Chapter Four

Construction of a biosensor for the screening of PQS quorum sensing inhibitors

4.1 Introduction

The discovery that many bacteria use quorum sensing (QS) to coordinate virulence has made it a viable target for the design and development of selective anti-infective compounds that can be potential antimicrobial drugs. The well-studied example of AHL QS inhibition originates from *Delisea pulchra*, a marine alga that produces several types of halogenated furanone compounds that are capable of interfering AHL-mediated signalling in bacteria (Rasmussen et al., 2000). AHLs are prevented from binding the *luxR* transcriptional regulator homologues, thus incapacitating quorum sensing. Synthetic furanone derivatives have been proven to attenuate *P. aeruginosa* infections via the inhibition of QS (Hentzer et al., 2002). A synthetic library of *P. aeruginosa* AHL analogs, with targeted variation at the homoserine lactone (HSL) moiety was screened for AHL QS inhibiting properties. Among the synthesized analogs, several antagonists were found to significantly reduce biofilm formation and several virulence factors (Smith et al., 2003).

These evidences prompted the construction of a collection of QS inhibitor selectors (QSIS) that is useful in the identification of novel QS inhibitors (QSI) of natural and synthetic origins (Rasmussen et al., 2005). There were essentially two main designs of QSIS; one employing the fusion of a QS-controlled promoter to a killing gene, while the other utilizes a repressor controlled antibiotic gene which is in turn controlled by a QS-regulated promoter. In essence, the presence of AHL leads to cell death and growth

inhibition, respectively. However, in the presence of an AHL QSI compound, the cells will then be allowed to grow (Rasmussen et al., 2005). The three QSIS systems are shown and further described in Figure 4.1. QSI compounds that were tested positive with these QSISs were eventually further evaluated for its potential as a human drug. A series of protocols detailing the *in vivo* validation of the AHL QSIs using mouse models was recently published (Bjarnsholt et al., 2010).

The AQ quorum sensing signals, HHQ and PQS plays important roles in activating and controlling the *P. aeruginosa* PqsR-dependent QS regulatory pathway. The primary precursor for the synthesis of AQ signalling molecules is anthranilic acid. Lesic et al., 2007 managed to identify halogenated anthranilic acid analogs that were shown to specifically inhibit AQ biosynthesis, thus restricting *P. aeruginosa* pathogenesis. These findings potentiate the search for novel AQ QSIs that may aid in complementing AHL-based quorum quenching in *P. aeruginosa*.

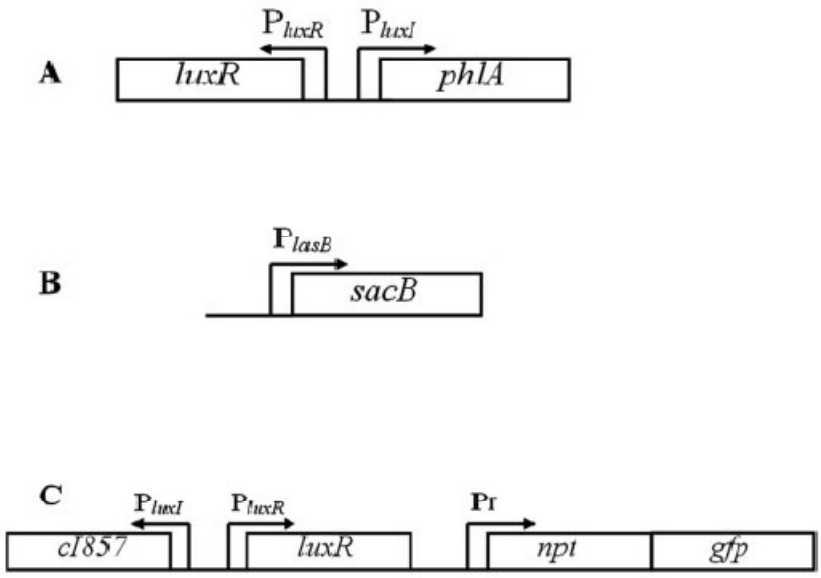


Figure 4.1 The three QSIS systems. (A) QSIS1: *phlA* encodes a toxic gene product of which expression is controlled by LuxR. The system was established in *E. coli.*(B) QSIS2: The LasR- and RhlR-regulated *lasB* promoter controls *sacB*-based killing of the host cell in the presence of sucrose. The system was established in a *lasI rhlI* double mutant of *P. aeruginosa* harbouring a plasmid containing a constitutively expressed *lux* operon, giving rise to bioluminescene expression in growing cells. (C) QSIS3: The *npt* and *gfp* genes, conferring kanamycin resistance and green fluorescence, respectively are controlled by the *c*I repressor. This repressor is regulated via the *luxI* promoter, with the system being established in *E. coli*.

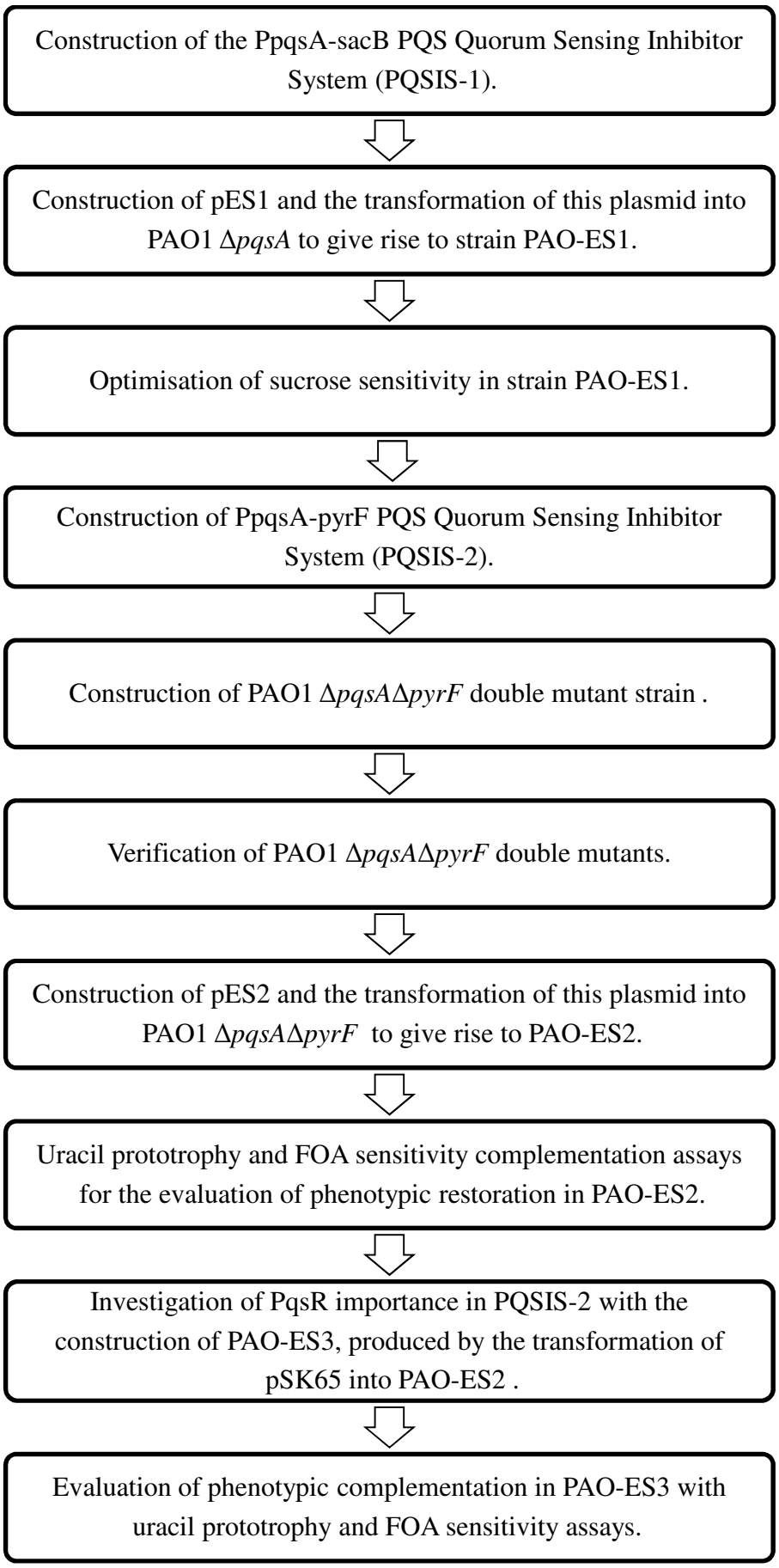


Figure 4.2 Work flow for the PQSIS biosensor construction.

