Gene expression of *Pseudomonas aeruginosa* in a mucin-containing synthetic growth medium mimicking CF lung sputum

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SUMMARY

Pseudomonas aeruginosa airway infection is the leading cause of morbidity and mortality in cystic fibrosis (CF). Various *in vitro* models have been developed to study *P. aeruginosa* pathobiology in the CF lung. We have produced a modified artificial sputum medium (ASMDM) more closely resembling CF sputum than previous models, and have extended previous work by using PAO1 arrays to examine global transcription profiles of *P. aeruginosa* UCBPP-PA14 under early exponential phase and stationary phase growth. In early exponential phase, 38 of 39 nutrition-related genes were upregulated in line with data from previous in vitro models using UCBPP-PA14. Additionally, 23 type III secretion system (T3SS), genes, several anaerobic respiration genes and 24 quorum sensing (QS)-related genes were upregulated in ASMDM suggesting enhanced virulence factor expression and a priming for anaerobic growth and biofilm formation. Under stationary phase growth in ASMDM, macroscopic clumps resembling microcolonies were evident in UCBPP-PA14 and CF strains, and over 40 potentially-important genes were differentially expressed relative to stationary phase growth in Luria-Broth (LB). Most notably, QS-related and T3SS genes were downregulated in ASMDM and iron acquisition and assimilatory nitrate reductase genes were upregulated, simulating the iron-depleted, microaerophilic/anaerobic environment of CF sputum. ASMDM thus appears highly suitable for gene expression studies of *P. aeruginosa* in CF.

INTRODUCTION

Pseudomonas aeruginosa is the major pathogen responsible for lung function decline and premature death of cystic fibrosis (CF) patients. It grows as free-swimming cells in the early stages of infection in CF lung airway surface liquid, but can progress to form ball-shaped micro-/macro-colonies that resemble biofilm form #2 (bacteria attached together and not to surfaces) (Hassett *et al.*, 2009) within hypoxic mucus zones of the airway lumen (Hassett *et al.*, 2002; Worlitzsch *et al.*, 2002). Biofilm and planktonic *P. aeruginosa* forms coexist in long-term infection (Garcia-Medina *et al.*, 2005).

The use of CF-patient sputum to study the pathobiology and growth characteristics of *P. aeruginosa* in the CF lung is impractical due to changes in consistency on sterilization, presence of highly-resistant yeasts, patient-to-patient variability and antibiotic use. Sputum provides amino acids as the major carbon source (Sriramulu *et al.*, 2005), however the particular carbon source used dramatically affects biofilm formation (Klausen *et al.*, 2003; Shrout *et al.*, 2006). Mucin is another important nutrient source and triggers changes in expression, reduces surface motility and enhances biofilm formation (Landry *et al.*, 2006; Sriramulu *et al.*, 2005; Wang *et al.*, 1996). Concentration of the principal mucins in sputum (MUC5AC and MUC5B) also increases greatly during periods of exacerbation (Henke *et al.*, 2007). The presence of high molecular weight DNA is important in the formation of mature multicellular biofilm structures (Barken *et al.*, 2008; Beatson *et al.*, 2002; Tetz *et al.*, 2009).

Various synthetic or semi-synthetic media have been developed in attempts to mimic the CF lung environment. Studies using the reference strain *P. aeruginosa* UCBPP-PA14 (Rahme *et al.*, 1995) grown in a medium containing 10% (v/v) CF sputum (sputum-containing medium) (Palmer *et al.*, 2005) showed upregulated expression of branched chain and aromatic amino acid catabolism genes, the **P**seudomonas **q**uinolone **s**ignal (PQS) molecule and repression of anabolism genes.

Subsequently this group demonstrated upregulation of nutritionally-controlled genes in a totally synthetic CF sputum medium (SCFM) (Palmer *et al.*, 2007a). However SCFM lacked DNA and mucin, while sputum-containing medium contained these components at below CF-sputum levels. DNA and mucin also help to form a biological matrix to facilitate *P. aeruginosa* biofilm formation. Studies using *P. aeruginosa* PAO1 in an artificial medium containing porcine mucin instead of human sputum (ASM+) showed that amino acids, salt, low iron, lecithin and DNA were necessary for the establishment of the macroscopically visible clumps seen in CF sputum and described as tight microcolonies (Sriramulu *et al.*, 2005). However, as far as we are aware there are no published studies of *P. aeruginosa* gene expression during stationary phase growth in an artificial CF sputum medium.

We have produced an artificial CF sputum medium (ASMDM) based on modifications of ASM+ that avoids use of CF sputum and contains other components including mucin, albumin and DNA at CF-sputum levels, and have extended previous studies by using PAO1 arrays to examine global *P. aeruginosa* gene expression in early exponential and stationary phase growth, mimicking the process of infection in the CF lung.

MATERIALS AND METHODS

All microarray experiments were performed using *P. aeruginosa* UCBPP-PA14, the strain used in expression studies in sputum-containing medium (Palmer *et al.*, 2005) and SCFM (Palmer *et al.*, 2007a), and sourced from the same research group (Rahme *et al.*, 1995). For exponential phase studies, growth protocols were as described for sputum-containing medium with MOPS-glucose medium used as reference (Palmer *et al.*, 2005) allowing comparisons of expression data from the two studies. Growth curves were used to determine the OD_{600} required for harvest in MOPS-Glucose, exponential phase ASMDM, and LB (**Fig. 1**). Phenotypic growth studies were carried out

using UCBPP-PA14 and two CF isolates, an Australian Epidemic Strain-1 isolate (AES-1R) and a non-epidemic isolate (34Bris).

Exponential growth for early gene expression

MOPS-Glucose medium

Two ml of MOPS-glucose medium (MOPS buffer (50 mM MOPS [pH 7.2], 93 mM NH_4Cl, 43 mM NaCl, 3.7 mM KH_2PO_4, 1 mM $MgSO_4$, and 3.5 M $FeSO_4$ $7H_2O$ with 6.3 mM glucose) in 5 ml screw-capped bottles was inoculated with culture (final concentration OD_{600} = 0.003 McFarland 0.5 Standard) (bioMerieux SA, France) and incubated with shaking (250 rpm) at 37°C. Cells were harvested at an OD_{600} = 0.3±0.1, ca. 6 h post-inoculation by comparison with growth curve readings (**Fig. 1**) and uninoculated MOPS-Glucose was used as a blank. (Palmer *et al.*, 2005), pelleted (5 min, 5000 *g*, 4 °C), resuspended in 1×PBS, and treated with RNAprotect™ (Qiagen).

