Transcriptome analyses and biofilm-forming characteristics of a clonal *Pseudomonas aeruginosa* from the cystic fibrosis lung

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Transmissible *Pseudomonas aeruginosa* clones potentially pose a serious threat to cystic fibrosis (CF) patients. The AES-1 clone has been found to infect up to 40 % of patients in five CF centres in eastern Australia. Studies were carried out on clonal and non-clonal (NC) isolates from chronically infected CF patients, and the reference strain PAO1, to gain insight into the properties of AES-1. The transcriptomes of AES-1 and NC isolates, and of PAO1, grown planktonically and as a 72 h biofilm were compared using PAO1 microarrays. Microarray data were validated using real-time PCR. Overall, most differentially expressed genes were downregulated. AES-1 differentially expressed bacteriophage genes, novel motility genes, and virulence and quorum-sensing-related genes, compared with both PAO1 and NC. AES-1 but not NC biofilms significantly downregulated aerobic respiration genes compared with planktonic growth, suggesting enhanced anaerobic/microaerophilic growth by AES-1. Biofilm measurement showed that AES-1 formed significantly larger and thicker biofilms than NC or PAO1 isolates. This may be related to expression of the gene PA0729, encoding a biofilm-enhancing bacteriophage, identified by PCR in all AES-1 but few NC isolates ($n=42$). Links with the Liverpool epidemic strain included the presence of PA0729 and the absence of the bacteriophage gene cluster PA0632–PA0639. No common markers were found with the Manchester strain. No particular differentially expressed gene in AES-1 could definitively be ascribed a role in its infectivity, thus increasing the likelihood that AES-1 infectivity is multifactorial and possibly involves novel genes. This study extends our understanding of the transcriptomic and genetic differences between clonal and NC strains of *P. aeruginosa* from CF lung.

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INTRODUCTION

In cystic fibrosis (CF) patients, chronic *Pseudomonas aeruginosa* infection and inflammation are associated with biofilm formation, a progressive decline in lung function

and premature death (Al-Aloul *et al.*, 2004; Koch & Hoiby, 1993; Kosorok *et al.*, 2001). Whilst most CF patients acquire non-clonal (NC) *P. aeruginosa* from the environment, genotyping has revealed the dissemination of *P. aeruginosa* clones within and between CF clinics in Britain, Australia (Armstrong *et al.*, 2003; McCallum *et al.*, 2002; Panagea *et al.*, 2005; Scott & Pitt, 2004) and Brazil

Abbreviations: CF, cystic fibrosis; NC, non-clonal; QS, quorum sensing.

(Pellegrino *et al.*, 2006). Most CF clonal strains, apart from clone C (Romling *et al.*, 1997), have not yet been found in the environment of the patient's home, the CF clinic or in the general environment, suggesting person-to-person spread. One such clone, the Australian epidemic strain-1 (AES-1, also designated Melbourne epidemic strain, m16 or PI), currently infects up to 40 % of patients across five CF centres in mainland eastern Australia (Armstrong *et al.*, 2002, 2003; O'Carroll *et al.*, 2004).

The mechanisms of increased infectivity associated with clonal strains have not been defined and there are no clear indications to date of conserved processes across clonal strains. Potential processes may include acquisition or loss of genetic elements that confer adaptive or fitness advantages on cells (Brockhurst *et al.*, 2005; Finnan *et al.*, 2004; Klockgether *et al.*, 2004). In an earlier study of the clonal CF Manchester strain (MA) (Lewis *et al.*, 2005), its transmissibility (infectivity) was linked to the bacteriophage gene cluster designated Pf4 (Webb *et al.*, 2004), highly homologous to the Pf1 bacteriophage of *P. aeruginosa* located on a novel genomic island integrated adjacent to gene PA1014. Mutants of the wound isolate PAO1 (Stover *et al.*, 2000) infected with Pf4 have shown significantly increased maximum biofilm thickness and microcolony area, compared with wild-type, in 3-day-old biofilms (Webb *et al.*, 2004). Whilst gene acquisition may be an important factor in infectivity, the effect of expression differences occurring in shared genes should not be overlooked. Recently, Mathee *et al.* (2008) compared the genomes of a chronically infecting NC strain (strain PA2192) and the MA strain, and found that each carried a comparatively modest number of unique ORFs, representing approximately 9 and 1.3 % of all ORFs, respectively. They thus inferred that, with over 90 % of the genome being conserved, there is ample opportunity for expression of core genes, such as those held in common with PAO1, to play a role in the infectivity of the MA clonal strain.

