

Chapter Two

Materials and Methods

2.1 BACTERIAL STRAINS

2.1.1 Laboratory bacterial strains

The strains of bacteria used in this study are listed in Table 2.1.

Table 2.1 Bacterial strains used in this study

Strain	Characteristics	Reference/Source
<i>Escherichia coli:</i>		
DH5 α	F ⁻ , ϕ 80dlacZ Δ M15, Δ (lacZYA-argF) _{U169} , <i>deoR</i> , <i>recA1</i> , <i>endA1</i> , <i>phoA</i> , <i>hsdR17</i> (r _K ⁻ , m _K ⁺), <i>supE44</i> , <i>thi-1</i> , <i>gyrA96</i> , <i>relA1</i>	GibcoBRL, Life Technologies
S17-1 λ pir	<i>thi</i> , <i>pro</i> , <i>hsdR</i> , <i>hsdM</i> ⁺ , <i>recA</i> , RP4-2-Tc::Mu-Km::Tn7, λ pir	(Simon et al., 1983)
<i>Pseudomonas aeruginosa:</i>		
PAO1	Wild-type, Nottingham strain	Holloway collection
PAO1 Δ <i>pqsA</i> CTX- <i>lux::pqsA</i>	Chromosomal deletion of the <i>pqsA</i> gene in PAO1 containing CTX- <i>lux::pqsA</i>	(Diggle et al., 2007)
PAO1 Δ <i>pqsA</i>	<i>pqsA</i> chromosomal deletion mutant derived from PAO1	(Aendekerk et al., 2005)
PAO-ES1	PAO1 Δ <i>pqsA</i> containing pES1	This study
PAO1 Δ <i>pqsA</i> Δ <i>pyrF</i>	<i>pyrF</i> chromosomal deletion mutant derived from PAO1 Δ <i>pqsA</i>	This study
PAO1-ES2	PAO1 Δ <i>pqsA</i> Δ <i>pyrF</i> containing pES2	This study
PAO-ES3	PAO1 Δ <i>pqsA</i> Δ <i>pyrF</i> containing pES2 and pME6032:: <i>pqsR</i>	This study

2.1.2 Soil bacterial isolates

The soil isolates were routinely maintained on Luria-Bertani Agar (LBA). Plates were incubated at 30 °C.

2.2 PLASMIDS

All the plasmids that are used in this study are listed in Table 2.2.

Table 2.2 Plasmids used in this study

Name	Description	Reference
pBLS	pBluescript II KS (+) cloning vector; ColE1 replicon (Amp ^R)	Stratagene
pDM4	Suicide vector carrying the <i>sacBR</i> genes for sucrose sensitivity (Cm ^R)	(Milton et al., 1996)
pLasB-SacB1	pUCP22 <i>NotI</i> containing <i>lasB</i> promoter fused to <i>sacB</i> gene (Amp ^R)	(Rasmussen et al., 2005)
pBLS:: <i>pqsA</i>	A 410 bp <i>XbaI</i> , <i>SphI</i> PAO1 chromosomal DNA fragment containing <i>pqsA</i> promoter in pBLS (Amp ^R)	This study
pES1	pUCP22 <i>NotI</i> containing <i>pqsA</i> promoter fused to <i>sacB</i> gene (Amp ^R)	This study
pBLS:: <i>pyrFU</i>	A 631 bp <i>SpeI</i> , <i>BamHI</i> PAO1 chromosomal DNA fragment containing <i>pyrF</i> upstream region in pBLS (Amp ^R)	This study
pBLS:: <i>pyrFD</i>	A 595 bp <i>BamHI</i> , <i>XhoI</i> PAO1 chromosomal DNA fragment containing <i>pyrF</i> downstream region in pBLS (Amp ^R)	This study
pBLSΔ <i>pyrF</i>	pBLS containing <i>pyrF</i> flanking regions and deleted <i>pyrF</i> gene (Amp ^R)	This study
pDM4Δ <i>pyrF</i>	pDM4 containing <i>pyrF</i> flanking regions and deleted <i>pyrF</i> gene (Cm ^R)	This study
pES2	pUCP22 <i>NotI</i> containing <i>pqsA</i> promoter fused to <i>pyrF</i> gene (Amp ^R)	This study
pME6032:: <i>pqsR</i>	Cloning and expression vector derived from pVS1 containing the PAO1 <i>pqsR</i> gene in pME6032 (Tc ^R)	Sarah Kuehne, unpublished

2.3 OLIGONUCLEOTIDE PRIMERS

Oligonucleotide primers were synthesised by Sigma (UK). Restriction sites were incorporated into the 5' end of the primer where necessary to aid cloning of the PCR product. Primer sequences are listed in Table 2.3.