ASMDM

ASMDM contains the following modifications compared to ASM+ (Sriramulu *et al.*, 2005): We added 10 mg ml^{-1} bovine serum albumin (Sigma) (not added to ASM+), since studies have shown CF patient sputum has higher albumin concentrations compared to the sputum of non-CF patients (Sagel *et al.*, 2001). This is probably due to vascular leakage that may be occurring as part of the inflammatory process (Reid *et al.*, 2004); We increased the concentration of porcine stomach mucin (10 mg ml^{-1} versus 5 mg ml^{-1}) to better reflect the findings of Henke et al (Henke *et al.*, 2007) who identified greatly increased mucin levels during pulmonary exacerbations and lowered the concentration of herring sperm DNA (Sigma) (1.4 mg ml^{-1} versus 4 mg ml^{-1}) to bring it closer to that of CF sputum as described by Brandt et al (Brandt *et al.*, 1995). Ingredients were stirred for 5 min and homogenized to dissolve mucin and DNA. As ASMDM could not be autoclaved without damage to the mucin, antibiotics (final concentration: 16 µg ml^{-1} tetracycline, 1 µg ml^{-1} penicillin and 1 µg ml^{-1} ampicillin) were added to inhibit contaminants. Volume was made up to 100 ml with

dH_2O and pH adjusted to 6.5, the estimated pH of CF airway mucus (Yoon *et al.*, 2006). Ten ml of ASMDM in 30 ml screw–cap clear glass bottles (e.g. McCartney bottles) with loosened caps to provide adequate aeration was inoculated with a starting culture as for MOPS-Glucose (above) and incubated at 37°C with shaking (250 rpm). Uninoculated ASMDM was used as a blank. Cells were harvested at OD_{600} = 0.3±0.1, ca. 14 h post-inoculation by comparison with the growth curve readings (**Fig. 1**). Cells were processed for RNA as above.

Stationary phase growth

Luria broth

LB (25 mg ml^{-1}) (Oxoid) was used as reference medium for stationary phase planktonic growth as it has been widely used as a non-specialized growth medium for *P. aeruginosa* transcriptomics in both CF and non-CF studies (Alvarez-Ortega & Harwood, 2007; Juhas *et al.*, 2005; Schuster *et al.*, 2003; Waite *et al.*, 2005). Cells were incubated at 37°C with a loose lid and slow rotation (50rpm) to circulate nutrients and prevent settling, and harvested at mid stationary phase (OD_{600}=1.1±0.1 – ca. 11 h post inoculation - determined by growth curves (**Fig. 1**).

ASMDM

Overnight cultures were diluted in 1×PBS to an OD_{600} (ca. 1×10^8 CFU ml^{-1}). Ten ml ASMDM in McCartney bottles was inoculated with 50 µl of culture just under the surface of the medium and incubated statically at 37 °C with a loose lid. As it is not possible to determine the OD_{600} of the biofilm, we used our observations of growth patterns from 48 to 120 h to choose the 72 h time-point as indicator of stationary phase. At 72 h the pellicle and the deep anaerobic growth were harvested and washed 5× in PBS on ice. RNA was extracted and cDNA synthesized, purified, fragmented and labelled as described (Manos *et al.*, 2008; Palmer *et al.*, 2005; Schuster *et al.*, 2003).

Gene expression profiling

DNA fragmentation was assessed by bioanalysis and 7 µg of each suitable sample was used for hybridisation in a total volume of 300 µl hybridisation mix (Affymetrix). 80 µl of this was loaded into a Test3 array (Affymetrix-100 housekeeping genes) and hybridised at 45 ^{o}C for 16 h at 60 rpm to determine cDNA suitability for the full array. Of the remainder, 200 µl was hybridized to the Affymetrix *P. aeruginosa* PAO1 GeneChip® array as described (Manos *et al.*, 2008; Palmer *et al.*, 2005).

Data Analysis

Microarrays were performed in biological duplicate for each sample in each condition tested (same isolate; with different culture, RNA extraction, and microarray) to assess biological variability within cultures. Microarray data were analyzed with BIOCONDUCTOR (Gentleman *et al.*, 2004) using the robust multi-array average (RMA) method (Bolstad *et al.*, 2003; Gautier *et al.*, 2004) for data normalization, incorporating probe level background-correction, quantile normalization, and linear extraction of a final expression measure for each gene per array. The false discovery rate method (Benjamini & Hochberg, 1995) was controlled to reduce false positives. A positive B-statistic, where B-statistic is the log-odds that that gene is differentially expressed (Smyth, 2003), or $p<0.05$ was used as a guide for statistically significant differential expression. Additional differentially expressed biologically-relevant genes falling just outside these criteria ($B<0$ or $p>0.05$) have also been included. The microarray data are available on the Gene Expression Omnibus (GEO) website http://www.ncbi.nlm.nih.gov/projects/geo (series GSE18594).

Microarray Validation

Quantitative SYBR-green-PCR using Platinum SYBR Green qPCR Supermix-no UDG (Invitrogen Corp., Australia) and Real-Time amplification (Rotor-Gene6000, Qiagen, Australia) was performed on cDNA synthesized from RNA used for microarray analysis: six genes (*trpA, putA, dadX, oprB,*

exaB, exoT) were selected from the exponential growth array data and five *(aroQ2, aprE, phzD*, *aprD* and *pfeA*) from the stationary phase array data. Gene selection was based on differential gene expression and association with nutrition or virulence, and included genes with p>0.05 or B<0. Primers were designed using Oligo6 Version 6.67 (Molecular Biology Insights Inc., USA) and obtained from Sigma-Genosys Inc. (Australia). The genes *lpd3* and *recA* were used as endogenous controls in exponential and stationary phase RNA, respectively, because of uniform expression across arrays.

Microcolony observation

1ml ASMDM containing 0.1% (w/v) agar for better visualization of the microcolony structure, was added to wells of a 24-well polystyrene plate and after setting, 5 µl of diluted culture was inoculated under the surface. Plates were incubated with slow rotation (40 rpm) at 37 ^{o}C and growth monitored for 72 h by visual checking for formation of clusters of cellular growth (**Fig. 2A**). The extent of actively growing cells was ascertained by the addition of 2,3,5 triphenyltetrazolium chloride (Sigma) (5 % w/v) to the medium during preparation. Tetrazolium chloride turns red upon oxidation by living cells and does not affect growth. All experiments were carried out in triplicate and representative results are shown.

RESULTS AND DISCUSSION

Microarray expression levels

Excel files of array data from all biological replicates were checked for total number of genes showing expression (present - P) and no expression (absent - A), to determine replicate consistency. Transcript expression levels averaged 89 % for MOPS-glucose grown bacteria, 86 % for LB-grown cells and 88 % for ASMDM-grown organisms, (range 82.7 %-94.2 %). These results are in line with other studies (Manos *et al.*, 2009; Wagner *et al.*, 2003). Since a PAO1 array was used, the

differentially expressed genes were checked for homologues in UCBPP-PA14 on the Pseudomonas database v2.pseudomonas.com. All genes had homologues in the UCBPP-PA14 genome.