Evidence that infectivity in some strains relates more to upregulation or downregulation of critical genes rather than to novel genes has come from the Liverpool epidemic strain (LES). LES exhibits increased expression of alkaline protease and elastase (Salunkhe *et al.*, 2005). Changes in motility genes or genes that promote biofilm formation may also increase infectivity (O'Toole & Kolter, 1998; Van Alst *et al.*, 2007). Variants of *P. aeruginosa* possessing enhanced biofilm-forming characteristics have frequently been detected both *in vitro* and in CF patients' sputum (Drenkard & Ausubel, 2002), where *P. aeruginosa* exhibits microaerophilic or possibly anaerobic growth (Alvarez-Ortega & Harwood, 2007; Platt *et al.*, 2008). As infectivity is highly likely to be multi-factorial, both novel genes and differentially expressed genes present in other *P. aeruginosa* strains may be involved.

As a first step in characterizing AES-1, we compared the transcriptomes and biofilm-forming characteristics of AES-1 and NC isolates from chronically infected CF patients. Isolates were grown in planktonic culture and as biofilms, and were compared with each other and with PAO1.

METHODS

Strains and isolates used in this study. Biofilm-forming studies and gene expression microarray analyses using PAO1 chips (Affymetrix) were conducted on eight *P. aeruginosa* CF isolates from the sputum of eight chronically infected patients attending the adult CF clinic at the Royal Prince Alfred Hospital, Sydney, Australia. Four of these isolates had previously been typed as AES-1 and four as NC by *SpeI* macrorestriction followed by PFGE using established methods (Anthony *et al.*, 2002). A clonal strain is defined here as having up to three band differences, following the criteria of Tenover *et al.* (1995). NC strains are defined as having more than three band differences. AES-1 isolates were selected from patients with continuous infection with a *P. aeruginosa* strain for a minimum of 4 years and known infection with the same AES-1 isolate for at least 1 year. Patients with NC strains were matched as closely as possible with the AES-1 group for time of infection with the same *P. aeruginosa* NC strain and for gender and age (three female AES-1 patients of 28.6 ± 3.4 years, with two female NC patients of 30.0 ± 4.0 years; one male AES-1 patient of 22 years, with two male NC patients of 20.5 ± 1.5 years). The mean time of continuous infection with *P. aeruginosa* was 7.5 years (range 6–11 years) for the AES-1-infected patients and 6.5 years (range 4–10 years) for the NC-infected patients. Both planktonic and biofilm-grown PAO1 (ATCC 15692) (Stover *et al.*, 2000) were also analysed by microarray.

Planktonic and biofilm-growth methods. Cells were inoculated from isolated colonies into 2 ml Luria–Bertani (LB) broth. LB broth has been widely used as a non-specialized growth medium for planktonic *P. aeruginosa* transcriptomics in both CF and non-CF studies (Alvarez-Ortega & Harwood, 2007; Salunkhe *et al.*, 2005; Schuster *et al.*, 2003; Waite *et al.*, 2005). In accordance with the strategies adopted for transcriptome expression in other studies (Hentzer *et al.*, 2005; Wagner *et al.*, 2003), cells for planktonic growth were harvested at the mid-exponential phase ($OD_{600} = 0.5 \pm 0.05$; Beckman DU640 Spectrophotometer), whilst biofilm-growth cells were harvested at 72 h.