Table 2.3 Oligonucleotide primers used in this study M= A + C, W= A + T, and R= A + G

Primer	Sequence (5' to 3')	Function
27F	AGAGTTTGATCMTGGCTCAG	Forward primer used to amplify 16S rDNA
1525R	AAGGAGGTGWTCCARCC	Reverse primer used to amplify 16S rDNA
515F	GTGCCAGCMGCCGCGGTAA	Primer used for sequencing amplified 16S rDNA via primer walking
<i>PqsA</i> promF	TATTCTAGAAGCGCCTCGAACTGTGAG	Forward primer used to amplify <i>pqsA</i> promoter, containing <i>XbaI</i> restriction site
<i>PqsA</i> promR	TATGCATGCCAGAACGTTCCCTCTTCAGC	Reverse primer used to amplify <i>pqsA</i> promoter, containing <i>SphI</i> restriction site
<i>pyrF</i> F	TATGCATGCCCCGCCTGCCAGT	Forward primer used to amplify <i>pyrF</i> gene, containing <i>SphI</i> restriction site
<i>pyrF</i> R	TATAAGCTTTCAGCCCAGTTCGGCG	Reverse primer used to amplify <i>pyrF</i> gene, containing <i>HindIII</i> restriction site
<i>pyrF</i> DUF	TATACTAGTGGTGGACCTGGTG CAGGA	Forward primer used to amplify upstream region of <i>pyrF</i> , containing <i>SpeI</i> restriction site
<i>pyrF</i> DUR	TATGGATCCGCAGGCGGACATGGGCTC	Reverse primer used to amplify upstream region of <i>pyrF</i> , containing <i>BamHI</i> restriction site
<i>pyrF</i> DDF	TATGGATCCGAACTGGGCTGAGGCGGC	Forward primer used to amplify Downstream region of <i>pyrF</i> , containing <i>BamHI</i> restriction site
<i>pyrF</i> DDR	GATGGTCACCTGGCCTTCT	Reverse primer used to amplify downstream region of <i>pyrF</i> , containing <i>XhoI</i> restriction site

2.4 CHEMICAL REAGENTS

2.4.1 General Chemicals

All general chemicals were obtained from Sigma (UK) unless otherwise stated.

2.4.2 Synthetic AQs and *N*-Octanoylanthranilic acid

Synthetic AQs and *N*-Octanoylanthranilic acid were made by Alex Truman at the Centre for Biomolecular Sciences, University of Nottingham and kept as 10 mM stocks in methanol. All compounds were stored at -20 °C. Figure 2.1 shows the chemical structures of the synthetic compounds that were used in the project.