1. UCBPP-PA14 exponential growth gene expression in ASMDM

Genes differentially expressed ≥2-fold in ASMDM versus MOPS-glucose medium and sputum-containing medium are shown in **Table 1A**. Fifteen of the 39 nutrition-controlled genes reported to be upregulated in sputum containing medium (Palmer *et al.*, 2005) were also upregulated (B>0 or p<0.05) in ASMDM. Twenty three of the remaining 24 genes in this group were also upregulated in ASMDM, although below the cutoffs (B<0 and p>0.05). Data from SCFM showed similar findings (Palmer *et al.*, 2007a). In terms of expression levels, there were a few outliers, including PA0865 *hpd* (66-fold vs 2.3-fold) and PA2322-gluconate permease (-5.5-fold vs -35.5-fold). This is probably due to compositional differences between the media leading to different metabolic requirements. However, it should be noted that for nutrition-controlled genes, most fold differences for both media fell within a similar range (-5.5 to 20-fold for sputum containing medium and -12.3 to 10.8-fold for ASMDM). The early upregulation of nutrition-controlled genes is an important early step in the development of the dense multicellular biofilm-like phenotype seen in the sputum of chronically infected CF patients (Sriramulu *et al.*, 2005). The upregulation of these key nutrition-related genes suggests that ASMDM provides a very good mimic of the lung environment.

Notable among other genes upregulated in both ASMDM and sputum containing medium were *exaB*, coding for cytochrome c550 and part of the *exaAB* promoter controlling ethanol-oxidation, and the virulence-related genes *hcnB,* and *oprC*. *hcnA* and *phzAB* were also upregulated in ASMDM, as predicted by Palmer et al for CF sputum (Palmer *et al.*, 2005). However, 24 QS-related genes, 23 T3SS genes and several anaerobic metabolism genes were upregulated in ASMDM but not in sputum containing medium (**Table 1B**). Elevated QS and T3SS gene expression has been well documented in acute infection in vivo and in vitro (Berthelot *et al.*, 2003;

Roy-Burman *et al.*, 2001), thus ASMDM may have some advantages over sputum-containing medium. The upregulation of the T3SS in ASMDM does not reflect the low calcium environment, since calcium concentrations were the same as those in sputum containing medium (Palmer *et al.*, 2005). T3SS upregulation in ASMDM may have been mediated in part by upregulation of QS regulators in conjunction with the down-regulation of *trpA*, which suppresses the T3SS as the bacterium transitions from low to high density growth (Lin *et al.*, 2006). The upregulation of the QS system in exponential growth also promotes biofilm development (Singh *et al.*, 2000) and thus exponential phase UCBPP-PA14 in ASMDM is likely primed for biofilm growth.

One of the features of *P. aeruginosa* growth in CF mucus is its ability to switch to anaerobic or microaerophilic growth. An upregulation of anaerobic metabolism genes involved in nitrate, nitrite and nitrous oxide utilization was seen in exponential phase growth in ASMDM but not sputum-containing medium, suggesting that even in exponential phase growth, ASMDM may better mimic the hypoxic or anaerobic environment of the CF lower airway mucus plugs (Hassett *et al.*, 2002; Worlitzsch *et al.*, 2002). Furthermore, studies indicate CF sputum contains sufficient nitrate to support significant anaerobic growth of *P. aeruginosa* (Palmer *et al.*, 2007b) and the phenotypic characteristics of growth by CF isolates (**Fig. 2B**) showed deep widespread anaerobic growth in ASMDM. The upregulation of anaerobic respiration genes including *nirJ-S*, encoding the dissimilatory nitrite reductase and the oxygen-independent dehydrogenase *hemN*, may have contributed to T3SS upregulation, since nitric oxide produced via anaerobic metabolism of nitrite by the dissimilatory nitrite reductase is critical for the assembly of the entire T3SS (Van Alst *et al.*, 2009).

Iron-related genes, including pyochelin synthesis (*pchDCBA*), pyoverdine synthesis (*pvdE*) and ferric uptake (*fptA, tonB*) were downregulated in ASMDM but upregulated in sputum-containing medium. The downregulation of pyochelin (*pchDCBA*), pyoverdine synthesis (*pvdE*) and ferric

uptake genes (*fptA, tonB*) in exponential growth suggests that ASMDM contains adequate iron for exponential growth despite the presence of the chelator DPTA. In vivo *P. aeruginosa* utilises ferric enterobactin at the expense of pyochelin and pyoverdine because of its superior iron-chelating ability (Dean *et al.*, 1996). Thus the upregulation of pyochelin and pyoverdine synthesis genes seen in sputum-containing medium (Palmer *et al.*, 2005) may reflect the fact that sputum comprised only 10 % of the volume.

2. UCBPP-PA14 stationary phase growth and gene expression

Phenotypic characteristics

In 24-well plates, tight microcolony formation similar to that described by Sriramulu et al for PAO1 (Sriramulu *et al.*, 2005) was observed for *P. aeruginosa* UCBPP-PA14 grown in ASMDM (**Fig. 2A**). By 72 h the entire wells were red, indicating microcolony growth throughout (not shown). Similar observations were made for both CF isolates: the acute infection isolates of Australian Epidemic Strain-1 (AES-1R) and a non-epidemic strain (34Bris) **(Fig. 2A)**. A CF strain was previously demonstrated to form tight microcolonies in ASM+ (Sriramulu *et al.*, 2005), and this phenotype has now been confirmed here in both the epidemic and non-epidemic CF strains. Growth of AES-1R in McCartney bottles resulted in the formation of a thick pellicle and deep anaerobic growth by 24 h (**Fig. 2B**). The deep anaerobic growth of *P. aeruginosa* was more pronounced in AES-1R than in UCBPP-PA14, suggesting that the CF strain is better adapted to anaerobic growth in ASMDM than the wound isolate UCBPP-PA14.

Forty seven genes were differentially expressed (B>0 or p<0.05) in stationary phase ASMDM compared to LB growth **(Table 2)**. Another 24 genes of known function were differentially expressed but had a B-statistic or p-value just below the cutoff. Many QS-associated and T3SS genes were downregulated in ASMDM, including the regulatory gene *rhlR*, the *lasA* alkaline protease and phenazine (e.g. pyocyanin *phzC2*, *phzD2*, *phzG2*) (Brint & Ohman, 1995; Gupta *et al.*, 2009). While we cannot exclude the possibility that the presence of sub-inhibitory concentrations of

antibiotics influenced expression, a down-regulation of QS-related genes and the T3SS is consistent with chronic infection in the CF lung (Shen *et al.*, 2008). In vivo, reduced expression of QS-regulated virulence determinants likely reduces inflammation, limiting the robustness of the immune response. The downregulation of the QS regulator *rhlR* in ASMDM supports the finding by Sririamulu et al that it is not required for tight microcolony and hence biofilm formation (Sriramulu *et al.*, 2005). Of the structural component genes (*algD*, *pilB*, *fliC*) mutated by Sriramulu et al to test effects on microcolony formation, none were significantly differentially expressed during stationary phase growth in ASMDM, possibly because they were no longer required once the biofilm had become established.

