of the concentration of tryptophan in the medium (Fig. 5b). To further confirm the ability of PQS-dependent PhnAB expression to mitigate Δ*trpE* tryptophan auxotrophy, Δ*trpE* Δ*pqsA* was generated. Because the *pqsA* gene product is required for PQS biosynthesis, its deletion renders this strain unable to generate PQS endogenously (Bredenbruch *et al.*, 2005; Coleman *et al.*, 2008; Mashburn & Whiteley, 2005). In the absence of endogenous PQS, Δ*trpE* Δ*pqsA* growth yields were similar to those of Δ*trpE* Δ*phnAB*, and thus, were entirely dependent on the concentration of tryptophan in the medium (Fig. 5b). As a final line of evidence for the correlation of Δ*trpE* tryptophan auxotrophy and low levels of *phnAB* expression, RT-PCR was used to examine *phnA* expression levels during growth in the presence of tryptophan, compared with the constitutively expressed *rplU* gene. Consistent with other PQS-regulated genes (Cao *et al.*, 2001; Xiao *et al.*, 2006), *phnA* displayed low levels of expression in lag (OD$_{600}$ 0.07) and early exponential (OD$_{600}$ 0.20) phases of growth; however, expression increased substantially as density increased (OD$_{600}$ 1.0) (Fig. 5c). Importantly, as previously shown, *trpE* expression levels were high early in growth (OD$_{600}$ 0.15 and 0.30) and low upon reaching stationary phase (OD$_{600}$ 1.8) (Fig. 5d) (Essar *et al.*, 1990a). Taken together these results are consistent with cell-density-dependent PhnAB expression rescuing Δ*trpE* tryptophan auxotrophy.

Conclusions

P. aeruginosa has been known to possess two anthranilate synthases for over 20 years, and the unique phenotypes resulting from mutations in the anthranilate synthase genes suggested that TrpEG generates anthranilate exclusively for tryptophan biosynthesis while PhnAB generates anthranilate exclusively for quinolone biosynthesis (Essar *et al.*, 1990a, b). Here we extend this work by presenting additional evidence that *phnAB* was acquired through horizontal gene transfer following the diversification of fluorescent pseudomonads (Merino *et al.*, 2008; Xie *et al.*, 2004). Based on its organization in an operon and quinolone-dependent regulation, PhnAB has probably evolved to generate

anthranilate for quinolone biosynthesis (Essar *et al.*, 1990a, b; Merino *et al.*, 2008), and this is consistent with the ability of *P. aeruginosa* to produce more quinolones than its quinolone-producing relatives (Lépine *et al.*, 2004; Vial *et al.*, 2008). Furthermore, our data suggest that differential expression of *trpEG* and *phnAB* explains the observed anthranilate synthase mutant phenotypes. While our data do not rule out the physical sequestration of tryptophan and quinolone biosynthesis precursors via channelling or some other mechanism, the ability of exogenous anthranilate to restore PQS production in Δ*phnAB* suggests this is not the case (Fig. 3b) (Farrow & Pesci, 2007). Regardless, the importance of temporal expression of *phnAB* is clear, as *phnAB* expression levels are the primary determinant of Δ*trpE* tryptophan auxotrophy (Fig. 5). Additionally, *trpE* and *trpG* expression levels are decreased at high cell densities (Fig. 5d) (Essar *et al.*, 1990a), which leads to a model for *P. aeruginosa* anthranilate production in which density-dependent expression of PhnAB explains the apparent lack of redundancy between TrpEG and PhnAB. At low cell densities PhnAB expression is low and TrpEG is the primary anthranilate synthase enzyme present. As PQS-mediated *phnAB* expression is activated at higher cell densities, PhnAB becomes the primary anthranilate synthase enzyme present. The results presented here underscore the importance of studying basic microbial physiology and metabolism to understand bacterial signalling and pathogenesis.

REFERENCES

Ausubel, F. M. (2002). *Short Protocols in Molecular Biology: a Compendium of Methods from Current Protocols in Molecular Biology*, 5th edn. New York: Wiley.

Bredenbruch, F., Nimtz, M., Wray, V., Morr, M., Müller, R. & Häussler, S. (2005). Biosynthetic pathway of *Pseudomonas aeruginosa* 4-hydroxy-2-alkylquinolines. *J Bacteriol* **187**, 3630–3635.