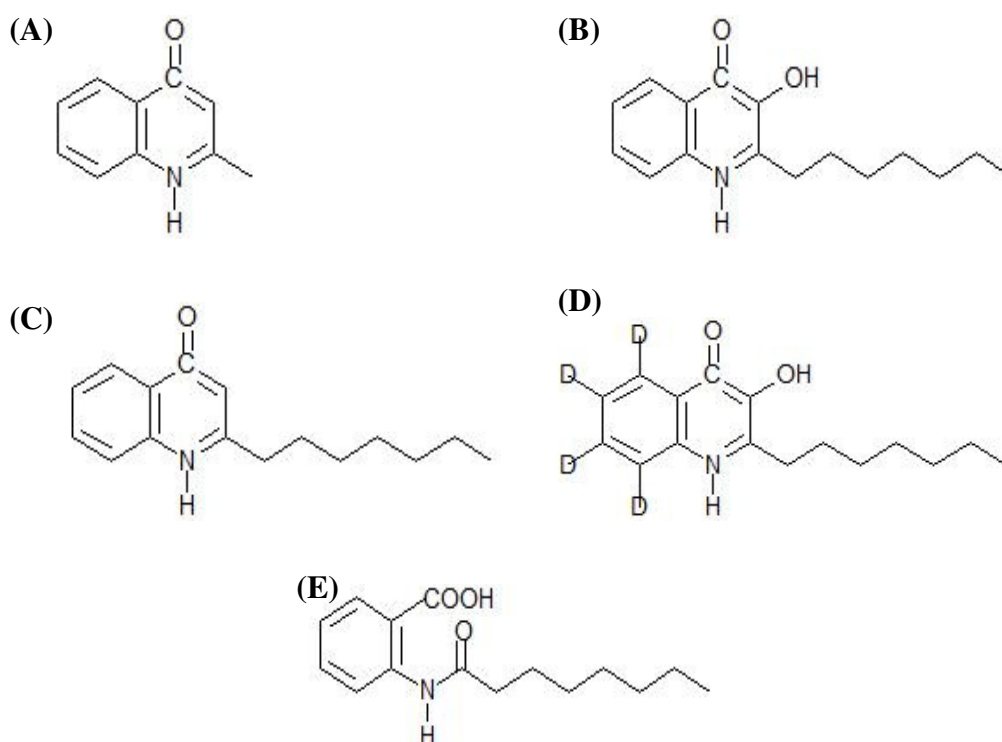


Figure 2.1 Synthetic AQ structures. (A) MPQS, (B) PQS, (C) HHQ, (D) Deuterium labelled PQS, (E) *N*-Octanoylanthranilic acid.

2.5 GROWTH MEDIA AND SOLUTIONS

All media were prepared using dH₂O and autoclaved at 121 °C for 20 min at 15 psi unless otherwise stated.

2.5.1 Luria Bertani media

Luria Bertani (LB) broth consisting of 10 g tryptone, 5 g yeast extract and 10 g NaCl in 1 L of dH₂O was prepared as described previously (Sambrook et al., 1989). LB agar was prepared by adding 1.5 % (w/v) Technical Agar No. 3 (Oxoid) to LB broth. All strains were routinely grown in LB broth or on LB agar unless stated otherwise.

2.5.2 Pseudomonas Isolation Agar

Pseudomonas isolation agar (PIA) (Difco) was prepared according to the manufacturer's instructions and consisted of peptone 20 g; MgCl₂ 1.4 g; potassium sulfate 10 g; irgasan 0.025 g; agar 13.6 g in 1 L of dH₂O. PIA was used for the selection of *P. aeruginosa* strains from the rain forest soil samples.

2.5.3 Antibiotics/ X-Gal/ IPTG

Stock solutions of antibiotics were prepared and stored at -20°C, according to Sambrook et al., (1989). All reagents were filter sterilised with the 0.22 µm-Sartorius Ministart filter before use. Carbenicillin (Cb) was utilized from a 100 mg/ml stock in dH₂O, tetracycline (Tc) from 50 mg/ml in MeOH and chloramphenicol (Cm) from a 50 mg/ml stock in EtOH. The final concentration of antibiotics that were added to media for the

selection as well as maintenance of plasmids was: Cb 100 µg/ml (*E. coli*) and 300 µg/ml (*P. aeruginosa*); Tc 5 µg/ml (*E. coli*) and 200 µg/ml (*P. aeruginosa*); Cm 30 µg/ml (*E. coli*) and 350 µg/ml (*P. aeruginosa*). To induce expression of *pqsR* in pSK65, isopropylthio-β-D-galactoside (IPTG) was added at a concentration of 1mM.