4.2 Results

4.2.1 PpqsA-sacB PQS Quorum Sensing Inhibitor System (PQSIS-1)

4.2.1.1 Determination of the extent of the *pqsA* promoter region that is to be fused to *SacB*

Based on the primer extension analysis in (McGrath et al., 2004) the *pqsA* transcriptional start site is located 71 bp upstream (indicated by a bent arrow) of the gene's translational start codon (Figure 4.3). The putative *LysR* box, with a perfect dyad symmetry is shown in blue in Figure 4.3. The *LysR* box is critical for *pqsA* transcriptional regulation via the binding of PqsR protein in the presence of PQS (Xiao et al., 2006). Primers *PqsA*promF and *PqsA*promR were designed to amplify the *pqsA* promoter fragment that contains the aforementioned critical regions for maximal expression. Figure 4.4 shows the 410 bp PCR product of the pqsA promoter region.

GCCCCCTTGGAGCCCAGGCCGAGCGCCTCGAACTGTGAGATTTGGGAGGCGATTTGCCGAGCAA

AGTGGGTTGTCATTGGTTTGCCATCTCATGGGTTCGGACGAGGCCTCGAGCAAGGGTTGTAACG

GTTTTTGTCTGGCCAATGGGCTCTTGCGTAAAAAGGCTGCCGCCCTTCTTGCTTGGTTGCCGTT

CTCGGATCCCGCGCAGCCCGGTGGGTGTGCCAAATTTCTCGCGGTTTGGATCGCGCCGATTGCC

GCGGCCTACGAAGCCCGTGGTTCTTCTCCCCGAAACTTTTTCG**TTCGGACTCCGAA**TATCGCGC

TTCGCCCAGCGCCGCTAGTTTCCCGTTCCTGACAAAGCAAGCGCTCTGGCTCAGGTATCTCCTG

ATCCGGATGCATATCGCTGAAGAGGGAACGTTCTGTC**ATG**->pqsA

Figure 4.3 Promoter region of *pqsA*. The underlined sequences indicate the position of the primers used to amplify the promoter. The boxed sequences indicate the -10 and -35 putative promoters found using the promoter prediction tool in www.softberry.com. The sequence in red, within the reverse primer, indicates the Shine-Dalgarno sequence whereas the *pqsA* start codon is shown in boldface.

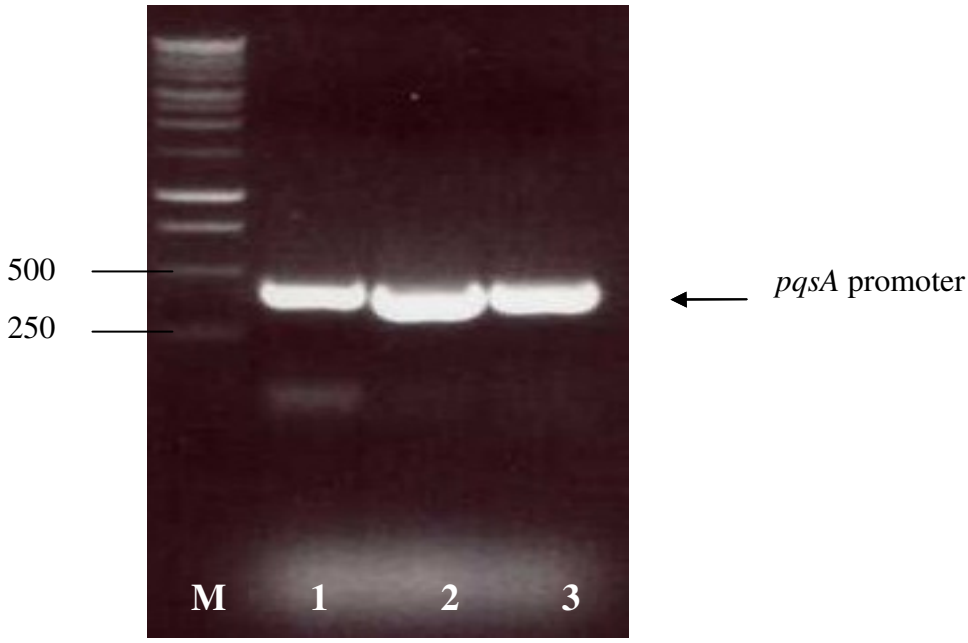


Figure 4.4 PCR product of *pqsA* promoter. Agarose gel, visualised under UV showing the 410 bp amplified region using primers PqsApromFw and PqsApromRv at different annealing temperatures. Lane (1) 58 °C, (2) 60°C and (3) 62°C. M= 1 kb DNA marker.

4.2.1.2 Construction of PQSIS-1 system

The SacB PQS quorum sensing inhibitor system (PQSIS-1) was constructed by a two-step cloning procedure, with use of the existing pLasB-SacB1 plasmid (Figure 4.5) from QSIS2 (Rasmussen et al., 2005). As shown in Figure 4.6, the pLasB-SacB1 plasmid was first digested with different combinations of the restriction enzymes in order to confirm that the cassette is flanked by *Not*I restriction sites as well as to check for the presence of the 348 bp *LasB* promoter, flanked by *Xba*I and *Sph*I. The plasmid was also singly digested with *Xba*I or *Sph*I to verify that it is only cut once by these two restriction enzymes (Figure 4.6). Using PAO1 template DNA, a 410 bp fragment upstream of the *pqsA* start codon was amplified using the primer pair *pqsA*promF, containing an *Xba*I restriction site and *pqsA*promR, containing a *Sph*I restriction site. The P*pqsA-sacB* translational fusion was made by digesting the pLasB-SacB1 plasmid with *Xba*I and *Sph*I to release the *lasB* promoter. Similarly digested *pqsA* promoter PCR products were inserted into the corresponding sites of pLasB-SacB1, giving rise to pES1 (Figure 4.7) which carries the translational P*pqsA-sacB* followed by translational stop codons in all three reading frames and two strong transcriptional terminators (Andersen et al., 1998). The construct was first transformed into *E. coli* DH5α and the resulting clones containing the plasmids with correct sized inserts, as determined by digestion with *Xba*I and *SphI* were subjected to sequencing analysis. The pES1 plasmid with correct *pqsA* promoter sequence was subsequently transformed into PAO1 with chromosomally deleted *pqsA* gene giving rise to PAO-ES1.

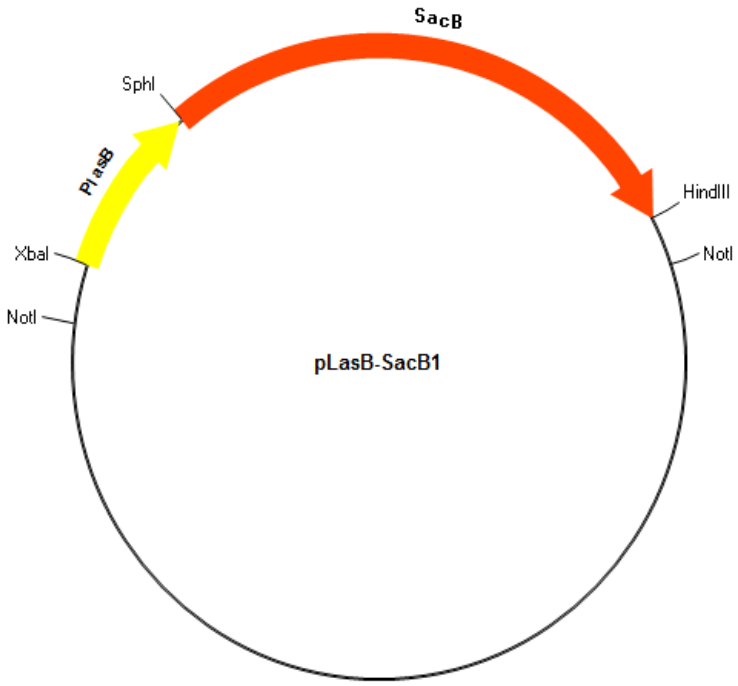


Figure 4.5 Map of pLasB-SacB1. The plasmid contains the s*acB* gene fused to the l*asB* promoter. Only unique restriction sites are shown.