Conversely, the rhamnolipid regulator *rhlG* was upregulated in stationary phase growth in ASMDM, indicating that rhamnolipid production probably facilitates biofilm development and the acquisition of hydrophobic carbon sources (Davey *et al.*, 2003; Lequette & Greenberg, 2005). Also upregulated were the ferric enterobactin siderophore receptor and transport protein (*pfeA*, *fepC*) and the assimilatory nitrate reductase genes (*nasC*, *nirD*). The upregulation of the siderophore receptor probably reflects the iron-depleted conditions in ASMDM which in turn mimic those of CF sputum (Sriramulu *et al.*, 2005). *nirD* and *nasC* are in the same operon and form part of the assimilatory nitrate reduction pathway, involving the reduction of nitrate to ammonia. Nitrate utilization is vital for growth and survival in the microaerophilic and anaerobic environment of CF sputum (Schreiber *et al.*, 2007). We propose to study the utilization of nitrate by creating *nirS*-gfp and *nasC*-gfp mutants and investigating their growth characteristics in ASMDM.

3. Validation of differential expression data by qRT-PCR

Validation studies using quantitative SYBR-green RT-PCR showed that all 11 genes (including those with $B<0$ and $p>0.05$) were up or downregulated in the same manner as in microarray analysis, and the correlation plot (**Fig. 3**) yielded a correlation coefficient of $R^2 = 0.7629$.

CONCLUSIONS

This study represents the first assessment of global gene expression of a *P. aeruginosa* strain in an artificial sputum medium under both exponential and stationary phase conditions. Overall, the results show a switch from upregulation of nutrition-related genes, QS, and T3SS genes in early exponential phase to upregulation of iron transport, fimbrial biogenesis and alginate genes, with concomitant downregulation of virulence-related genes and QS regulators in stationary phase. Upregulated anaerobic gene expression is present in both early exponential and stationary phases. The differential gene expression patterns in exponential phase confirm conclusions drawn from other acute infection in vitro model systems and CF sputum (De Kievit *et al.*, 2001; Manos *et al.*, 2008; Manos *et al.*, 2009; Palmer *et al.*, 2005). Gene expression in stationary phase is consistent with findings in other in vitro models (De Kievit *et al.*, 2001; Sarkisova *et al.*, 2005; Wagner *et al.*, 2003), while phenotypic growth characteristics compare well with those found in sputum from patients with established infection (Bjarnsholt *et al.*, 2009). Therefore ASMDM provides a physiologically relevant picture of *P. aeruginosa* growth in CF sputum. However UCBPP-PA14 is a wound-derived isolate, with likely differences in its gene expression pattern compared to CF isolates. Furthermore, component concentrations in ASMDM may have to be adjusted to account for variations in patients' CF sputum based on their disease stage. Nonetheless, the results obtained herein are a valid starting point for further studies of the pathobiology of *P. aeruginosa* in the CF lung and for investigations of how individual components of ASMDM affect gene expression in both CF and non-CF isolates.

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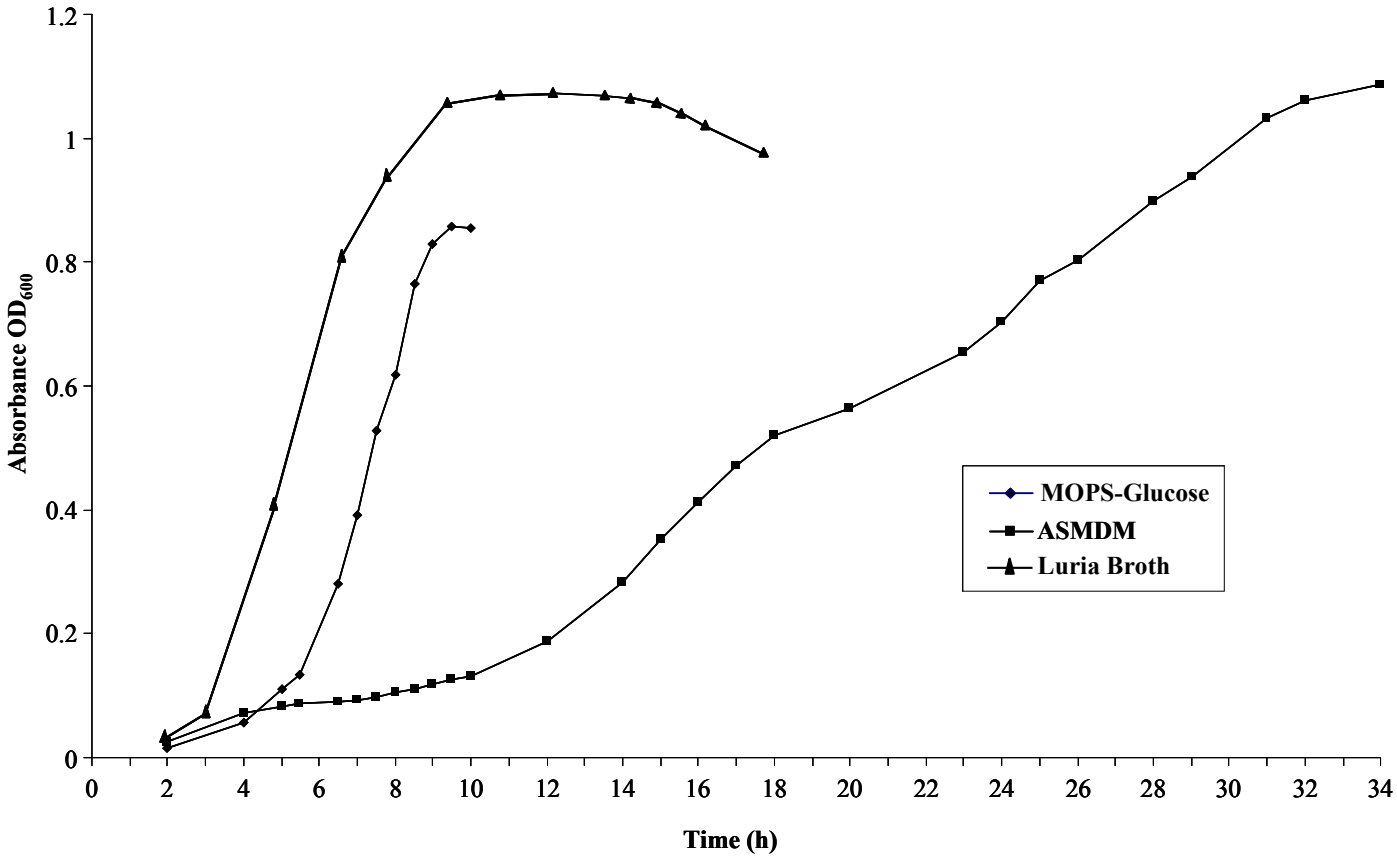
FIGURE LEGENDS

Fig. 1: Growth curves of *P. aeruginosa* UCBPP-PA14 in MOPS-Glucose, ASMDM and LB. Test tubes containing 2 ml of media were inoculated from a single colony and grown with shaking at 250 rpm for MOPS-Glucose and ASMDM, and 50 rpm for LB. Readings were taken periodically at OD_{600}.