2.5.4 PQS enrichment media

A basal medium was prepared according to (Chan et al., 2009) with slight modifications, containing (in grams per L of dH₂O) NaCl 1.2 g; KCl 0.8 g; Na₂SO₄ 0.25 g; KH₂PO₄ 0.25 g; MgCl₂ 0.5 g; CaCl₂ 0.25 g; NH₄Cl 0.3 g and 2-(*N*-morpholino)-ethanesulfonic acid (MES) 1.0 g. The pH was adjusted to 6.5 with 1M NaOH. The basal medium was autoclaved and cooled. Next, FeCl₃ and MnCl₂ were added at a final concentration of 5 µg and 2.5 µg per L of medium respectively. A vitamin stock solution containing (per L of dH₂O) 2 mg biotin, 20 mg nicotinic acid, 10 mg thiamine-HCl • 2H₂O, 5 mg 4-aminobenzoate, 10 mg calcium pantothenate, 50 mg pyridoxine-HCl, 10 mg vitamin B₁₂, 10 mg riboflavin, and 1 mg folic acid; was added to the at 1 ml/L of basal medium. PQS was added as the sole carbon source at 10 µM or 20µM, depending on assay requirements. For cultivation on solid media, agarose (Sigma, UK) was incorporated at a final concentration of 1.5 % (w/v). Where necessary, sodium succinate dibasic hexahydrate (C₄H₄Na₂O₄•6H₂O) was supplemented as a co-substrate.

2.5.5 Soft top agar

LB soft top agar consisted of 10 g tryptone, 5 g NaCl and 6.5 g Technical Agar No. 3 (Oxoid) in 1 L of dH₂O.

2.5.6 Phosphate buffer saline

Phosphate buffer saline (PBS) solution consists of 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 , and 0.24 g KH_2PO_4 , in 1L dH_2O . The pH of the solution was adjusted to pH 6.5 and autoclaved before being stored at room temperature.

2.6 LONG TERM STORAGE OF BACTERIAL STRAINS

Glycerol stocks of bacterial strains were prepared by adding 0.75 ml of an overnight bacterial culture to 0.75 ml of a sterile solution of 50 % (v/v) glycerol in dH_2O . The suspension of cells and glycerol were mixed thoroughly and transferred into 2 ml Eppendorf tubes before storing at $-80\text{ }^\circ\text{C}$.

2.7 SOIL SAMPLING

A sampling location in Rimba Ilmu, University of Malaya (Kuala Lumpur) was selected and the weather condition during sampling was recorded. Next, a thermometer was placed at approximately 1 cm into the soil surface to measure the soil temperature of the sampling site. Surface soil was collected with a sterile metal spatula and stored in a sterile plastic bag. Soil sample was transported to the laboratory to be processed immediately.

2.8 ENRICHMENT OF PQS METABOLISING BACTERIA

The soil was disrupted with a sterile metal spatula until all particles were dispersed, and the remaining large particles were removed. Next, 1 g of the loose soil particles were added into 5 ml of PBS buffer and vortexed at high speed for 10 min. Subsequently, 100 μ l of the mixture was added to 50 ml of the enrichment medium containing 20 μ M PQS as the sole source of carbon. Succinate was not added in the enrichment experiments. Cells were incubated at 30 °C with shaking (220 rpm). After 7 days, a 1% v/v transfer was made to 50 ml of fresh enrichment medium. For the entire procedure, a cell-free control as well as a PQS-free control was included. To monitor PQS degradation, aliquots were taken at every 48 h for the extraction and assessment of residual PQS as described in section 2.16. A portion of these aliquots were also stored as glycerol stocks. Growth in the enrichment culture was observed by the appearance of turbidity compared to the PQS-free controls. This observation was confirmed by the determination of OD₆₀₀ at every 48 h. Once PQS has been detected to be depleted by the consortium via TLC and PAO1 Δ pqsA CTX-lux::pqsA biosensor overlay (methods described in section 2.17), the culture was plated on LBA and solid enrichment medium. These plates were incubated at 30 °C until visible colonies were observed. Colonies with distinct morphology were picked from both type of medium and restreaked on fresh LBA to obtain pure bacterial isolates.

2.9 BACTERIAL GROWTH CONDITIONS

Liquid cultures were routinely grown in LB broth at 30 °C with agitation at 200 rpm in a Gallenkamp static incubator (Gallenkamp Ltd., UK) unless otherwise stated. Growth

of bacterial cultures was monitored by absorbance at a wavelength of 600 nm (optical density, OD₆₀₀) using a Novospec II visible spectrophotometer (Pharmacia LKB Ltd., Cambridge, UK), or at OD_{495/600} using the TECAN (GENios Pro™). When performing growth curves, consortium/individual strains were grown in identical flasks containing the same volume of media.