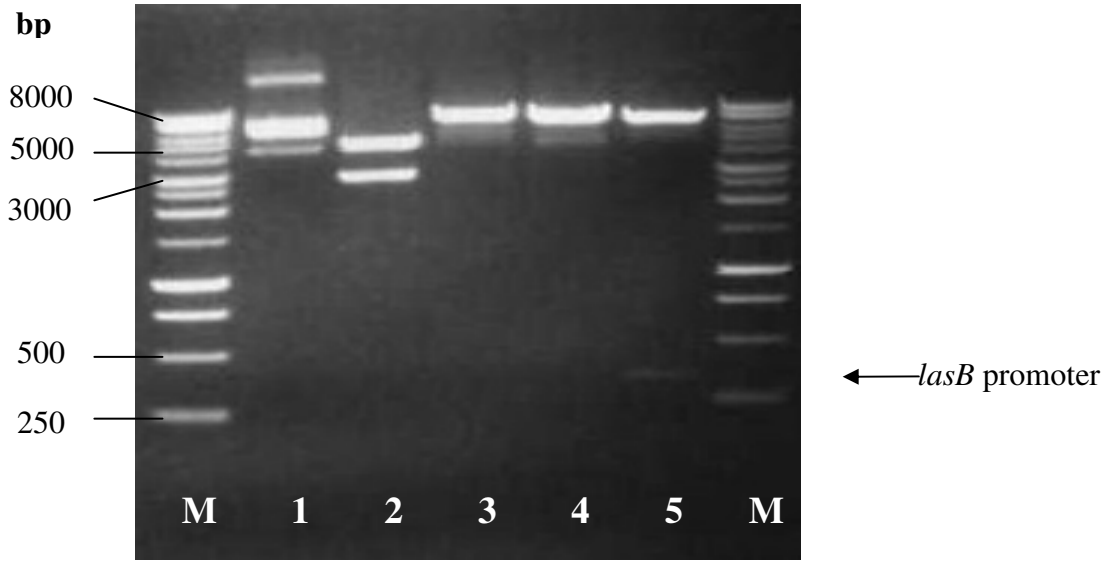


Figure 4.6 Digested pLasB-SacB1 plasmid. Agarose gel, visualised under UV showing the undigested plasmid (1) and the following corresponding lanes digested with NotI (2), SphI (3), XbaI (4), SphI and XbaI (5). M= 1kb DNA marker (Promega).

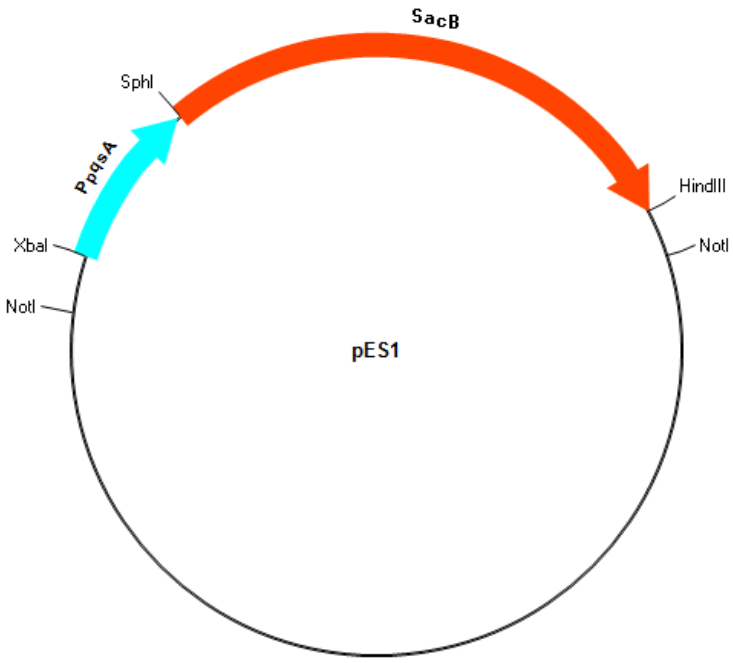


Figure 4.7 Plasmid map of pES1. The *lasB* promoter in pLasB-SacB1 has been substituted with the *pqsA* promoter.

4.2.1.3 Optimisation of sucrose sensitivity in the PQSIS-1 system

PQSIS-1 system works by inducing the killing of PAO-ES1 cells when grown in the presence of both PQS quorum sensing signal and sucrose. The exogenously added PQS signal binds to the *pqsA* promoter, which leads to the induction of *sacB*. Cell death is induced when sucrose in the media is converted to a toxic compound by the *sacB* encoded enzyme, levansucrase. In the presence of a PQS quorum sensing inhibiting compound, the cells will thus be 'rescued'. Sucrose sensitivity assay was initially performed to make sure that cell death can be successfully induced in this system, which aids in proving that the construct is in working order. The assay was carried out by plating PAO-ES1 on LB agar supplemented with PQS and sucrose at various concentrations. The omission of sucrose in the medium was included as a negative control. Unfortunately, the PAO-ES1 cells grew in all replicates, with equal robusity as the control. Similar results were obtained even when PAO-ES1 was grown overnight in LB broth in the presence of 5% and 15% of sucrose before plating on LB agar with various concentrations of PQS and sucrose.

4.2.2 PpqsA-pyrF PQS Quorum Sensing Inhibitor System (PQSIS-2)

We consequently decided to employ a different cell killing system, using the *P. aeruginosa* encoded PyrF system. The *pyrF* gene, also known as URA3 in *Pseudomonas putida* encodes a uracil biosynthesis enzyme, orotidine-5'-phosphate decarboxylase (ODCase). In the presence of 5-fluoroorotic acid (FOA, a uracil analogue), this enzyme can catalyze the transformation of it into a highly toxic compound (Boeke et al., 1984). The presence of intact pyrF in wildtype *P. aeruginosa* confers sensitivity to FOA, while pyrF-negative cells (pyrF mutants) are FOA resistant. In addition to that, pyrF mutant is unable to grow on uracil-deplete medium since it now lacks the ODCase enzyme. In developing the PpqsA-pyrF PQS Quorum Sensing Inhibitor System, PQSIS-2, we first constructed a *pyrF* knockout mutant using a PAO1 Δ*pqsA* genetic background, producing a PAO1Δ*pqsA*Δ*pyrF* double mutant. A plasmid containing the *pqsA* promoter fused to a complete *pyrF* gene is then transformed into the double mutant. The PQSIS-2 counter selection works on the basis that the presence of both PQS and FOA in the media complements FOA sensitivity, thus killing the cells. A PQS quorum sensing inhibitor will hamper *pyrF* expression and allow cell growth.

4.2.2.1 PAO1 Δ*pqsA* Δ*pyrF* construction

PCR primers were designed to amplify the regions immediately upstream and downstream of the *pyrF* gene based on the genome sequence of *P. aeruginosa* strain PAO1 (http://www.pseudomonas.com). The primer pairs amplifying a 631 bp fragment of the upstream region of *pyrF* were *pyrF*DUF and *pyrF*DUR, with *Spe*I and *BamH*I sites incorporated respectively. The 595 bp fragment of the downstream region were amplified using the primer pair *pyrF*DDF, containing a *BamH*I restriction site and *pyrF*DDR, containing an *Xho*I restriction site (Table 2.3). Figure 4.8 shows the

appropriately digested pBluescript II KS (+) vector and the respective inserts prior to ligation. The ligated DNA were transformed into competent *E. coli* DH5α cells generating pBLS::*pyrF*U and pBLS::*pyrF*D plasmids. Plasmid DNA were extracted from successful transfomants to check for the correct inserts by digesting with the correct combination of restriction enzymes (Figure 4.9). The plasmids with correct inserts were subjected to sequencing analysis to ensure that there are no errors in the *pyrF*U and *pyrF*D fragments that are to be subjected to downstream work. Both *pyrF*U and *pyrF*D fragments were recovered in the same pBluescript vector generating pBLSΔ*pyrF* (Figure 4.10). The 1226 bp Δ*pyrF* fragment was excised from the vector using *Spe*I and *Xho*I and inserted into similarly digested suicide vector pDM4 (Milton *et al*., 1996) (Figure 4.11), resulting in the plasmid pDM4Δ*pyrF*. Figure 4.12 shows the digested pDM4Δ*pyrF* plasmids, with the released Δ*pyrF* fragments from successful *E. coli* S17-1 λ-pir transfomants. Allelic exchange using pDM4Δ*pyr* contained in *E. coli* S17-1 λ-pir into the PAO1 Δ*pqsA* recipient resulted in an in-frame deletion of the *pyrF* gene.

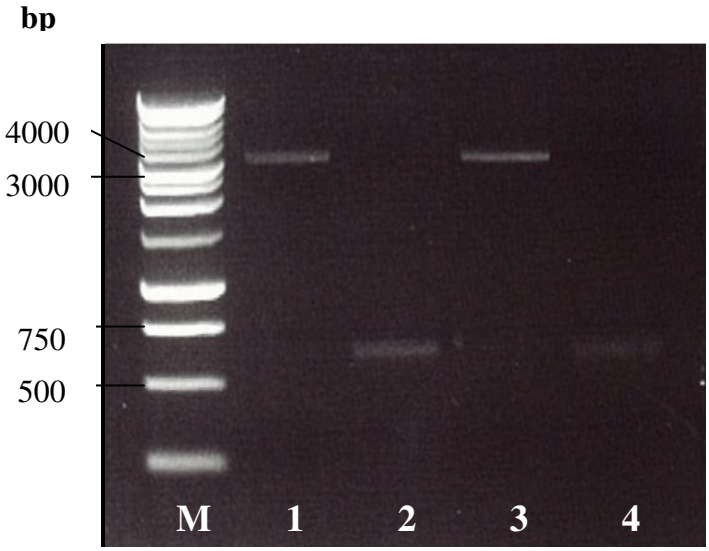


Figure 4.8 Agarose gel containing digested pBluescript II KS (+) vector and pyrFDU/DD inserts. M= 1kb DNA marker ladder (Promega), 1= *Spe*I and *Bam*HI digested pBluescript, 2= *Spe*I and *Bam*HI digested pyrFDU, 3= *Bam*HI and *Xho*I digested pBluescript, 4= *Bam*HI and *Xho*I digested pyrFDD.