Calfee, M. W., Coleman, J. P. & Pesci, E. C. (2001). Interference with *Pseudomonas* quinolone signal synthesis inhibits virulence factor expression by *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **98**, 11633–11637.

Cao, H., Krishnan, G., Goumnerov, B., Tsongalis, J., Tompkins, R. & Rahme, L. G. (2001). A quorum sensing-associated virulence gene of *Pseudomonas aeruginosa* encodes a LysR-like transcription regulator with a unique self-regulatory mechanism. *Proc Natl Acad Sci U S A* **98**, 14613–14618.

Coleman, J. P., Hudson, L. L., McKnight, S. L., Farrow, J. M., III, Calfee, M. W., Lindsey, C. A. & Pesci, E. C. (2008). *Pseudomonas*

aeruginosa PqsA is an anthranilate-coenzyme A ligase. *J Bacteriol* **190**, 1247–1255.

de Lorenzo, V. & Timmis, K. N. (1994). Analysis and construction of stable phenotypes in Gram-negative bacteria with Tn*5*- and Tn*10*-derived minitransposons. *Methods Enzymol* **235**, 386–405.

Delhaes, L., Monchy, S., Fréalle, E., Hubans, C., Salleron, J., Leroy, S., Prevotat, A., Wallet, F., Wallaert, B. & other authors (2012). The airway microbiota in cystic fibrosis: a complex fungal and bacterial community–implications for therapeutic management. *PLoS ONE* **7**, e36313.

Déziel, E., Gopalan, S., Tampakaki, A. P., Lépine, F., Padfield, K. E., Saucier, M., Xiao, G. & Rahme, L. G. (2005). The contribution of MvfR to *Pseudomonas aeruginosa* pathogenesis and quorum sensing circuitry regulation: multiple quorum sensing-regulated genes are modulated without affecting *lasRI, rhlRI* or the production of *N*-acyl-L-homoserine lactones. *Mol Microbiol* **55**, 998–1014.

Diggle, S. P., Lumjiaktase, P., Dipilato, F., Winzer, K., Kunakorn, M., Barrett, D. A., Chhabra, S. R., Cámara, M. & Williams, P. (2006). Functional genetic analysis reveals a 2-alkyl-4-quinolone signaling system in the human pathogen *Burkholderia pseudomallei* and related bacteria. *Chem Biol* **13**, 701–710.

Emerson, J., Rosenfeld, M., McNamara, S., Ramsey, B. & Gibson, R. L. (2002). *Pseudomonas aeruginosa* and other predictors of mortality and morbidity in young children with cystic fibrosis. *Pediatr Pulmonol* **34**, 91–100.

Essar, D. W., Eberly, L., Hadero, A. & Crawford, I. P. (1990a). 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.

Essar, D. W., Eberly, L., Han, C. Y. & Crawford, I. P. (1990b). DNA sequences and characterization of four early genes of the tryptophan pathway in *Pseudomonas aeruginosa*. *J Bacteriol* **172**, 853–866.

Farrow, J. M., III & Pesci, E. C. (2007). Two distinct pathways supply anthranilate as a precursor of the *Pseudomonas* quinolone signal. *J Bacteriol* **189**, 3425–3433.

Gallagher, L. A. & Manoil, C. (2001). *Pseudomonas aeruginosa* PAO1 kills *Caenorhabditis elegans* by cyanide poisoning. *J Bacteriol* **183**, 6207–6214.

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.

Harrison, F. (2007). Microbial ecology of the cystic fibrosis lung. *Microbiology* **153**, 917–923.

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.

Hoiby, N., Flensborg, E. W., Beck, B., Friis, B., Jacobsen, S. V. & Jacobsen, L. (1977). *Pseudomonas aeruginosa* infection in cystic fibrosis. Diagnostic and prognostic significance of *Pseudomonas aeruginosa* precipitins determined by means of crossed immunoelectrophoresis. *Scand J Respir Dis* **58**, 65–79.