2.10 DNA MANIPULATION

2.10.1 Isolation of plasmid DNA

Small scale plasmid DNA isolation, approximately 1-5 ml culture, was performed using the Qiagen Miniprep kit (Qiagen, UK) according to the manufacturer's protocol. In brief, cells from an overnight bacterial culture were lysed under alkali conditions, neutralised and centrifuged at 13,000 rpm for 10 min to remove denatured and precipitated cellular debris. The crude cell lysates were then loaded onto a silica-gel filter, washed, and finally, plasmid DNA was eluted using dH₂O.

2.10.2 Preparation of chromosomal DNA

Genomic DNA was extracted using the phenol chloroform method. Briefly, 1.5 ml overnight culture in LB broth was pelleted and resuspended in 400 µl of TE. After the addition of 50 µl of 10 % (w/v) SDS, 50 µl of 2.5 mg/ml proteinase K and 10 µl of 10 mg/ml RNase A, the mixture was then incubated at 37°C for 3 h. The volume of the mixture was topped up to 700 µl with dH₂O before adding 700 µl of phenol: chloroform (1:1) to extract the DNA. The mixture was centrifuged at 14,000 rpm for 5 min at 4°C to separate the phases. The top layer was transferred to a new tube, carefully avoiding the

interface. Extraction was carried out twice with equal volume of chloroform. DNA was subsequently precipitated with cold absolute ethanol (mix by inverting) and centrifuged at 14,000 rpm for 10 min at 4°C. The ethanol was aspirated away and the DNA pellet air-dried for 30 min. The pellet was finally resuspended with 70 µl of dH₂O and DNA purity was checked by gel electrophoresis.

2.10.3 Digestion of DNA with restriction enzymes

Restriction enzymes were purchased from Promega and used according to the manufacturer's instructions. Restriction digests usually contains 0.5-1 µg DNA, 0.5-1 µl restriction endonuclease(s) and 1 × of the appropriate restriction buffer. The entire mixture was made to a final volume of 20 µl with sterile dH₂O and subsequently incubated at 37°C for a minimum of 1 h or until digestion was complete. Reactions were analysed on 0.8 % (w/v) agarose gels and the appropriate sized bands cut out before proceeding to DNA extraction using the GeneClean III Kit.

2.10.4 DNA agarose gel electrophoresis

DNA loading buffer (Promega, UK) was added to DNA samples and analysed on 0.8 % (w/v) agarose gels using a horizontal gel apparatus (Biorad, UK). The gels were prepared as described by Sambrook *et al.*, (1989) using analytical grade agarose (Promega, UK) in 1 × TAE buffer with the addition of ethidium bromide (EtBr) at a concentration of 10 µg/ml (2 µl EtBr per 50 ml gel). All gels were run in 1 × TAE buffer (40 mM Tris base; pH 8.0; 50 mM EDTA; 0.1142 % (v/v) glacial acetic acid). Electrophoresis was performed at 80-120 V and DNA fragments were visualised on a UV transilluminator (UVP, USA).

2.10.5 DNA molecular weight markers

In order to establish the size of the DNA fragments, 1 kb DNA ladder (Promega, UK) was loaded on agarose gels.

2.10.6 Extraction and purification of DNA from agarose gels

DNA fragments were purified from agarose gel slices using the GeneClean[®] III Kit (MP Biomedicals, UK), according to the manufacturer's instructions. DNA was routinely eluted in 20 µl sterile dH₂O.

2.10.7 DNA ligation

DNA ligations were performed using 1:3 ratio of vector to insert. Briefly, reactions were carried out at 4 °C overnight using 0.5-1 µl T4 DNA ligase (Promega, USA) and 2 µl T4 ligation buffer in a final volume of 20 µl. When required, control ligations containing no insert were also carried out.

2.11 TRANSFORMATION

2.11.1 Preparation of electrocompetent *E. coli* cells

Electrocompetent *E. coli* cells were prepared by adding 1 % (v/v) inoculum from an overnight culture to 100 ml of sterile LB broth in a 1 L conical flask and grown at 37°C, 200 rpm to and OD₆₀₀ of 0.4-0.8 (approximately 6 h). Cells were harvested by

centrifugation at 10,000 rpm (Beckman) for 10 min at 4 °C and washed twice in sterile ice cold 10 % (v/v) glycerol containing 1 mM 4-morpholinepropanesulfonic acid (MOPS) before resuspending in 1 ml of the same buffer. Finally, 50 µl aliquots of the electrocompetent DH5α cells were flash frozen in liquid nitrogen and stored at -80 °C in microcentrifuge tubes.