Fig. 2: Growth of *P. aeruginosa* UCBPP-PA14 and two strains isolated from CF patients, in ASMDM. Cell growth was identified by the oxidation of 2,3,5 triphenyltetrazolium chloride (5% w/v) added to the medium. **Fig. 2A:** Growth of *P. aeruginosa* UCBPP-PA14, the Australian Epidemic Strain-1 isolate AES-1R and the non-epidemic isolate 34Bris in 24-well plates at 24 and 48 h post-inoculation, showing evidence of microcolony formation through the increasing density of the stained regions. The red color of the indicator oxidized by growing cells demarcates the boundaries of the expanding region of cell to cell attachment leading to microcolony formation. By 72 h the entire wells were colored red in all strains tested (not shown). **Fig. 2B:** Growth of *P. aeruginosa* UCBPP-PA14 and AES-1R in McCartney bottles: 24 h: A pellicle of varying thickness has developed. 48 h: Pellicle has thickened and deeper growth is evident in the form of finger-like projections (circled). 72 h: Projections coalesce to form an almost continuous growth in the upper two-thirds of the medium.

Fig. 3: Correlation plot of microarray and quantitative RT-PCR fold value data for 11 genes (*trpA, putA, dadX, oprB, exaB, exoT, aroQ2, phzD, pfeA, aprE and pchD*) used in the validation of the microarray results. The plot had a correlation coefficient $R^2 = 0.7629$.

Fig. 1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ASMDM
Luria Broth
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Fig. 2A