For biofilm measurement, mid-exponential-phase cells were inoculated (1:100) into 800 ml LB broth in a Centers for Disease Control and Protection (USA) Bioreactor and coupons were removed for staining with Syto9 (Molecular Probes) and image capture at 24, 48 and 72 h post-inoculation using fluorescence microscopy. Thickness measurements were conducted on the same biofilm samples from images taken using confocal microscopy. Ten randomly selected biofilms per isolate were measured (ImageJ software, version 1.38) for area and thickness, and the mean surface area and mean thickness covered by each isolate were calculated. Mean results for the two biological replicates per isolate were calculated and comparative statistical analyses between groups were performed (SPSS version 14.0) using an independent-samples *t*-test ($P < 0.01$). All isolates were grown and measured in duplicate, and viable cell counts were performed to ensure that consistent live cell numbers were inoculated into the bioreactor. To extract sufficient RNA for transcriptome analysis, coupons were replaced with attached glass slides to increase biofilm coverage. The assembly was incubated (37°C in a water bath) and stirred (100 r.p.m.). At 72 h the biofilm was washed off using ice-cold $1 \times$ PBS. Cells were pelleted (3 min, 5000 g, 4°C), washed in $1 \times$ PBS and resuspended in RNeasy Protect (Qiagen).

Motility tests. Observation of straight-line motility was employed to ascertain the presence or absence of flagella in the AES-1 and NC isolates. Ten fields of view per isolate were examined by the hanging drop method, and phase-contrast microscopy for non-Brownian motion and $\pm$ scoring was used to classify isolates.

RNA extraction and purification. Cells were treated with RNeasy Protect (Qiagen) and total RNA was extracted using an RNeasy mini

purification kit (Qiagen). RNA concentration was determined by measuring the absorbance at 260 nm, with a minimum of 500 ng RNA μL^{-1} required to proceed to cDNA synthesis. RNA quality and the presence of residual DNA were checked by formaldehyde/agarose gel electrophoresis (Ausubel *et al.*, 2003).

cDNA synthesis, fragmentation and labelling. cDNA was synthesized, fragmented and labelled following the instructions of the Affymetrix GeneChip expression analysis technical manual. cDNA was purified using a MinElute PCR purification kit (Qiagen), fragmented with DNase I (Amersham Biosciences) and the fragments 3'-end-labelled using GeneChip DNA Labelling Reagent (Affymetrix). Fragmented DNA was quality checked (Bioanalyser 2100; Agilent) and hybridized to the *P. aeruginosa* genome array following the Affymetrix protocol. A 'test3' array (Affymetrix-100 housekeeping genes) was used to determine DNA suitability for the full array. Array conditions were as follows: hybridization was carried out at 50 °C for 16 h, at 60 r.p.m.; washing was carried out using an Affymetrix Fluidics Station 450; and scanning was carried out using an Affymetrix GeneChip Scanner 3000 at 532 nm for excitation and 570 nm for emission. CEL and CHP files were generated using the scanner program GCOS.

Replicates for microarray analysis. The four AES-1 isolates and four NC isolates were treated as biological replicates in a two-group differential expression analysis. Two technical replicates (same isolate, same culture, same RNA extraction, different microarrays) of one AES-1 biofilm (isolate 1) and two planktonic NC isolates (isolates 6 and 7) were also analysed by microarray to assess technical variability. A comparison of fluorescence values for all genes showed no significant difference between the technical replicates.

Three isolates were analysed by microarray as biological replicates to assess biological variability at the level of culture (same isolate, different culture, different RNA extraction and different microarray): one planktonic isolate (isolate 4) and one biofilm-grown AES-1 isolate (isolate 3) were analysed by microarray in duplicate, and one planktonic NC isolate (isolate 8) was analysed in triplicate. Substitution of different biological (culture) replicates had little or no effect on the prediction of differentially expressed genes. Pair-wise comparisons of the data from the two planktonic AES-1 replicates and the two biofilm AES-1 replicates gave R^2 values of 0.61 and 0.64, respectively, whilst the three NC replicates gave R^2 values of 0.55, 0.56 and 0.59, reflecting the inherent variability between isolates. The microarray data are available on the GEO (Gene Expression Omnibus) website at <http://www.ncbi.nlm.nih.gov/projects/geo> (GEO accession no. GSE6122).

Data analysis. Microarray data were analysed using BIOCONDUCTOR (Gentleman *et al.*, 2004). Data normalization used the robust multi-array average method (Gautier *et al.*, 2004; Irizarry *et al.*, 2003) incorporating probe level background correction, quantile normalization (Bolstad *et al.*, 2003) and linear extraction of a final expression measure for each gene per array. These expression measures were used to determine differential expression using the empirical Bayes method (Smyth, 2004). The false discovery rate method (Benjamini & Hochberg, 1995) was controlled to reduce false positives. A positive B-statistic was used as a guide for statistically significant differential expression (Smyth, 2003). Data were combined with the latest information from the *P. aeruginosa* sequence annotation project (PseudoCAP) at <http://www.pseudomonas.com>.