2.11.2 Electroporation of *E. coli*

Electroporation was performed in 0.2 cm electrode gap Gene Pulser cuvettes (BioRad, UK) containing 50 µl of competent cells and 2-3 µl of DNA. An electroporation pulse of 2.5 kV (200 Ω) was delivered using the BioRad Gene Pulser connected to a BioRad pulse controller (BioRad, UK). The electroporated cells were recovered by adding 1 ml of LB broth to the cells and incubating at 37 °C for 1 h in the absence of antibiotics. The cells were subsequently plated onto LB agar plates containing appropriate antibiotics to select for transformants and grown overnight at 37 °C. Negative controls of electroporated cells with no plasmid were similarly prepared.

2.11.3 Preparation of electrocompetent *P. aeruginosa* cells

Competent *P. aeruginosa* were prepared by pelleting 1.5 ml of culture grown overnight in 42 °C. The pelleted cells were washed three times in 1 ml ice cold 10 % (v/v) glycerol with 1 mM MOPS. The pellet was then resuspended in 50 µl ice cold 10 % (v/v) glycerol with 1 mM MOPS.

2.11.4 Electroporation of *P. aeruginosa*

Transformation of plasmids into electrocompetent *P. aeruginosa* cells was performed the same way as for *E. coli*.

2.12 CONJUGATION

2.12.1 Gene replacement in *P. aeruginosa* PAO1-derived strains using *E. coli*/pDM40derived suicide plasmids (pDM4-X)

The plasmid transfer from *E. coli* donor to *P. aeruginosa* recipient cells were carried out by bacterial matings. Both donor and recipient cells were grown by inoculating 100 ml of LB with a 1% (v/v) overnight culture and incubated overnight. *P. aeruginosa* recipient strains were grown at 42°C to inactivate the restriction enzyme system, which degrades incoming foreign DNA whilst *E. coli* donor strains were grown at 37°C. Donor and recipient cells (1.5 ml each) were pelleted and washed twice with 1 ml of fresh LB broth. Cells were then resuspended in 500 µl of LB broth. Conjugation was achieved by pooling the resuspended donor and recipient cells into a single tube, briefly mixed and pelleted. Most of the supernatant was discarded, leaving an equivalent volume of liquid and cells at the bottom of the tube. Same preparations were done for the controls, without the mixing of donor and recipient cells. The pellet was resuspended with gentle pipetting and transferred onto an LB agar plate.

2.13 POLYMERASE CHAIN REACTION (PCR)

2.13.1 PCR amplification

PCR amplifications were performed according to Sakai *et al.*, (1988) in a final volume of 50µl. For general reactions, approximately 1 µM of each primer was included in the reaction mix containing 1.5 mM MgCl₂, 2 mM dNTPs, 0.5 µl Taq DNA polymerase and 1 × buffer (GoTaq™ Promega, UK). The DNA template was either from whole cells transferred from a fresh colony or 1 µg of purified DNA from a chromosomal or plasmid preparation. A total of 30 cycles were carried out for each reaction in the Progene PCR Thermocycler (Techne). Briefly, the DNA template was denatured at 95°C for 5 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 60-65°C for 30 s and then extension at 72°C for 1-2 min depending on the length of the DNA to be amplified (1 min per kb). The last step finished with a final extension stage at 72°C for 5 min to ensure completion of all strands followed by incubation at 4 °C indefinitely. The annealing temperature was varied according to the primers used as well as the required stringency level. The amplicon was quantified using the NanoDrop ND-1000 (Nanodrop Technologies) and samples were checked for integrity using the Agilent Bioanalyzer 2100 with 2100 Expert software (Agilent technologies).

2.13.2 Cloning of PCR products

PCR products were analysed on 0.8 % w/v agarose gels and purified using the GeneClean® III Kit (MP Biomedicals, UK) as per the manufacturer's instructions. The purified PCR product was then digested at 37°C for 1-3 h with the relevant restriction

enzyme(s) and appropriate buffer. Digested PCR fragments were ligated into similarly digested vector and electroporated in *E. coli* DH5 α . Transformants containing recombinant clones were picked from LB agar plates containing the appropriate selective antibiotics and subjected to PCR to confirm the presence of the insert.