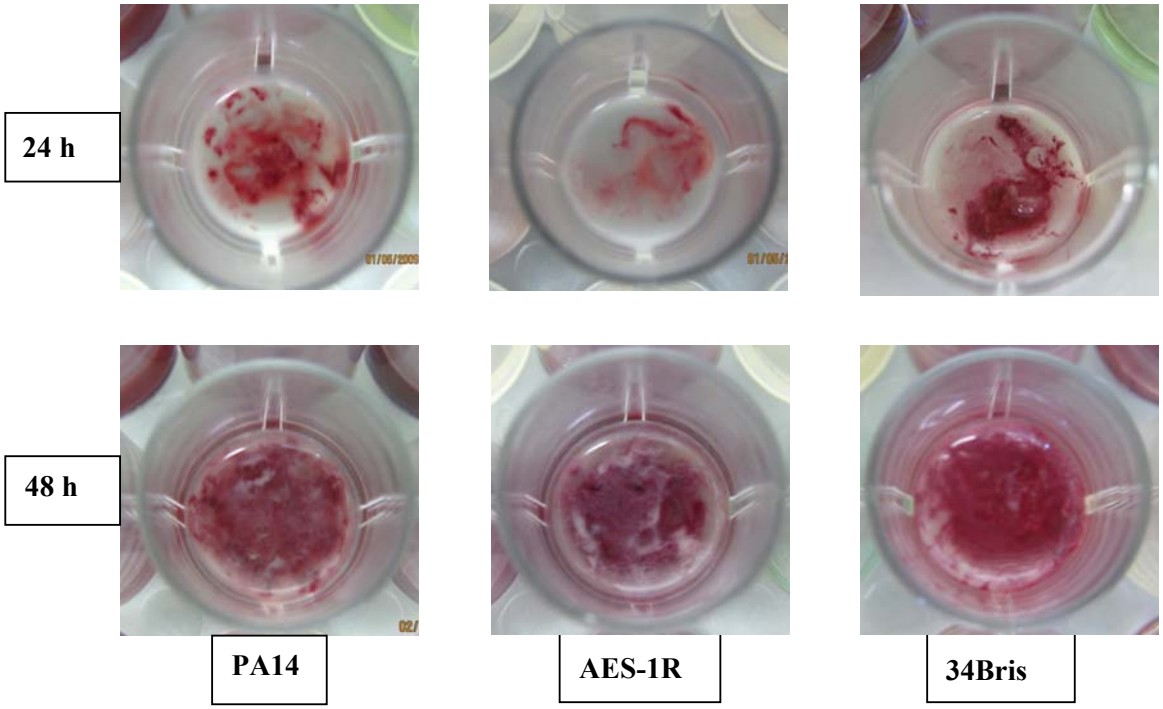


Fig. 2B

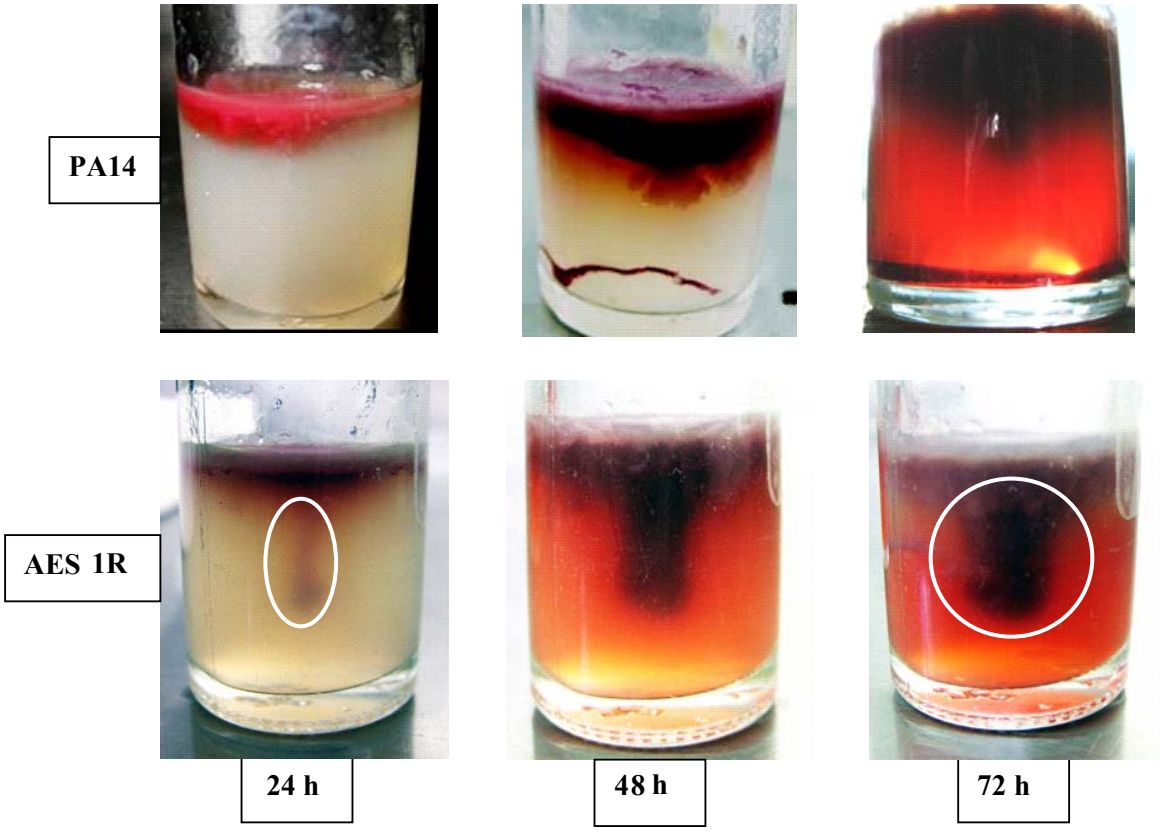
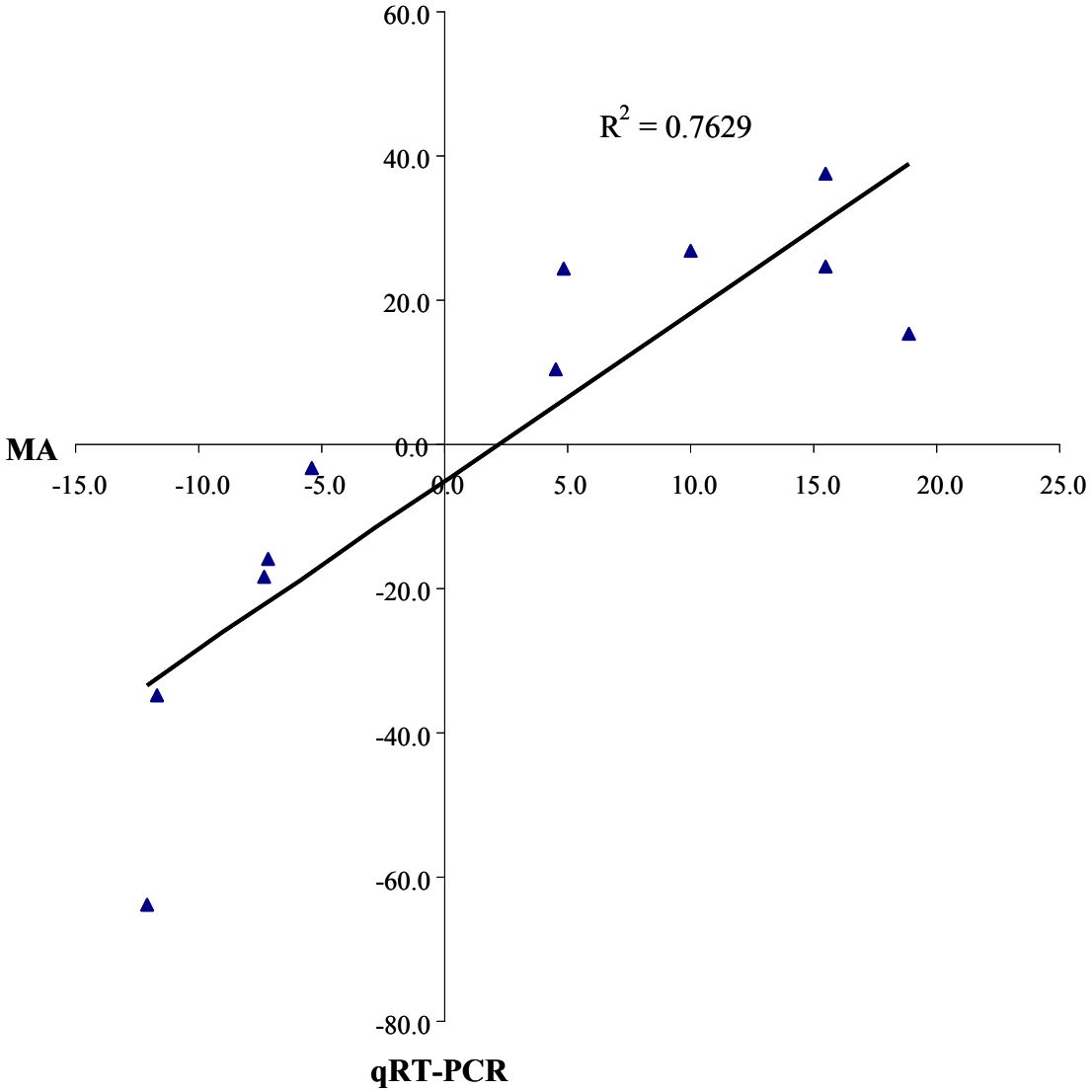


Fig. 3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MA
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5.0
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15.0
20.0
25.0
R2 = 0.7629
qRT-PCR

Table 1A: Genes differentially expressed during early log-phase growth (OD_{600} = 0.3±0.1) in both ASMDM and CF sputum-containing medium (≥2-fold).

Gene ID	Description	Fold Change	
		CF Sputum medium vs glucose †	ASMDM vs glucose #
Nutrition-controlled genes			
Amino Acid Biosynthesis			
PA0035 *trpA*	Tryptophan synthase alpha chain	-7	-7.3
PA0036 *trpB*	Tryptophan synthase beta chain	-9	-8.6
PA4695 *ilvH*	Acetolactate synthase isozyme III small subunit	-2.6	-2.5
Amino Acid Transport and Degradation			
PA0782 *putA*	Proline dehydrogenase PutA	4.3	10.0
*PA0865 *hpd*	4-Hydroxyphenylpyruvate dioxygenase	66	2.3
*PA0870 *phhC*	Aromatic amino acid aminotransferase	9.0	2.4
*PA0871 *phhB*	Pterin-4-α-carbinolamine dehydratase	5.0	2.6
*PA0872 *phhA*	Phenylalanine-4-hydroxylase	32	2.9
*PA0898 *aruD*	Succinylglutamate-5-semialdehyde dehydrogenase	2.7	2.4
PA2001 *atoB*	Acetyl coenzyme A acetyltransferase	16	10.8
PA2007 *maiA*	Maleylacetoacetate isomerase	8	3.0
PA2008 *fahA*	Fumarylacetoacetase	9	4.9
PA2009 *hmgA*	Homogenitisate 1,2-dioxygenase	11	8.9
*PA2249*bkdB*	Branched chain α-keto acid	13	2.5

	dehydrogenase		
*PA2250 *lpdV*	Lipoamide dehydrogenase-Val	19	3.0
PA4470 *fumC1*	Fumarate hydratase	6	-8.7
PA5302 *dadX*	Catabolic alanine racemase	9	4.5
	D-amino acid dehydrogenase, small		
PA5304 *dadA*	subunit	20	5.7
Glucose Transport and Metabolism			
PA2322	Gluconate permease	-5.5	-35.0
	Probable glyceraldehyde-3-phosphate		
PA2323	dehydrogenase	-3.8	-9.6
	2-Keto-3-deoxy-6-phosphogluconate		
PA3181	aldolase	-3	-12.3
	Carbohydrate outer membrane porin		
PA3186 *oprB*	OprB	-2.7	-11.7
	Glyceraldehyde-3-phosphate		
*PA3195 *gapA*	dehydrogenase	-3.2	-4.8
Other genes differentially expressed in CF sputum-containing medium and ASMDM			
PA0034	Probable two-component response regulator	-7	-7.3
PA0672	Heme oxygenase	6.0	-6.9
*PA0730	Probable transferase	-7	-5.3
PA1983 *exaB*	Cytochrome c550	-16	-5.4
PA1999	Probable coenzyme A tranferase, subunit A	28	48.2
PA2000	Probable coenzyme A transferase, subunit B	22	22.2
*PA2194 *hcnB*	Hydrogen cyanide synthase HcnB	5	8.0
PA2426 *pvdS*	Sigma factor PvdS	10	-68.1
*PA3790 *oprC*	Outer membrane porin protein C	14	2.5