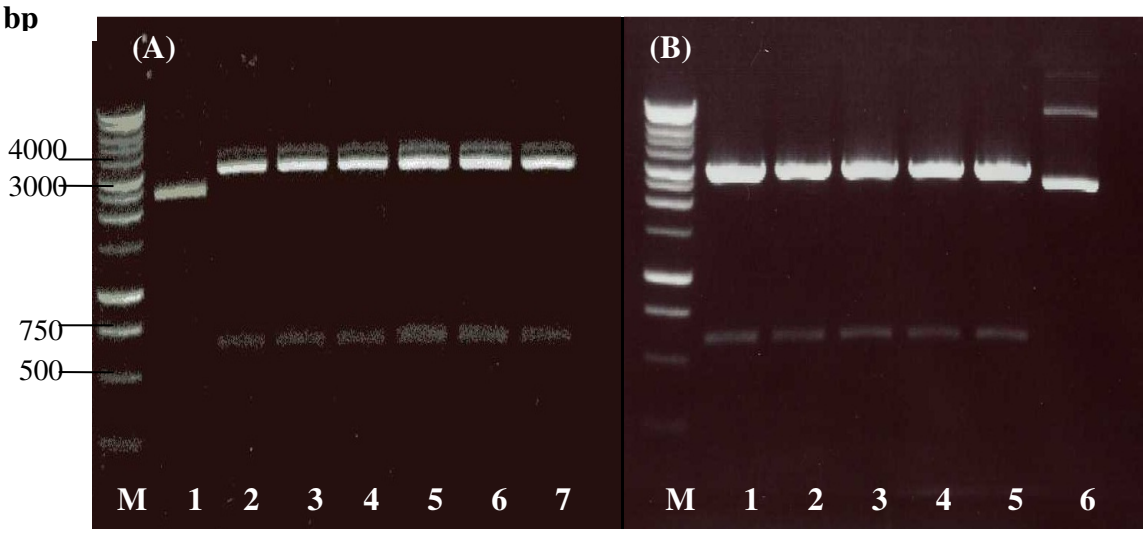


Figure 4.9 Verification of the presence of correct inserts in the pBluescript recombinant plasmids via restriction enzyme digestion. (A) Agarose gel, visualised under UV shows (1) undigested recombinant plasmid, (2) – (7) are six different recombinant plasmids subjected to *Spe*I and *Bam*HI digestion to release the 631 bp *pyrFDU* insert. (B) (1)-(5) *Bam*HI and *Xho*I digested recombinant plasmids releasing the 595 bp pyrFDD insert, and (6) undigested recombinant plasmid. These plasmids containing the correct sized inserts were subsequently sent for sequencing analysis. M= 1kb DNA marker (Promega).

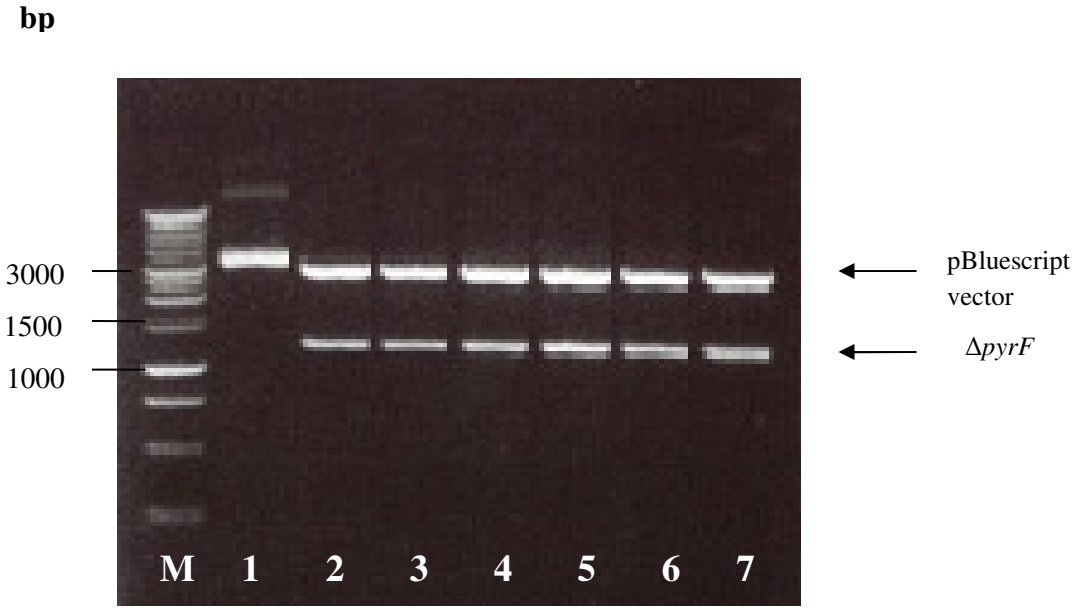


Figure 4.10 Verification of the presence of Δ*pyrF* fragment in the pBluescript recombinant plasmids via SpeI and XhoI digestion. M= 1 kb DNA ladder (Promega), 1= Undigested recombinant plasmid , 2-7= six different *Spe*I and *Xho*I digested recombinant plasmids containing the ~1.2 kb Δ*pyrF* fragment.

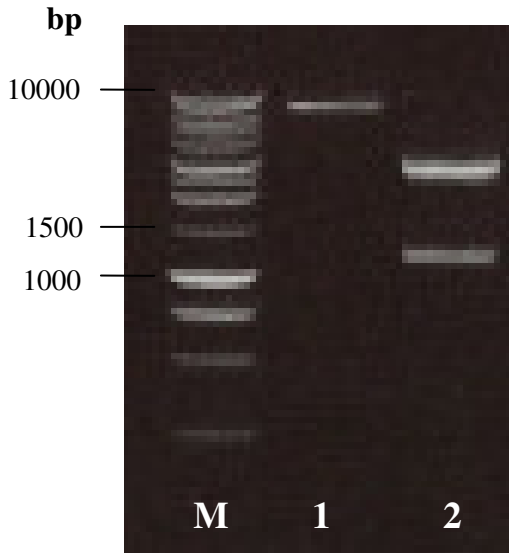


Figure 4.11 Agarose gel containing *Spe*I and *Xho*I digested products. M= 1kb DNA ladder (Promega), 1= digested pDM4 vector , 2= digested pBluescript vector and released Δ*pyrF* fragment. The pDM4 vector and Δ*pyrF* were ligated and transformed into *E. coli* S17-1 λ-pir cells.