2.14 DNA SEQUENCE ANALYSIS

DNA sequencing was carried out using the 3130xl ABI PRISM Genetic Analyzer in the DNA sequencing facility of the School of Biomedical Sciences (University of Nottingham, UK). DNA sequences analyses were performed using the Lasergene computer package (DNASTar, Ltd) in combination with the BLAST programs available from the NCBI website (<http://www.ncbi.nlm.nih.gov/>). *P. aeruginosa* sequences were analysed using the *P. aeruginosa* Genome Sequence Database (<http://www.pseudomonas.com>).

2.15 PHYLOGENETIC ANALYSIS

Phylogenetic and molecular evolutionary analyses were performed with the Molecular Evolution Genetics Analysis (MEGA) software version 4.0 (Tamura *et al.*, 2007) obtained from <http://www.megasoftware.net>. The trees were generated from 16S rDNA gene sequences using the Neighbour-Joining algorithm. Bootstrap analyses for 1,000 resamplings were always carried out as it is a statistical method to evaluate the reliability of the tree. An appropriate outgroup was selected to produce a rooted tree to show the evolutionary direction. The outgroup selected has to be a taxon that is distantly related nevertheless sufficiently conserved or homologous to each of the ingroup taxa.

2.16 AQ EXTRACTION

Overnight bacterial cultures were standardised to OD₆₀₀ 1.0 before being diluted 1 in 100 in 25 ml LB and grown in 250 ml flasks at 37°C with shaking at 200 rpm for 8 h. Whole cell cultures were triple-extracted with 30 ml acidified ethyl acetate, vortexed vigorously and centrifuged at 10,000 rpm for 5 min. The organic solvent was evaporated to dryness using a rotary evaporator (R-114, Buchi). The organic extract was subsequently resuspended in 1.5 ml methanol and transferred to a fresh vial before being dried to completion using the SpeedVac® (Thermo Scientific, UK). The residue was resuspended in 100 µl methanol for subsequent analysis.

2.17 DETECTION OF AQS IN BIOLOGICAL CULTURE EXTRACTS USING LUX-BASED BIOREPORTERS

2.17.1 TLC analysis of AQs

A 20 × 20 cm aluminium plate (silica gel 60 F254, Merck) was prepared by soaking in 5 % w/v KH₂PO₄ for 30 min before being air-dried and activated in a hybridisation oven (Stuart Scientific) at 100°C for 1 h. Next, 2 µl of 10 mM PQS and HHQ synthetic standards were spotted onto a plate along with 5 µl of each sample. The plate was run in a solvent system of dichloromethane (DCM) to methanol (MeOH) 95:5 until the solvent front reaches the top of the plate. The plate was dried and viewed immediately using a UV transilluminator (UVP, USA) and photographed.

2.17.2 Overlay of TLC plates with bioreporter

TLC plates used to detect AQs were overlaid with 0.3 % w/v LB soft top agar seeded with a 0.5 % (v/v) of overnight culture of the PAO1 Δ *pqsA* CTX-*lux::pqsA* bioreporter. Plates were incubated at 37°C and bioluminescence examined after 6-8 h using a Luminograph LB 980 photon video camera (EG&G Berthold). Detection of pyocyanin on plates was observed by visual examination after 24 h incubation at 37°C.

2.17.3 Determination of bioluminescence and optical density using luminometer-spectrometer

Bioluminescence and optical density were monitored simultaneously in 96 well microtitre plates (Black Isoplates, Perkin Elmer Life Sciences) using a combined, automated luminometer-spectrometer (Genios Pro; TECAN Ltd). Overnight cultures of *P. aeruginosa* bioreporter strains PAO1 Δ *pqsA* CTX-*lux::pqsA* (*pqsA::lux* bioreporter-Diggle et al., 2007), were diluted 1 in 1000 in fresh LB medium, and 0.3 ml cultures were grown in microtiter plates (Appleton Woods, UK). Luminescence and turbidity were automatically determined every 30 min. Where required, cell culture extracts and/or AQs were added in the wells and dried thoroughly prior to the inoculation of the biosensor. Luminescence was given in RLU divided by OD₄₉₅. All assays were carried out in triplicate in three independent experiments. Error bars represent 2 × SE of the mean (95 % Confidence Interval).