Lépine, F., Milot, S., Déziel, E., He, J. & Rahme, L. G. (2004). Electrospray/mass spectrometric identification and analysis of 4-hydroxy-2-alkylquinolines (HAQs) produced by *Pseudomonas aeruginosa*. *J Am Soc Mass Spectrom* **15**, 862–869.

Liberati, N. T., Urbach, J. M., Miyata, S., Lee, D. G., Drenkard, E., Wu, G., Villanueva, J., Wei, T. & Ausubel, F. M. (2006). An ordered, nonredundant library of *Pseudomonas aeruginosa* strain PA14 transposon insertion mutants. *Proc Natl Acad Sci U S A* **103**, 2833–2838.

Mashburn, L. M. & Whiteley, M. (2005). Membrane vesicles traffic signals and facilitate group activities in a prokaryote. *Nature* **437**, 422–425.

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.

Merino, E., Jensen, R. A. & Yanofsky, C. (2008). Evolution of bacterial *trp* operons and their regulation. *Curr Opin Microbiol* **11**, 78–86.

Newman, J. R. & Fuqua, C. (1999). Broad-host-range expression vectors that carry the L-arabinose-inducible *Escherichia coli araBAD* promoter and the *araC* regulator. *Gene* **227**, 197–203.

Palmer, K. L., Mashburn, L. M., Singh, P. K. & Whiteley, M. (2005). Cystic fibrosis sputum supports growth and cues key aspects of *Pseudomonas aeruginosa* physiology. *J Bacteriol* **187**, 5267–5277.

Palmer, K. L., Aye, L. M. & Whiteley, M. (2007). Nutritional cues control *Pseudomonas aeruginosa* multicellular behavior in cystic fibrosis sputum. *J Bacteriol* **189**, 8079–8087.

Palmer, G. C., Palmer, K. L., Jorth, P. A. & Whiteley, M. (2010). Characterization of the *Pseudomonas aeruginosa* transcriptional response to phenylalanine and tyrosine. *J Bacteriol* **192**, 2722–2728.

Palmer, G. C., Schertzer, J. W., Mashburn-Warren, L. & Whiteley, M. (2011). Quantifying *Pseudomonas aeruginosa* quinolones and examining their interactions with lipids. *Methods Mol Biol* **692**, 207–217.

Pesci, E. C., Milbank, J. B., Pearson, J. P., McKnight, S., Kende, A. S., Greenberg, E. P. & Iglewski, B. H. (1999). Quinolone signaling in the cell-to-cell communication system of *Pseudomonas aeruginosa*. *Proc Natl Acad Sci U S A* **96**, 11229–11234.

Rumbaugh, K. P., Griswold, J. A., Iglewski, B. H. & Hamood, A. N. (1999). Contribution of quorum sensing to the virulence of *Pseudomonas aeruginosa* in burn wound infections. *Infect Immun* **67**, 5854–5862.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: a Laboratory Manual*, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schuster, M. & Greenberg, E. P. (2006). A network of networks: quorum-sensing gene regulation in *Pseudomonas aeruginosa*. *Int J Med Microbiol* **296**, 73–81.

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.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M. & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**, 2731–2739.

Vial, L., Lépine, F., Milot, S., Groleau, M. C., Dekimpe, V., Woods, D. E. & Déziel, E. (2008). *Burkholderia pseudomallei, B. thailandensis*, and *B. ambifaria* produce 4-hydroxy-2-alkylquinoline analogues with a methyl group at the 3 position that is required for quorum-sensing regulation. *J Bacteriol* **190**, 5339–5352.

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.

Whiteley, M., Parsek, M. R. & Greenberg, E. P. (2000). Regulation of quorum sensing by RpoS in *Pseudomonas aeruginosa. J Bacteriol* **182**, 4356–4360.

Xiao, G., He, J. & Rahme, L. G. (2006). Mutation analysis of the *Pseudomonas aeruginosa mvfR* and *pqsABCDE* gene promoters demonstrates complex quorum-sensing circuitry. *Microbiology* **152**, 1679–1686.

Xie, G., Bonner, C. A., Song, J., Keyhani, N. O. & Jensen, R. A. (2004). Inter-genomic displacement via lateral gene transfer of bacterial *trp* operons in an overall context of vertical genealogy. *BMC Biol* **2**, 15.

Edited by: W. J. Quax