Microarray validation. Array data were validated by quantitative SYBR Green PCR using a Rotor-Gene 6000 real-time amplification system (Corbett Life Sciences) and performed on cDNA synthesized from microarray RNA. Eight genes (*exoT*, *pvdA*, *lasB*, *bdhA*, *aprA*, *prtB*, PA0986 and *P. aeruginosa* PAK strain *flaA*) were chosen based

on high differential expression, association with virulence and/or known function. Expression of the test genes was measured in biological duplicates, and ratios were calculated using the *recA* gene as endogenous control. Measurements were made under all conditions for all isolates. Oligonucleotide primers were designed using Primer Express (Applied Biosystems). Reverse transcription using 50 U SuperScriptII reverse transcriptase (Invitrogen) and 1 μg total RNA was carried out as recommended by the manufacturer (Invitrogen).

Follow-up studies: PCR detection of non-expressing genes

For PCR detection of selected genes showing no fluorescence in the arrays, a further 17 AES-1 and 17 NC isolates were chosen from our bank of 300 *P. aeruginosa* isolates to reflect all adult age groups and both genders. The aim was to differentiate genes that were absent or extensively mutated from those that were present in the genome but not expressed. Thirteen genes showing no expression by microarray analysis (fluorescence absent) in all AES-1 or all NC isolates were chosen for PCR analysis to determine gene absence or mutation (see Table 2). Genes were chosen based on their putative virulence or infectivity characteristics.

RESULTS AND DISCUSSION

This study used PAO1 arrays to identify a core set of genes that were differentially expressed in the AES-1 strain compared with NC strains and revealed differences in biofilm-forming characteristics between AES-1 and NC strains.

Biofilm formation

Biofilms from all four AES-1 isolates exhibited significantly more surface coverage than NC and PAO1 biofilms at 24 and 48 h ($P<0.01$) and at 72 h ($P<0.05$) post-inoculation (Fig. 1a). AES-1 biofilms were also significantly thicker than NC and PAO1 biofilms at 72 h ($P<0.05$) (Fig. 1a). Representative PAO1, AES-1 and NC biofilms at all time points are shown for surface area (Fig. 1b). No observable difference in shape was apparent between the four AES-1 isolates. A common feature in all four AES-1 isolates was filament formation (Fig. 1b), though its contribution to increased surface coverage is unknown. The propensity of AES-1 to form larger biofilms than either PAO1 or NC isolates may influence this clonal strain's infectivity. Recent *in vitro* biofilm studies of LES strain B58 also showed that this clonal isolate produced more biofilm than PAO1 (Kukavica-Ibrulj *et al.*, 2008).

Transcriptome analyses

Mean transcript expression levels were 89% for planktonically grown isolates and 85% for biofilm isolates. These values are comparable to other studies (Ochsner *et al.*, 2002; Wagner *et al.*, 2003). Initially, AES-1 and NC expression profiles from planktonic and biofilm-grown cultures were each compared with those for PAO1. Subsequently, comparisons were made between AES-1 and NC isolates. Table 1 shows the number of genes that were significantly differentially expressed between PAO1 and all AES-1 and NC isolates in planktonic and biofilm culture.

Fig. 1. (a) Comparison of mean biofilm areas for *P. aeruginosa* PAO1, AES-1 and NC isolates at 24 h (white bars), 48 h (grey bars) and 72 h (hatched bars). Each isolate was grown twice (biological replicate) in a Centers for Disease Control and Protection Bioreactor and individual rods removed at the specified times for Syto9 staining of cells and image acquisition. The areas of ten randomly selected biofilm images were measured for each replicate of each isolate ($n=20$). The mean biofilm areas for each isolate at each time point and the mean biofilm thickness at 72 h (black bars) are shown (mean $\pm$ SEM). AES-1 biofilm areas were significantly larger than those of NC isolates and PAO1 at 24 and 48 h ($P<0.01$). AES-1 biofilms were also significantly larger and thicker than NC isolates and PAO1 at 72 h ($P<0.05$). (b) Biofilm development of *P. aeruginosa* AES-1 (isolate 2), NC (isolate 7) and PAO1 (ATCC 15692) at 24, 48 and 72 h showing the difference in biofilm area in representative isolates. Biofilm images were selected from ten randomly chosen biofilms measured for size for each isolate.