	Probable permease of ABC taurine		
PA3936	transporter	-8	-5.1
PA3938	Probable periplasmic taurine-binding protein precursor	-5	-9.3
PA4131	Probable iron-sulfur protein	7	5.6
PA4221 *fptA*	Fe(III)-pyochelin outer membrane receptor precursor	44	-67.9
PA4224 *pchG*	Pyochelin biosynthetic protein PchG	96	-26.3
PA4225 *pchF*	Pyochelin synthetase	59	-19.9
PA4226 *pchE*	Dehydroaeruginoic acid synthetase	75	-29.8
PA4229 *pchC*	Pyochelin biosynthesis protein PchC	80	-44.3
PA4230 *pchB*	Salicylate biosynthesis protein PchB	139	-108.1
PA4231 *pchA*	Salicylate biosynthesis isochorismate synthase	121	-33.2
PA4514	Probable OM receptor for iron transport	-10	-22.5
PA5303	Conserved hypothetical protein	21	9

[†](Palmer *et al.*, 2005)

[#] Fold change of UCBPP-PA14 grown in ASMDM compared to growth in MOPS-glucose medium.

***** Fold change below cutoff, i.e. B<0 or p>0.05.

Table 1B: Genes of known function differentially expressed in ASMDM but not CF sputum-containing medium during early log-phase growth (OD_{600} = 0.3±0.1) (≥3-fold).

Gene ID	Description	Fold †
Upregulated		
PA0044 *exoT*	Exoenzyme T	15.5
PA0265 *gabD*	Succinate-semialdehyde dehydrogenase	5.0
PA0447 *gcdH*	Glutaryl-CoA dehydrogenase	11.3
PA0511 *nirJ*	Heme d1 biosynthesis protein NirJ	4.6
PA0514 *nirL*	Heme d1 biosynthesis protein NirL	4.8
PA0516 *nirF*	Heme d1 biosynthesis protein NirF	6.4
PA0517 *nirC*	Probable c-type cytochrome precursor	12.5
PA0518 *nirM*	Cytochrome c-551 precursor	14.7
PA0519 *nirS*	Nitrite reductase precursor	14.8
PA0783 *putP*	Sodium/proline symporter	3.3
PA0796 *prpB*	Carboxyphosphoenolpyruvate phosphonomutase	8.2
PA1477 *ccmC*	Heme exporter protein CcmC	3.2
PA1480 *ccmF*	Cytochrome C-type biogenesis protein CcmF	5.1
PA1546 *hemN*	Oxygen-independent coproporphyrinogen III oxidase	4.8
PA1693 *pscR*	Translocation protein in type III secretion	7.1
PA1694 *pscQ*	Translocation protein in type III secretion	5.3
PA1695 *pscP*	Translocation protein in type III secretion	9.4
PA1696 *pscO*	Translocation protein in type III secretion	11.3
PA1698 *popN*	Outer membrane protein PopN	4.8
PA1704 *pcrR*	Transcriptional regulator protein PcrR	3.2
PA1706 *pcrV*	Type III secretion protein PcrV	23.2

PA1707 *pcrH*	Regulatory protein PcrH	41.0
PA1708 *popB*	Translocator protein PopB	19.9
PA1709 *popD*	Translocator outer membrane protein PopD precursor	17.2
PA1710 *exsC*	ExsC, exoenzyme S synthesis protein C precursor	13.1
PA1712 *exsB*	Exoenzyme S synthesis protein B precursor	9.3
PA1713 *exsA*	T3SS transcriptional regulator ExsA	8.3
PA1715 *pscB*	Type III export apparatus protein	13.0
PA1716 *pscC*	Type III secretion outer membrane protein PscC precursor	8.9
PA1717 *pscD*	Type III export protein PscD	5.7
PA1718 *pscE*	Type III export protein PscE	12.9
PA1719 *pscF*	Type III export protein PscF	9.9
PA1720 *pscG*	Type III export protein PscG	7.3
PA1721 *pscH*	Type III export protein PscH	11.7
PA1722 *pscI*	Type III export protein PscI	8.1
PA1723 *pscJ*	Type III export protein PscJ	7.5
PA1724 *pscK*	Type III export protein PscK	3.8
PA1725 *pscL*	Type III export protein PscL	4.6
PA1871 *lasA*	LasA protease precursor	5.3
PA2003 *bdhA*	3-hydroxybutyrate dehydrogenase	4.5
PA2191 *exoY*	Adenylate cyclase ExoY	8.7
PA2193 *hcnA*	Hydrogen cyanide synthase HcnA	3.5
PA2279 *arsC*	ArsC protein	3.4
PA2300 *chiC*	Chitinase	3.8
PA2442 *gcvT2*	Glycine cleavage system protein T2	6.4
PA2444 *glyA2*	Serine hydroxymethyltransferase	36.9
PA2445 *gcvP2*	Glycine cleavage system protein P2	25.7

PA2446 *gcvH2*	Glycine cleavage system protein H2	43.6
PA2755 *eco*	Ecotin precursor	3.8
PA2830 *hptX*	Heat shock protein HptX	3.8
*PA3478 *rhlB*	Rhamnosyltransferase chain B	5.9
PA3479 *rhlA*	Rhamnosyltransferase chain A	5.4
PA3569 *mmsB*	3-hydroxyisobutyrate dehydrogenase	5.9
PA3570 *mmsA*	Methylmalonate-semialdehyde dehydrogenase	10.8
PA4210 *phzA1*	Phenazine biosynthesis protein A	9.1
PA4211 *phzB1*	Phenazine biosynthesis protein B	4.5
PA4587 *ccpR*	Cytochrome c551 peroxidase precursor	64.4
PA4865 *ureA*	Urease gamma subunit	4.6
PA5098 *hutH*	Histidine ammonia-lyase	5.0
PA5100 *hutU*	Urocanase	6.8
PA5170 *arcD*	Arginine/ornithine antiporter	4.1
PA5172 *arcB*	Ornithine carbamoyltransferase	5.1
PA5415 *glyA1*	Serine hydroxymethyltransferase	8.7
PA5427 *adhA*	Alcohol dehydrogenase	21.1
Downregulated		
PA0281 *cysW*	Sulfate transport protein CysW	-5.3
PA0282 *cysT*	Sulfate transport protein CysT	-3.8
PA1178 *oprH*	PhoP/Q and low Mg^{2+} inducible outer membrane protein H1 precursor	-9.7
PA1493 *cysP*	Sulfate-binding protein of ABC transporter	-3.0
PA2386 *pvdA*	L-ornithine N5-oxygenase	-21.4
PA2397 *pvdE*	Pyoverdine biosynthesis protein PvdE	-10.9
PA2398 *fpvA*	Ferripyoverdine receptor	-28.9