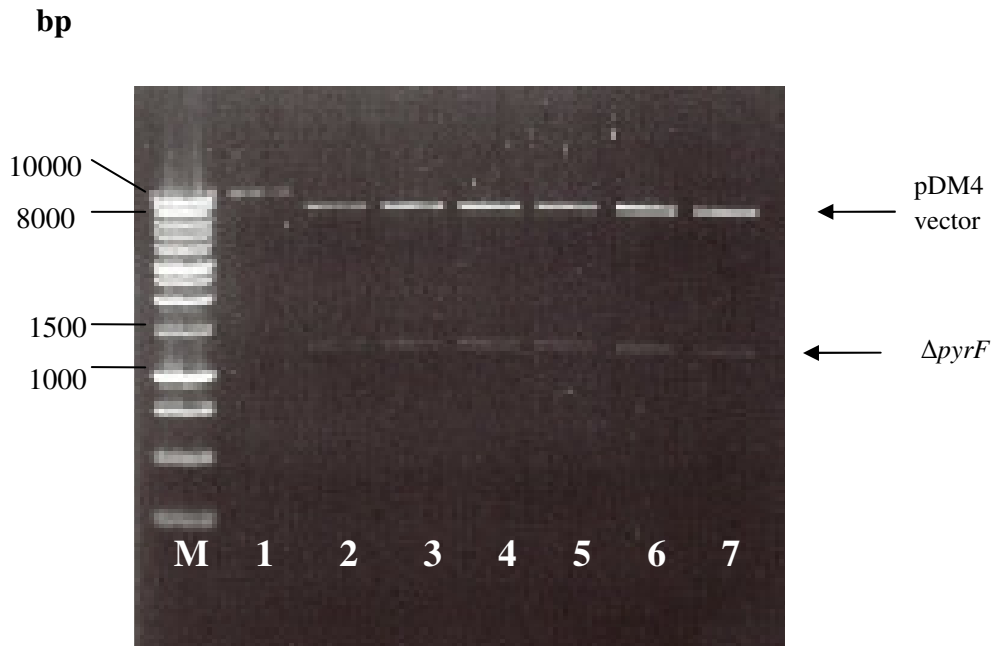


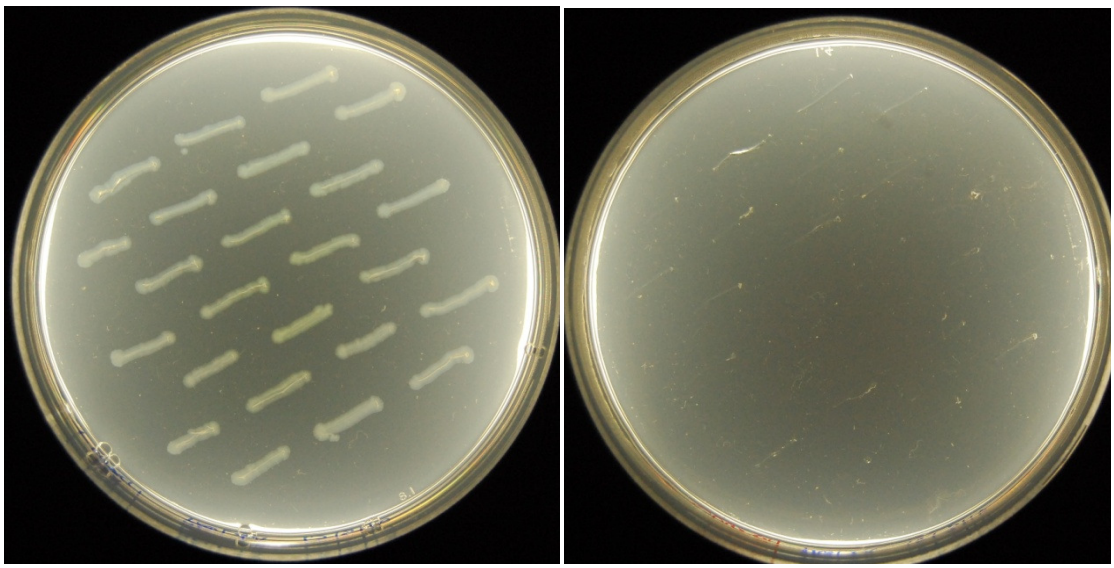
Figure 4.12 Verification of the presence of Δ*pyrF* fragment in the pDM4 recombinant plasmids via *Spe*I and *Xho*I digestion. M= 1 kb DNA ladder (Promega), 1= Undigested recombinant plasmid , 2-7= six different *Spe*I and *Xho*I digested recombinant plasmids containing the ~1.2 kb Δ*pyrF* fragment

4.2.2.2 Verification of PAO1Δ*pqsA*Δ*pyrF* mutants

PCR analysis and phenotypic assays were performed on the *pqsA pyrF* double mutant to verify the construct. After performing allelic exchange between pDM4Δ*pyrF* contained in *E. coli* S17-1 λ-pir with PAO1Δ*pqsA*, the PAO1Δ*pqsA*Δ*pyrF* mutant was selected by plating on minimal medium without uracil, and this resolution can be forced by replating on medium containing FOA. Figure 4.13 shows that PAO1Δ*pqsA*Δ*pyrF* does not grow without uracil in the media since the absence of the *pyrF* gene confers it to be uracil auxotrophic.

Figure 4.14 shows the PCR verification of the double mutant. The primer pair *pyrF*DUF and *pyrF*DDR were used to amplify a stretch of DNA, using the chromosomal DNA of suspected *pyrF* mutants as template. When the PCR reactions were separated on agarose gel, the wild type strain as well as the PAO1Δ*pqsA* gives rise to a stretch of DNA approximately 1.9 kb, comprising the wild type gene (699 bp) and approximately 600 bp at both upstream and downstream of the gene. The successfully constructed mutant should result in amplified DNA of approximately 1.2 kb due to the loss of *pyrF* gene.

A second PCR using primer pair's *pyrF*F and *pyrF*R to amplify the *pyrF* gene was carried with chromosomal DNA from the same colony that gave rise to the PCR result (lane 4 of Figure 4.14). When run on an agarose gel (Fig. 4.15), the wild type strain and the PAO1Δp*qsA* resulted in the 699 bp *pyrF* gene product, whereas the deletion mutant does not yield a PCR product of that size. This confirmed double mutant was subsequently selected for downstream work.



(a) **(b)**

Figure 4.13 Uracil auxotrophy conferred by *pyrF* deletion in PAO1Δ*pqsA*. PAO1Δ*pqsA*Δ*pyrF* is able to grow in the presence of uracil (20 µg/ml) in the medium (a) but not in its absence, (b).

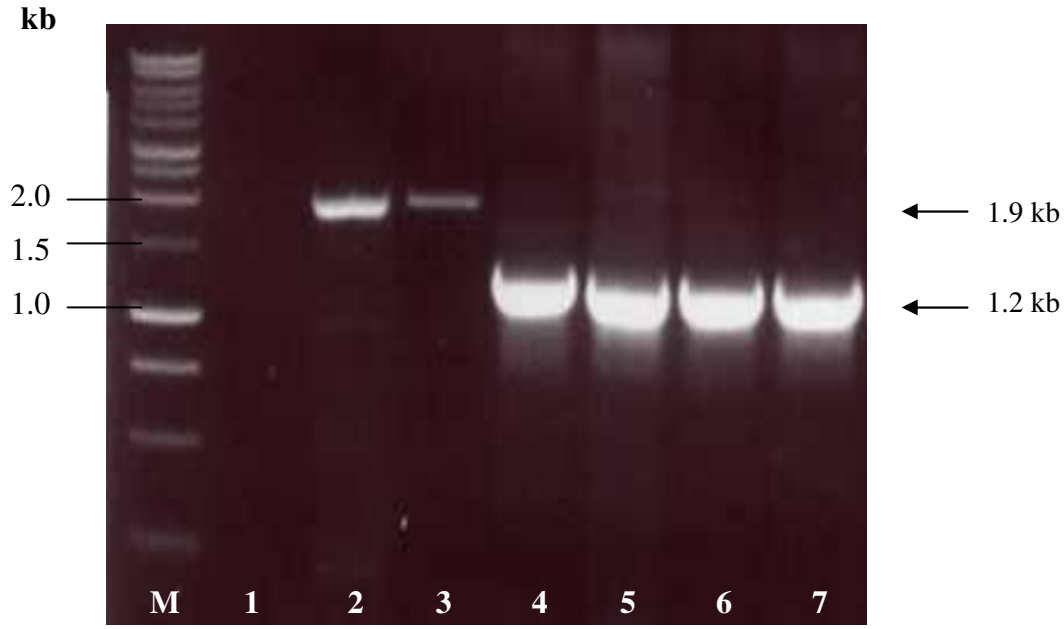


Figure 4.14 Agarose gel containing PCR reactions run using the primer pair *pyrF*DUF/*pyrF*DDR and 4 different colonies of suspected chromosomal deletion mutant of PAO1Δ*pqsA*Δ*pyrF* to provide template DNA. M= 1kb DNA marker ladder (Promega), 1= negative control (no template), 2= Wild type PAO1 (expect band around 1.9 kb, arrow), 3= PAO1Δp*qsA* (expect band around 1.9 kb, arrow), 4-7= Suspected PAO1Δ*pqsA*Δ*pyrF* chromosomal deletion mutant (expect band around 1.2 kb, arrow).

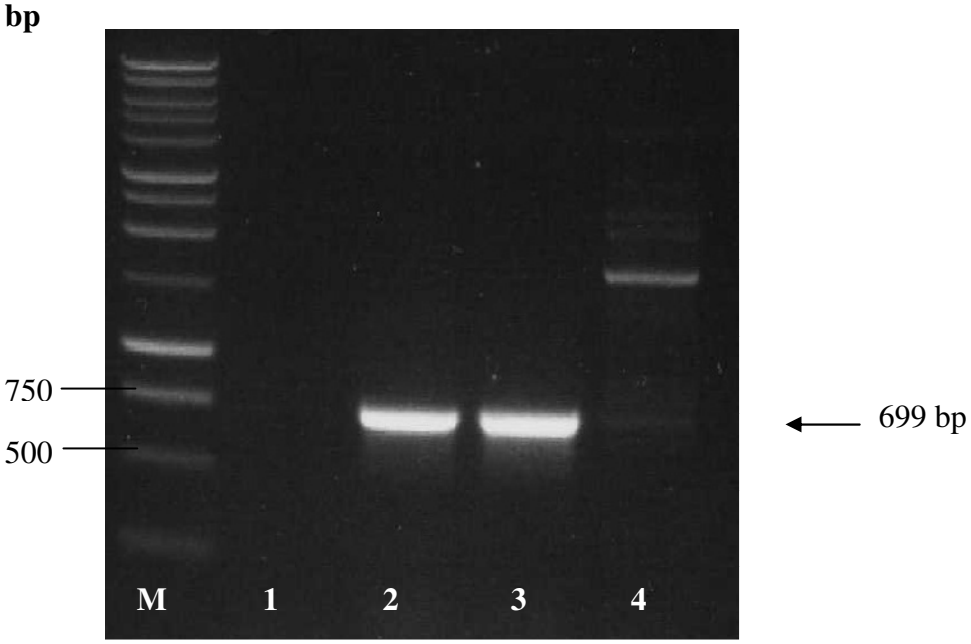


Figure 4.15 Agarose gel containing PCR reactions performed with the primer pair *pyrF*F/*pyrF*R and a colony of suspected chromosomal deletion mutant of PAO1Δ*pqsA*Δ*pyrF* to provide template DNA. M= 1 kb DNA marker ladder (Promega), 1= negative control (no template), 2= Wild type PAO1 (expected band around 699 bp, arrow), 3=PAO1Δ*pqsA* (expected band around 699 bp, arrow), 4= PAO1Δ*pqsA*Δ*pyrF* (no expected band).