2.18 PQS INACTIVATION ASSAY

2.18.1 Preparation of resting cells

A single colony of the bacteria isolate was picked and grown overnight in 5 ml of LB broth (30°C, 200 rpm). The cells were harvested by centrifuging at $10,000 \times g$ for 10 min. The optical density after incubation will be $OD_{600} \approx 3.5 - 4.0$, corresponding to 10^9 CFUml⁻¹. Next, the pelleted cells were resuspended in equal volume of PBS (pH6.5) and washed twice in the same buffer. After the second wash step, the supernatant was discarded and the pellet was resuspended in 5 ml of enrichment medium. The resulting concentrated cell suspension was used directly as a source of resting cells for the *in vitro* PQS inactivation assay.

2.18.2 PQS inactivation assay

For the whole cell inactivation assay, 10 -20 μ M of AQ, depending on assay requirements were directly added into the resting cell suspension as the primary carbon source. The culture was incubated at 30°C, 200 rpm for 24 h. The cell-free control was always performed concomitantly. Residual PQS was detected via TLC separation coupled with PAO1 Δ *pqsA* CTX-*lux::pqsA* biosensor overlay and/or LC-MS/MS analysis. For the time course experiment, samples were withdrawn at set time points in triplicates and stored at -20 °C until all samples have been collected for analysis. Extraction of residual PQS was carried out, similar to the AQ extraction method described in Section 2.16.

2.18.3 Supplementation of co-substrate in PQS degradation

The assessment of the most suitable co-substrate for the biotransformation of 20 μ M PQS was carried out with a series of centrifuge tubes, each containing 5 ml of resting cells in the enrichment media, with the supplementation of glucose, succinate or glycerol at various concentrations. The experimental process was the same as the PQS inactivation assays, with detection of residual PQS via TLC analysis coupled with biosensor overlay.

2.18.4 Purification of PQS degradation product by TLC

The PQS inactivation assay was scaled up to 50 ml total volume with 20 μ M PQS when purification of the bioconversion product was to be carried out. The ethyl acetate extracted metabolites were concentrated and separated by preparative TLC. After development, the plate was viewed under UV light and the prominent spot with the major metabolite was circled with a pencil. This spot was scraped from the plate with a spatula and silica particles containing the compound were collected in a 2 ml Eppendorf tube. The metabolite was extracted from the silica gel with three 1 ml washes of DCM, and silica gel was removed by centrifugation at 13,000 rpm for 10 min. The pooled sample was evaporated to dryness under sterile nitrogen gas. Dried samples were stored at -20 °C prior to LC-MS/MS analysis.

2.19 LIQUID CHROMATOGRAPHY- MASS SPECTROSCOPY (LCMS)

The instrument was primed and equilibrated for an hour prior to the chromatographic run. Using the Agilent 1200 series HPLC, comprising degasser, binary pump, column heater and autosampler the mobile phase was built using formic acid 0.1% in water (A) and formic acid 0.1% in acetonitrile (B) as a gradient run over 30 minutes at a flow rate of 0.2 ml/minute. The mobile phase was maintained at 90% A for 1 minute and reduced to 0 by 10 minutes. This was maintained for 12 minutes before returning to 90% by 23 minutes. The column was an Ascentis Express C18 150 x 2.1 mm internal diameter, 2.7 um particle size, maintained at 50°C. Following each 10 ul injection the needle was washed 3 times in methanol. The LC column was couple with MS (Bruker HCT Plus ion trap) in multiple reaction monitoring (MRM) mode and Hystar software. Ions were introduced using positive/ negative ion electrospray from the Agilent HPLC system, depending on the type of analysis to be carried out. The trap was set to isolate from full scan and then fragment up ions at the required mass using the Smartfrag software. The ion charge control was used to prevent charge overload in the trap. The quantity of each ion is proportional to the area under the curve (AUC) representing the detected mass-to-charge ratio for each ion over time.