Table 1. Differential gene expression in AES-1, NC and PAO1

Only genes showing expression in all isolates and with a B statistic >0 are shown, with the exception of the bacteriophage genes PA0729 and PA0632–PA0639 (identified with an asterisk).

Gene	Planktonic AES-1 vs PAO1	Biofilm AES-1 vs PAO1	Planktonic NC vs PAO1	Biofilm NC vs PAO1	Planktonic AES-1 vs NC	Description of protein
Upregulated genes						
PA0197				2.9		Transport of small molecules
PA0729	2.9*					Bacteriophage Pf4 protein
PA0979		3.9				Related to phage/transposon/plasmid
PA0986	4.7				7.2	Probable transposase
PA1414			4.4			Hypothetical protein
PA1673			3.4			Hypothetical protein
PA2103 (<i>moeB</i>)					3.4	Probable molybdopterin biosynthesis protein MoeB
Downregulated genes						
PA0044 (<i>exoT</i>)		−20.4		−17.9		Exoenzyme T
PA0610 (<i>prtN</i>)				−6.1		Transcriptional regulator PrtN
PA0612 (<i>prtB</i>)		−7.1			−2.3	DksA, DnaK suppressor protein
PA0632–PA0639	−3.4 to −11.2*					Bacteriophage N15 assembly proteins
PA1244			−9.6			Hypothetical protein
PA1245			−10.0			Hypothetical protein
PA1246 (<i>aprF</i>)			−5.4			Alkaline protease transport protein
PA1247 (<i>aprE</i>)			−4.1			Alkaline protease transport protein
PA1249 (<i>aprA</i>)			−4.7			Alkaline protease
PA1713 (<i>exsA</i>)		−14.4				Transcriptional regulator ExsA
PA1714		−15.1				Hypothetical protein
PA1939		−5.2				Hypothetical protein
PA1947 (<i>rbsA</i>)		−6.3		−5.2		Ribose transport protein RbsA
PA1959 (<i>bacA</i>)				−3.4		Bacitracin resistance protein
PA2003 (<i>bdhA</i>)	−5.1					3-Hydroxybutyrate dehydrogenase
PA2008 (<i>fahA</i>)	−4.6					Fumarylacetoacetase
PA2009 (<i>hmgA</i>)	−4.9					Homogentisate 1,2-dioxygenase
PA2386 (<i>pvdA</i>)	−6.9					L-Ornithine N5 oxygenase
PA2460			−3.2			Hypothetical protein
PA2482		−3.1		−2.9		Probable cytochrome c
PA2565	−19.9					Hypothetical protein
PA3371		−4.3				Hypothetical protein
PA3488			−6.6			Hypothetical protein
PA3528 (<i>rnt</i>)					−2.3	RNase T
PA3841 (<i>exoS</i>)		−20.1				Exoenzyme S
PA4443	−3.5					ATP sulfurylase small subunit
PA4918		−4.7		−6.3		Hypothetical protein
PA5167		−3.2				Probable c4-dicarboxylate-binding protein
PA5374 (<i>betI</i>)				−6.2		Transcriptional regulator BetI
Pae tRNA (<i>Met</i>)		−17.30		−11.3		tRNA Methionine, 5242026–5242102 (+) strand
Non-PAO1 genes expressed in AES-1						
Pae_M57501cdsg	+	+				Flagellin-encoding gene <i>flaA</i> strain PAK
Pae_AF035937cds6	+	+				WbpQ (<i>P. aeruginosa</i> IATS06)
Pae_AF035937cds8	+	+				WbpR (<i>P. aeruginosa</i> IATS06)
Pae_AF035937cds11	+	+				WbpU (<i>P. aeruginosa</i> IATS06)
Pae_AF035937cds12	+	+				WbpV (<i>P. aeruginosa</i> IATS06)
Pae_L81176cds3		+				Flagellar cap protein FliD strain PAK
Pae orfG	+	+				Putative acetyl transferase from glycosylation island genes in strain PAK

Table 1. cont.