4.2.2.3 Construction of PAO1-ES2

The plasmid containing the PpqsA-pyrF fusion, pES2 was constructed with use of the previously constructed pES1 plasmid. The *pyrF*F primer was designed to contain a *Sph*I restriction site and *pyrF*R, containing a *Hind*III restriction site. The pES1 plasmid was first digested with *Sph*I and *Hind*III to release the *sacB* gene fragment (Figure 4.16). The resulting plasmid backbone was subsequently ligated to similarly digested *pyrF* PCR product (699 bp), resulting in the pES2 plasmid. The construct was first transformed into *E. coli* DH5α and the clones containing the plasmids with correct sized inserts as determined by digestion with *Sph*I and *Hind*III (Figure 4.17) were subjected to sequencing analysis. As shown in Figure 4.18, pES2 carries the translational PpqsA-pyrF. The pES2 plasmid with correct *pyrF* sequence was subsequently transformed into PAO1Δ*pqsA*Δ*pyrF* double mutant, giving rise to PAO-ES2.

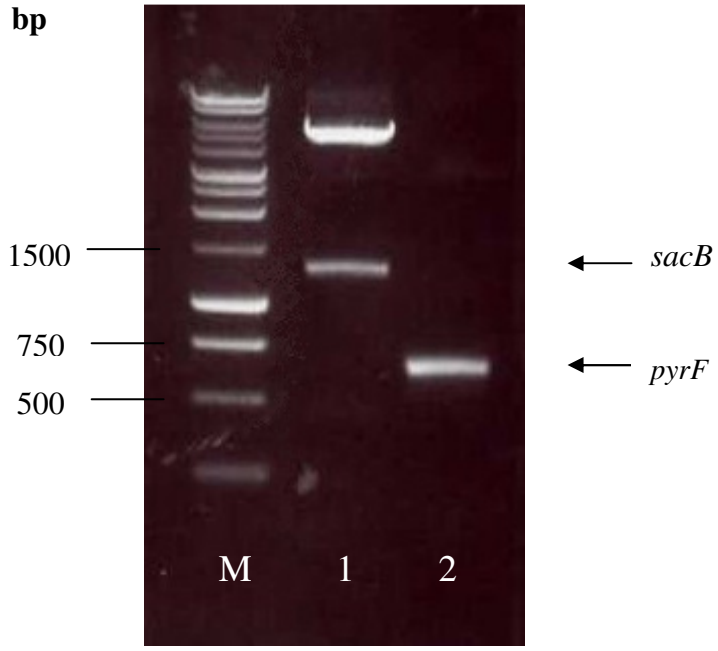


Figure 4.16 *SphI* and *Hind*III digested pES1 plasmid and *pyrF* gene PCR product. Agarose gel, visualised under UV shows pES1 with released 1.4 kb *sacB* gene (1) and the 699 bp *pyrF* gene (2). M= 1kb DNA marker.

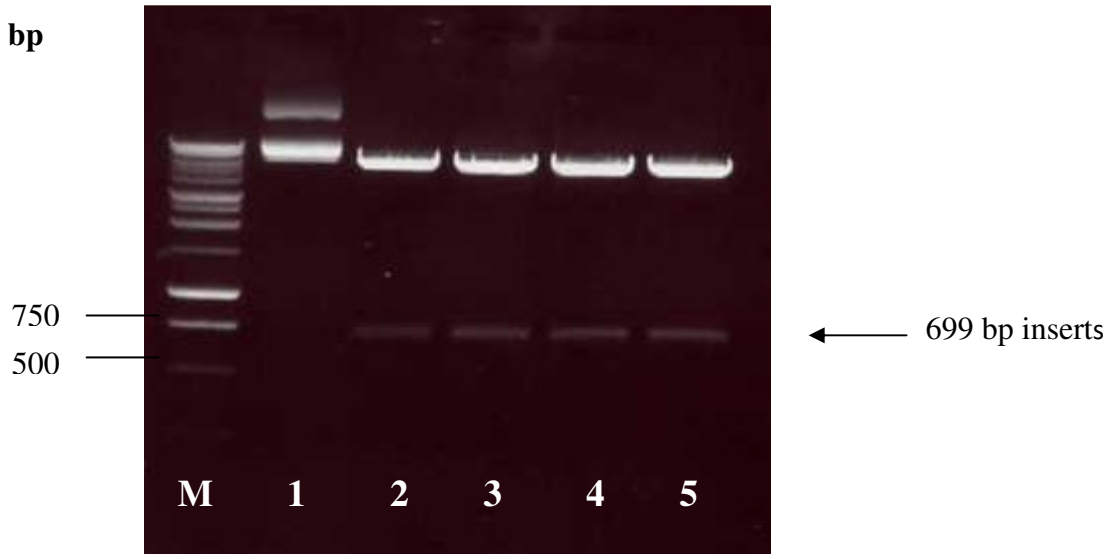


Figure 4.17 Verification of the presence of *pyrF* gene inserts in the clones via *Sph*I and *Hind*III digestion. Agarose gel, visualised under UV shows (1) Undigested plasmid, (2) – (5) are four different recombinant plasmids containing the correct sized inserts and subsequently sent for sequencing analysis. M= 1kb DNA marker (Promega).

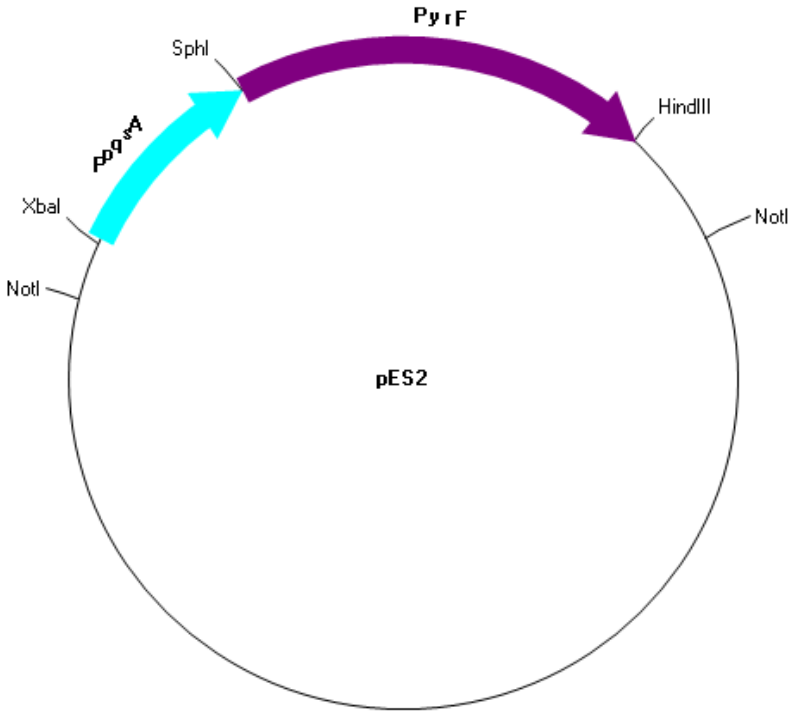


Figure 4.18 Plasmid map of pES2. The *sac*B gene has been substituted with *pyr*F.