PA2507 *catA*	Catechol 1,2-dioxygenase	-4.4
PA2508 *catC*	Muconolactone delta-isomoerase	-3.8
PA2513 *antB*	Anthranilate dioxygenase small subunit	-3.7
PA2687 *pfeS*	Two-component sensor PfeS	-5.4
PA3192 *gltR*	Two-component response regulator GltR	-3.9
PA3193 *glk*	Glucokinase	-3.2
PA3603 *dgkA*	Diacylglycerol kinase	-3.0
PA3935 *tauD*	Taurine dioxygenase	-10.6
PA3937	Probable ATP-binding component of ABC taurine transporter	-6.9
PA4442 *cysN*	ATP sulfurylase GTP-binding subunit/APS kinase	-4.7
PA4468 *sodM*	Superoxide dismutase	-7.4
PA4687 *hitA*	Ferric iron-binding periplasmic protein HitA	-7.1
PA4688 *hitB*	Iron (III)-transport system permease HitB	-5.6
PA5531 *tonB*	TonB protein	-7.1

† Fold change of UCBPP-PA14 grown in ASMDM compared to growth in MOPS-glucose medium.

* Fold change below cutoff, i.e. $B<0$ or $p>0.05$.

Table 2: Genes differentially expressed in ASMDM after 72 h compared to stationary phase growth in LB (≥2-fold).

Gene ID	Description	Fold
Upregulated		
PA0013	Conserved hypothetical protein	5.3
PA0245 *aroQ2*	3-dehydroquinate dehydratase AroQ2	5.2
PA0491	Probable transcriptional regulator	3.2
PA0685	Probable type II secretion system protein	5.5
PA0824	Hypothetical protein	3.2
PA0886	Probable C4-dicarboxylate transporter	6.8
PA0987	Conserved hypothetical protein	3.7
*PA1251	Probable chemotaxis transducer	3.2
PA1286	Probable MFS transporter	3.9
*PA1635 *kdpC*	Potassium-transporting ATPase	3.2
PA1779 *nasC*	Assimilatory nitrate reductase	3.0
PA1780 *nirD*	Assimilatory nitrate reductase small subunit	2.6
PA1962	Conserved hypothetical protein	3.8
PA2688 *pfeA*	Ferric enterobactin receptor PfeA	3.8
PA2780	Hypothetical protein	7.6
*PA3387 *rhlG*	Beta-ketoacyl reductase	2.9
*PA3545 *algG*	alginate-c5-mannuronan-epimerase AlgG	2.7
*PA3547 *algL*	poly(beta-d-mannuronate) lyase precursor AlgL	3.8
PA4033	Hypothetical protein	12.3
PA4072	Probable amino acid permease	4.5
PA4084	Probable fimbrial biogenesis usher protein	5.3
PA4158 *fepC*	Ferric enterobactin transport protein FepC	3.0

PA4574	Conserved hypothetical protein	4.8
PA4629	Hypothetical protein	3.2
PA4823	Hypothetical protein	5.6
*PA4901 *mdlC*	benzoylformate decarboxylase	2.7
PA5469	Conserved hypothetical protein	5.4
Downregulated		
PA0399	Cystathionine beta-synthase	-4.5
PA0400	Probable cystathionine gamma-lyase	-3.4
PA0572	Hypothetical protein	-5.1
PA0587	Conserved hypothetical protein	-6.2
PA0620	Probable bacteriophage protein	-5.8
PA0622	Probable bacteriophage protein	-9.2
PA0625	Probable bacteriophage protein	-5.7
PA0631	Probable bacteriophage protein	-3.9
PA0633	Probable bacteriophage protein	-7.6
PA0634	Probable bacteriophage protein	-7.6
PA0635	Probable bacteriophage protein	-7.2
PA0744	Probable enoyl-CoA hydratase/isomerase	-3.7
PA0745	Probable enoyl-CoA hydratase/isomerase	-4.4
PA0746	Probable acyl-CoA dehydrogenase	-3.7
*PA0958 *oprD*	Outer membrane porin protein OprD	-3.2
*PA0996	Probable coenzymeA ligase	-2.6
PA0997 *pqsB*	Beta-keto-acyl-acyl-carrier protein synthase B	-5.3
PA0998 *pqsC*	Beta-keto-acyl-acyl-carrier protein synthase C	-5.4
*PA0999 *fabH1*	3-oxoacyl-[acyl-carrier-protein] synthase III	-4.4
*PA1246 *aprD*	Alkaline protease secretion protein AprD	-2.8

*PA1247 *aprE*	Alkaline protease secretion protein AprE	-3.2
*PA1250 *aprI*	Alkaline proteinase inhibitor AprI	-2.7
PA1431 *rsaL*	Regulatory protein RsaL	-6.1
*PA1587 *lpdG*	Lipoamide dehydrogenase G	-5.9
*PA1871 *lasA*	LasA protease precursor	-7.2
*PA1901 *phzC2*	Phenazine biosynthesis protein PhzC	-7.0
PA1902 *phzD*	Phenazine biosynthesis protein PhzD	-9.1
PA1903 *phzE*	Phenazine biosynthesis protein PhzE	-5.0
*PA1904 *phzD2*	Probable phenazine biosynthesis protein PhzD2	-6.0
PA1905 *phzG2*	Probable pyridoxamine 5'-phosphate oxidase	-5.4
PA1999	Probable CoA transferase, subunit A	-5.7
PA2000	Probable CoA transferase, subunit B	-4.1
*PA2007 *maiA*	Maleylacetoacetate isomerase	-5.7
*PA2195 *hcnC*	Hydrogen cyanide synthase	-3.7
*PA2247 *bkdA1*	2-oxoisovalerate dehydrogenase (alpha subunit)	-4.5
*PA2249 *bkdB*	Branched-chain alpha-keto acid dehydrogenase	-5.1
*PA2250 *lpdV*	Lipoamide dehydrogenase V	-6.7
PA2303	Hypothetical protein	-3.4
PA2553	Probable acyl-CoA thiolase	-4.7
PA3101 *xcpT*	General secretion pathway protein G	-3.5
*PA3103 *xcpR*	General secretion pathway protein E	-4.0
PA3190	Conserved hypothetical protein	-6.0
PA3477 *rhlR*	Transcriptional regulator RhlR	-4.4
PA3719	Hypothetical protein	-4.4
PA4208	Probable outer membrane efflux protein precursor	-5.8
*PA4236 *katA*	Catalase	-6.4

*PA5173 *arcC*	Carbamate kinase	-7.5

* Fold change below cutoff, i.e. B<0 or p>0.05.

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