Gene	Planktonic AES-1 vs PAO1	Biofilm AES-1 vs PAO1	Planktonic NC vs PAO1	Biofilm NC vs PAO1	Planktonic AES-1 vs NC	Description of protein
Pae orfI	+	+				Unknown ORF from glycosylation island genes in strain PAK
Pae orfK	+	+				Hypothetical protein (glycosylation island, strain PAK)
Pae orfM	+	+				Putative aldolase (glycosylation island, strain PAK)
Pae orfN	+					Putative glycosyl transferase (glycosylation island, strain PAK)

Table 2. Oligonucleotide primers used in PCR amplification of selected genes

Gene/strain	Primer (5'→3')	Product (bp)	AES-1	NC
PA0632-PA0633	CGGTACAACGTAGTCATGCTT GTAGCGACCAGCGTGTG	666	Absent	Present
PA0634-PA0635	CCTTCACCAATATGGAGAAC GGCAAGGTCCAGAGGAAC	844	Absent	Present
PA0636-PA0637	CTGTGCCACTGGTGCATCAC GGATAGTCCGGAAGCTCCAAAC	662	Absent	Present
PA0729	CGGTCGTCATTCGTTTTACTG CGGGCAGTAGCGGATCAATTG	323	Absent	Present
PA0729-PA0730	GTTACCCAGTCAGCCAGCAG CCTGGTCAACGAAACCATCG	775	Present	Absent
PA0724	GAAGTTTGCGAGCCTGATTC CGGTGGAGCAGTTCTCTTTC	926	Absent	Absent
PA2032	CGAAGTCCGGCTATTTCGT ACCCAGTCGATCATCAGGTC	970	Present	Present in three isolates
<i>flgK</i> (PA1086)	CTCTCGATAGGCCTGTCCG CGTTTTGCGTGGAGTCG	785	Present	Absent
<i>flgL</i> (PA1087)	CAGGCTTTCAACAACCTCGG GCTGCTGGCGATCTGTACC	492	Absent	Absent
<i>fliC</i> (PA1092)	CAACGGCTCCAACAGCGA CTGCGAAGCCTGGGTGC	822	Absent	Present in isolates 5 and 6
<i>flaA</i> (strain PAK): primer set 1	CAACGGCTCCAACAGCGA CCGGTGTCAGCGATGACAA	506	Absent*	Present in isolates 7 and 8
<i>flaA</i> (strain PAK): primer set 2	GCCAACGACGGTATCTCCC GCAGCAGGCTCAGAACCG	978	Present	Present in isolates 7 and 8
<i>fliD</i> (PA1094)	GACAACGTCAAGAAGTTCGTC CCTTGTCGAGCTTCTTCTTCTC	265	Absent	Present in isolates 5 and 6
<i>fpvA</i> (PA2398)	GTCACCCGCCAGAACATGGA CAGCTCGTGCTCGCGAC	909	Present	Present

*Not detected due to mutations in reverse primer-binding region. These were detected by sequencing the larger PCR product (978 bp) amplified using primer set 2.

Overall features

Overall, the proportion of genes showing differential expression during biofilm growth for both AES-1 and NC isolates was ~0.3 %. This is low compared with other studies (Waite *et al.*, 2005; Whiteley *et al.*, 2001), where it ranged between 1 and 20 %, and probably reflects the inherent variability of the NC isolates as well as differences

in methods used to generate biofilms and varying harvest time points. However, the general trend seems to be a decrease in the number of differentially expressed genes compared with planktonic growth, as the biofilm matures (Davies *et al.*, 1998; De Kievit *et al.*, 2001; Sauer *et al.*, 2002; Whiteley *et al.*, 2001). The most prominent gene groups identified in the various comparisons are discussed below.