4.2.2.4 Optimisation of the restoration of phenotypes

The PAO-ES2 was assessed for the complementation of uracil prototrophy and FOA sensitivity in the presence of PQS and FOA. Wild type PAO1, PAO1Δ*pqsA* and PAO1Δ*pqsA* Δ*pyrF* were analysed as controls. Table 4.1 summarises the first set of experimental results from uracil prototrophy and FOA sensitivity restoration assay. All strains were grown overnight in LB, washed twice with M9 minimal medium and finally plated on M9 minimal medium plates containing citrate as a carbon source. Depending on which phenotype was being evaluated, uracil (20 µg/ml) and/or FOA (250 µg/ml) were added into medium. PQS concentrations ranging from 200 nM to 100 µM were used in the assay in an attempt to identify the ideal concentration for activating the PpqsA-pyrF fusion. Uracil prototrophy and FOA sensitivity was not complemented in PAO-ES2 in the presence of various concentrations of PQS, even with the co-addition of 3-oxo-C12-HSL, which was supposed to aid in the upregulation of *pqsA*.

Table 4.1 Summary of result for uracil prototrophy and FOA sensitivity assay. PAO-ES2 does not appear to revert to wild type PAO1 phenotype even with the addition of 200nM to 100µM PQS. Uracil= 20 µg/ml, FOA= 250 µg/ml.

Strain / Medium composition	WT PAO1	PAO1 Δ *pqsA*	PAO1 Δ*pqsA* Δ*pyrF*	*PAO-ES2
+ uracil	+ve	+ve	+ve	+ve
- uracil	+ve	+ve	-ve	-ve
+ uracil + FOA	-ve	-ve	+ve	+ve

* Medium used for assaying the construct must contain 400 µg/ml carbenicillin and PQS.

4.2.2.5 Evaluation of the importance of PqsR

We next set out to investigate the importance of providing additional PqsR for binding to the exogenously added PQS. This was carried out to observe whether an abundance of PqsR in the system will lead to improved PqsA expression, thus complementing FOA sensitivity. The pSK65 plasmid was obtained with courtesy from Dr. Sarah Kuehne (University of Nottingham). This plasmid is essentially a pME6032::*pqsR* construct that contains an IPTG-inducible promoter and His tag for protein purification, expressed from a *tac* promoter. We proceeded to transformed pSK65 into PAO-ES2 to produce PAO-ES3. Complementation of uracil prototrophy and FOA sensitivity was assayed for PAO-ES3 as per for PAO-ES2, with the additional step of 1mM IPTG induction for 3 h.

4.2.2.6 Uracil prototrophy complementation

A 24 h growth assay in M9 minimal medium with citrate as a carbon source (2mg/ml) but without uracil was carried out to investigate uracil prototrophy of PAO-ES3 in the presence of PQS, after IPTG induction. The assay was carried out in 96-well microtitre plates with half-hourly readings taken by the TECAN (GENios Pro™). According to Figure 4.19, uracil prototrophy was restored in PAO-ES3 in the presence of 20 µM of PQS. This shows that a higher amount of PqsR is indeed required for PpqsA-pyrF expression, thus successfully complementing the double mutant. Wildtype PAO1, PAO1Δ*pqsA*Δ*pyrF* and PAO-ES2 were added as controls. The lack of growth of PAO-ES2 in the presence of the same concentration of PQS as PAO-ES3 shows that the

presence of pSK65 in the strain is indeed making a difference. This observation confirms that over-expression of PqsR protein aids in better expression of *pqsA*. Only wildtype PAO1 was able to grow in the absence of PQS, proving that exogenous PQS has to be provided to PAO-ES3 in order for PQSIS-3 to function correctly.

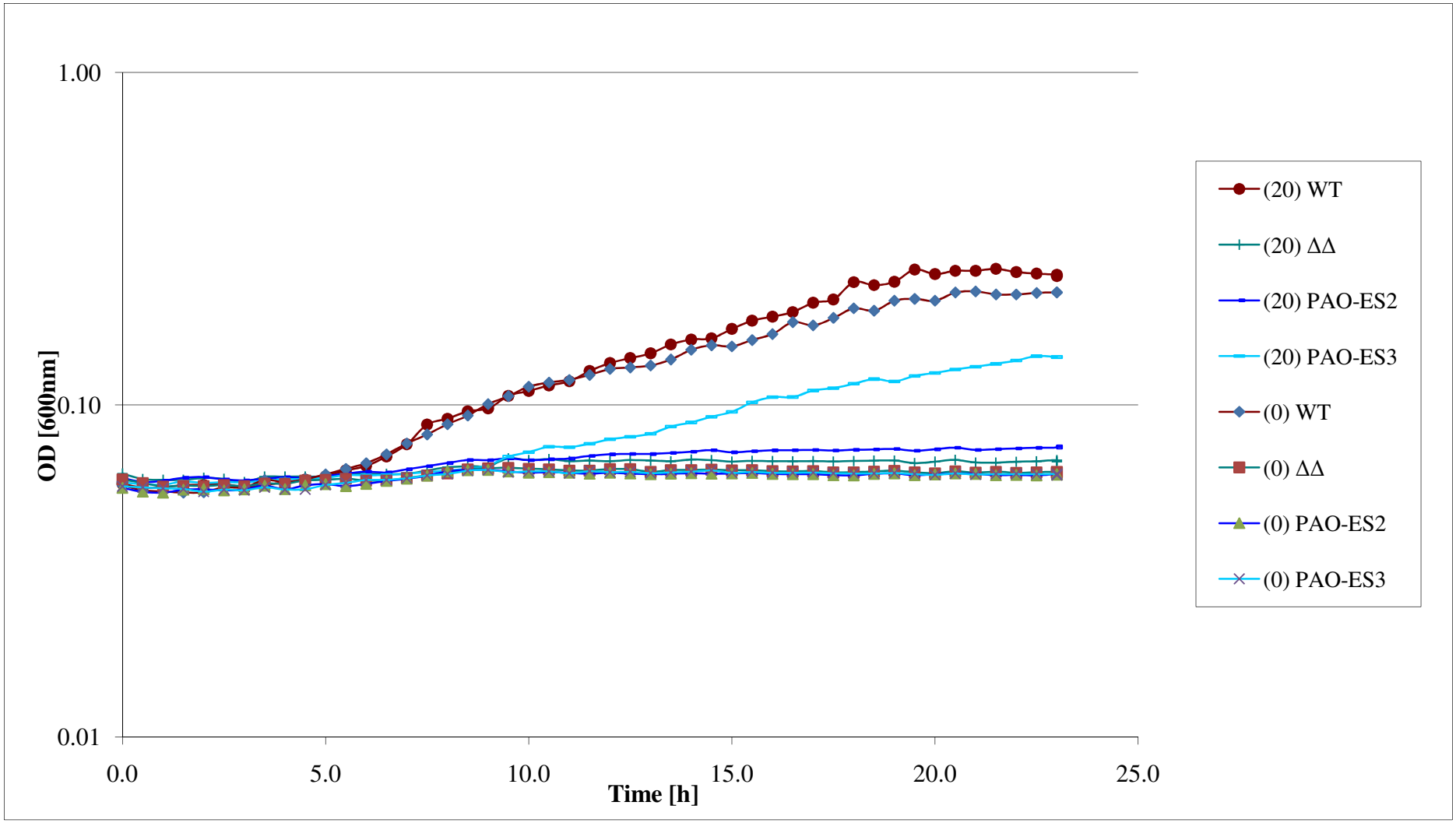


Figure 4.19 Growth curve of PAO-ES3 in M9 minimal medium in the absence of uracil to determine uracil prototrophy restoration. In the legend, (0) indicates 0 µM or no addition of PQS whereas (20) indicates that 20 µM PQS was provided in the medium.

4.2.2.7 FOA sensitivity complementation

Restoration of FOA sensitivity in PAO-ES3 was determined by plating the IPTG induced culture on sets of FOA medium containing 1 µg/ml, 0.8 µg/ml or 0.4 µg/ml uracil in the absence and presence of FOA. This was carried out to identify the ideal uracil concentration that demonstrates a good contrast between growing and non-growing cells. Two batches of PAO-ES3 cells were prepared; one batch was subjected to 3 h of induction in the presence of 1 mM IPTG and no IPTG induction for the other batch. Figure 4.20 shows that 1 µg/ml of uracil shows that best contrast for FOA sensitivity as there is no growth in the presence of FOA and relatively robust growth in the absence of FOA. In addition to that, there was no significant difference in the expression of PpqsA-pyrF with or without IPTG induction of the *pqsR* gene. This suggests that even without IPTG, PqsR is still being produced at amounts sufficient to activate the PpqsA-pyrF fusion.