Bacteriophage-related genes

The bacteriophage-related genes PA0729 and PA0986 were upregulated 2.9-fold and 4.7-fold, respectively, whilst the bacteriophage-related cluster PA0632–PA0639 was down-regulated 3.4-fold to 11.2-fold in all AES-1 isolates compared with NC (Table 1). The PA0729 gene was subsequently found to be absent by PCR in all NC isolates, whilst PA0632–PA0639 was absent in all AES-1 isolates. PA0729 is part of the integrated Pf4 prophage in PAO1, and the PA0729 gene product probably influences *P. aeruginosa* biofilm development (Hentzer *et al.*, 2004; Kim *et al.*, 2003; Webb *et al.*, 2004; Whiteley *et al.*, 2001). This may help explain the enhanced biofilm-forming capacity of AES-1 compared with NC strains, which produced significantly smaller biofilms in this study and do not have the PA0729 gene. PA0729 and the adjacent gene PA0730 together have 79 % nucleotide sequence identity with the 'prevent host death' (*phd*) toxin–antitoxin-encoding genes of *Pseudomonas syringae* (GenBank accession no. NP_790091.1) (Buell *et al.*, 2003; Webb *et al.*, 2004). Toxin–antitoxin genes, often located on mobile cassettes, may aid in phage retention through elimination of non-phage-containing cells (Lewis *et al.*, 2005). They are probably important in stress-resistant and persister phenotypes, where induction of cellular stress responses by the toxin–antitoxin locus renders cells more resistant to antimicrobials (Pandey & Gerdes, 2005). The gene cluster PA0632–PA0639 has high nucleotide identity to the tail proteins of bacteriophage N15 (Ravin *et al.*, 2000). N15 has been identified as having a functional stabilization system, which acts on the toxin–antitoxin principle described above (Dziewit *et al.*, 2007).

In order to further explore putative associations, we felt it was important to establish the prevalence of these genes in a large number of AES-1 and NC isolates. This may also establish them as potential markers of AES-1 infection. The prevalence of genes PA0729 and PA0632–PA0639 was analysed in 21 AES-1 and 21 NC isolates by PCR, which revealed that PA0729 was present in all 21 AES-1 isolates compared with only 6 NC isolates ($P < 0.001$), whilst PA0632–PA0639 was amplified in 13 NC isolates compared with 3 AES-1 isolates ($P < 0.001$). Whilst several PCR primer sets (Table 2) were used to conclude the absence/presence of genes, there is a small possibility that they all failed to amplify a product due to mutations in the target regions of the templates. Confirmation of the sequence was obtained by sequencing of the PCR products. These findings demonstrated a link between AES-1 and the LES clonal strains LES400 and LES431, which also lack the genes PA0632–PA0648 whilst retaining PA0729 (Salunkhe *et al.*, 2005). Another Pf4 gene, PA0724, was not expressed in any isolates (AES-1 or NC) and PCR failed to amplify it. This is interesting as it indicates that the presence or expression of other Pf4 genes is not essential to PA0729 expression.

Whilst PA0729 was expressed in PAO1, it is worth noting that the mean expression of PA0729 across the four planktonic AES-1 was threefold higher than in planktonic

PAO1. In mature (72 h) biofilms, the mean PA0729 expression in AES-1 was still approximately 1.5-fold higher than PAO1. Subsequent microarray analysis of two of the six NC isolates containing the PA0729 gene (from PCR analysis) showed no and very low expression, respectively. Thus, the higher expression of PA0729 in AES-1 compared with PAO1 and NC strains indicates that planktonic AES-1 may be primed for enhanced biofilm formation.

Another differentially expressed bacteriophage gene, PA0986, was upregulated compared with both PAO1 and NC isolates. PA0986 has 95 % nucleotide similarity to IS407, a gene-activating transposase of *Burkholderia cenocepacia*. Virulent, multi-resistant epidemic strains of *B. cenocepacia* are highly pathogenic to CF patients. Studies by Mack & Titball (1998) showed that two strains of *B. cenocepacia* that contained IS407 (C1576 and J2315) have been associated with epidemic outbreaks, whilst the avirulent E27 and E82 strains did not contain IS407, suggesting an association between this transposase and virulence in *B. cenocepacia*.