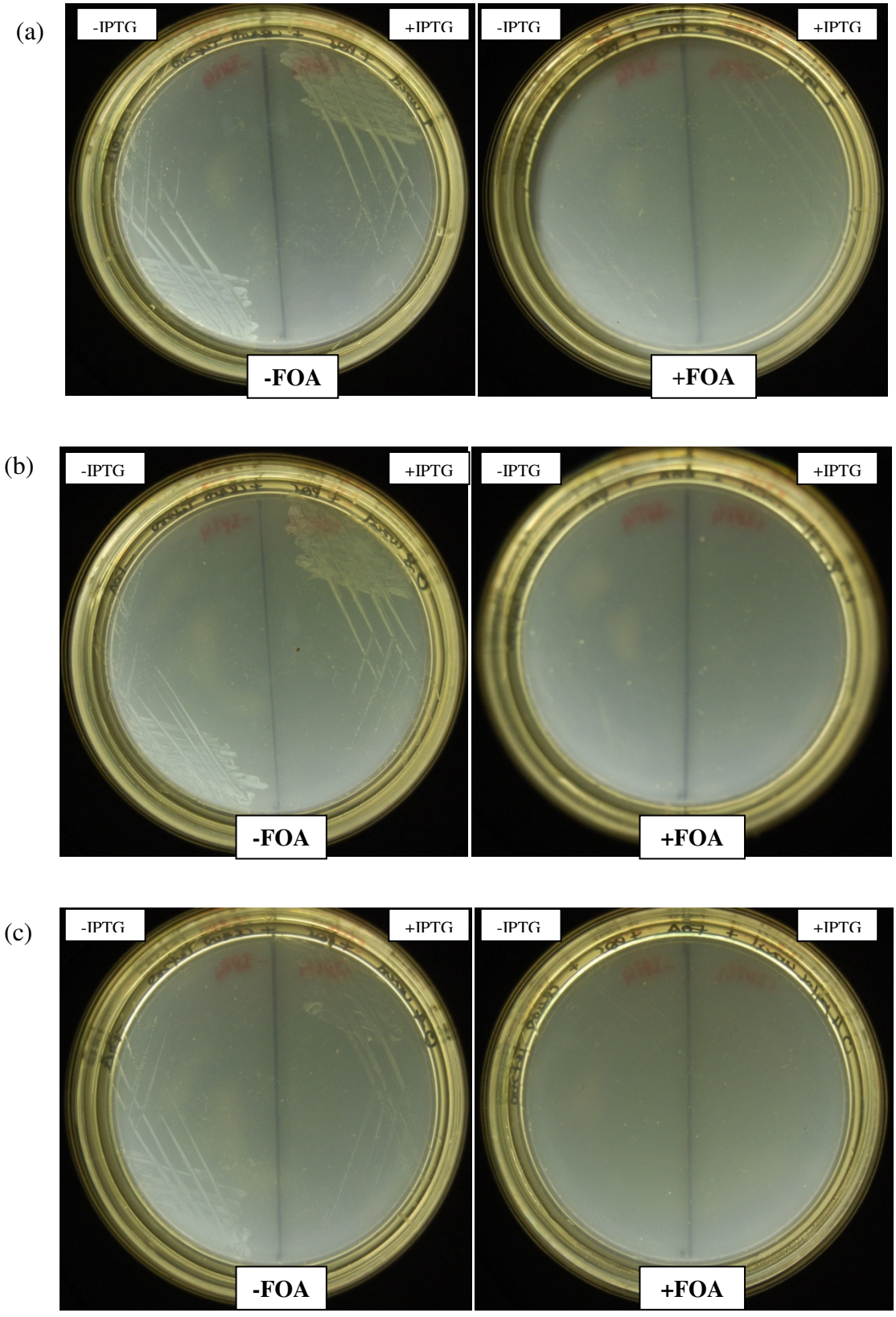


Figure 4.20 FOA sensitivity assay for PAO-ES3 with and without IPTG induction and in the presence and absence of FOA. (a) 1.0 µg/ml uracil (b) 0.8 µg/ml uracil (c) 0.4 µg/ml uracil.

4.3 Discussion

4.3.1 PQSIS-1

The genetic construct of this bioreporter for the induction of cell death in the presence of exogenously added PQS signal molecule relies on the presence of a promoter that can be induced by AQs (Fletcher et al., 2007). Fletcher and co workers introduced a *pqsA::luxCDABE* reporter gene fusion into a *P. aeruginosa pqsA* mutant which cannot synthesize AQs (Fletcher et al., 2007). This biosensor produces bioluminescence in response to AQs. The PQSIS-1 was also developed in a *P. aeruginosa pqsA* mutant. In determining the extent of *pqsA* promoter to be fused to *sacB*, the crucial regions within the promoter were included in the construct. The fusion retains the lysR box, critical for *pqsA* transcriptional regulation; as well as the native ribosome-binding site (RBS) of the *pqsA* promoter. No RBS was found upon checking the *sacB* gene sequence of the pLasB-SacB1 plasmid (Rasmussen et al., 2005). This indicates that in order for transcription of *sacB* to occur, the RBS needs to be present in the promoter that is to be fused to the *sacB* gene.

The *Bacillus subtilis sacB* gene is a well known counter-selectable marker for gram-negative bacteria that codes for the levansucrase enzyme. In the presence of sucrose, the bacteria will synthesise levans, which is a high molecular weight fructose polymer. The accumulation of levans in the periplasm is toxic to the bacteria, thus leading to cell death (Gay et al., 1983, Reyrat et al., 1998). However, even with the introduction of up to 20% w/v of sucrose supplemented with 100 µM PQS in the media, no induction of *sacB* that leads to killing was observed in PQSIS-1. Nonetheless, it has been highlighted by Galvao and Lorenzo (2004) that *sacB* is not an optimal gene for counter-selection because if depends on sucrose transport. Successful induction is often forced by the

addition of high extracellular concentrations of sucrose, often 5% w/v or more. This causes undue stress onto the bacteria thus favouring the rise of mutations which can bypass selection stringency. Furthermore, in the lasB-sacB system (QSIS 2), false positive results whereby the cells survive from *sacB* based killing is often obtained when screening extracts with high carbohydrate content (Rasmussen et al., 2005). This suggests that glucose may interfere with the sucrose killing system. Hence, other counter-selective genes to be incorporated in the construct were considered in an effort to improve the PQSIS construct.

4.3.2 PQSIS-2 and PQSIS-3

The complementation of *pyrF* encoded phenotypes was not successful in PAO-ES2. This complementation was also unsuccessful when pES2 was transformed into a *P. aeruginosa pyrF* single mutant, which is able to produce PQS on its own accord. This observation indicates that the lack of complementation in PAO-ES2 was not due to the lack of basal levels of PQS. Primer extension and RT-PCR analysis have shown that *pqsA* is part of a five-gene operon (*pqsABCDE*) important for PQS biosynthesis (McGrath et al., 2004). Several researchers have proven that the *pqsR* gene is a master regulator of the PQS biosynthetic gene cluster. This gene encodes a LysR-type regulator that has been shown to affect *pqsABCDE* expression in a positive manner (McGrath et al., 2004, Wade et al., 2005, Xiao et al., 2006). PqsR binds to the *pqsA* promoter and this binding was shown to increase dramatically in the presence of PQS (Dubern and Diggle, 2008, Wade et al., 2005).

We postulated that there may be insufficient PqsR in PAO-ES2 to respond to exogenously added PQS. To investigate this theory, we proceeded to introduce additional PqsR into the system by transforming a multicopy, IPTG inducible plasmid

that contains the *pqsR* gene into PAO-ES2. This new construct was called PAO-ES3, developed for the PQSIS-3. Over expression of *pqsR* restored wildtype phenotype, as observed in the growth experiment carried out to examine uracil prototrophy complementation (Figure 4.19). Growth of PAO-ES3 was obtained in the presence of 20 µM PQS and PAO-ES3 was not viable when PQS was not provided. This indicates that in PAO-ES3, the presence of PQS is crucial in activating the PpqsA-pyrF fusion, aided by an abundance of PqsR that is provided by pSK65. However, this construct was not growing as robust as the wildtype *P. aeruginosa*. This suggests that overexpression of *pqsR* is rather taxing for the double mutant, especially when grown with citrate as the sole carbon source in the minimal medium. It may be helpful to provide a co-substrate in the medium to improve the growth of PAO-ES3. FOA sensitivity was also complemented in the construct even when not subjected to IPTG induction, suggesting that copious amount of the pSK65 plasmid was being produced in the strain.

4.4 Conclusion

A bioreporter that can be potentially used for high throughput screening of PQS quorum sensing inhibitors has been constructed. It is beneficial that the PQSIS-3 is based on the 'rescuing' of cells in the presence of an inhibitor because it allows us to immediately detect and rule out compounds that are lethal to the cells. This corresponds well with the aim of quorum quenching, which is to attenuate quorum sensing regulated virulence factors without affecting cell survivability. PQSIS-3 application is also cost effective as it does not require any specialised equipments.

Currently, a version of PAO-ES3 with regulatable expression of PqsR, in which the IPTG inducible *pqsR* gene cassette will be integrated into the chromosome of PAO-ES2 to ensure stable segregation and a constant gene dosage of *pqsR* is being constructed. The omission of a multicopy plasmid will make it a more refined system, allowing careful titration of expression levels. An optimized methodology with use of the final construct will also be developed for the high through-put screening of PQS quorum sensing inhibitors.