Motility genes

The AES-1 isolates were found to express the *flaA* flagellin-encoding gene originally identified in *P. aeruginosa* PAK (Pae M57501cdsg) (Arora *et al.*, 2001), rather than the PAO1 flagellin-encoding gene *fliC*. Confirmation was obtained by PCR (Table 2) and sequencing of flagellin genes from the eight AES-1 and NC isolates, which demonstrated that the AES-1 flagellin-encoding gene was approximately 95 % identical to *flaA* (PAK), with the remaining 5 % comprising two insertions totalling 21 bp and 40 single-base differences (including insertions/deletions and base switches). Sequencing also showed that the two NC isolates contained *fliA* whilst the other two contained *fliC*.

Expression studies and PCR confirmation also showed that AES-1 isolates had the PAK *fliD* (Pae L81176cds3), not the PAO1 variant, *fliD* (PA1094). The PAO1 *flgK* (PA1086) was also absent, but PAO1 *flgL* (PA1087) was found to be present by expression and PCR. This degree of genetic switching/variation within flagellar genes has been reported for other bacteria, and has been shown to affect flagellar shape and thus motility (Manos *et al.*, 2004). In qualitative tests of *P. aeruginosa* isolate motility, we found that planktonic AES-1 isolates displayed greater motility than NC isolates under the microscope (data not shown). With respect to *fliD*, a study by Scharfman *et al.* (2001) found that whether a strain expresses the PAO1 or the PAK FliD protein can affect its ability to bind to recognition sites on carbohydrate epitopes of human respiratory mucins, and concluded that FliD specificity is required for mucus colonization.

The two PAK genes *fliD* and *flaA* were also highly expressed in AES-1 biofilms. Studies indicate that motility may be significant in the early biofilm stage for some isolates (Head & Yu, 2004; Klausen *et al.*, 2003). Overall, these results suggested a different genetic response to biofilm formation between NC and AES-1 isolates that may provide clues to AES-1 infectivity.

Virulence and quorum-sensing (QS)-related genes
One interesting finding was the low number of differentially expressed virulence and QS-related genes in all of the CF isolates analysed by microarray compared with PAO1. A comparison of AES-1 results with those from LES400 and LES431 (Salunkhe *et al.*, 2005) showed a similar lack of differentially expressed virulence factors and QS-related genes. Exceptions included downregulation of the QS-regulated genes *fahA* (PA2008) and *hmgA* (PA2009), seen in both AES-1 and LES. The lack of differentially expressed virulence genes in both the AES-1 and LES isolates probably reflects the fact that they were isolated from chronically infected patients. It could also be that both the AES-1 and LES clonal strains have strain-specific virulence or QS-related genes that are not present on the PAO1 array.

Some virulence-related genes were downregulated in NC but not AES-1 isolates when compared with PAO1. These included *aprF* (PA1246) and *aprE* (PA1247) – genes encoding the ABC transport system that exports alkaline protease – and the protease itself, *aprA* (PA1249). Alkaline protease is a QS-regulated exoenzyme with optimal activity at alkaline pH and is an important *P. aeruginosa* virulence factor necessary for adaptation to the CF lung (Kim *et al.*, 2006). Its downregulation in NC may be an indicator of reduced virulence in these NC isolates, although the small number of isolates analysed by microarray does not allow a more detailed analysis. Two genes encoding hypothetical proteins adjacent to the alkaline protease cluster (PA1244

and PA1245) were also significantly downregulated, suggesting a putative link to the alkaline protease cluster. Other virulence genes such as *exoT* (PA0044) were downregulated in both AES-1 and NC isolates, and this downregulation is probably a consequence of chronic infection.

The virulence factor pyoverdine gene, *pvdA* (PA2386), was downregulated in AES-1. Pyoverdine aids growth in the iron-deprived environment of the CF lung (Lamont *et al.*, 2002) and its downregulation suggests that AES-1 may possess other iron-acquisition genes. Interestingly, the pyoverdine receptor gene, *fpvA* (PA2398), was not expressed in any AES-1 isolates. Deletion of *fpvA* in *P. aeruginosa* has been shown to reduce pyoverdine production (Shen *et al.*, 2002). PCR (Table 2) and sequencing of *fpvA* were conducted, and showed that all AES-1 and NC isolates had the intact *fpvA* gene.

Differential gene expression in the switch from planktonic to biofilm growth

Not surprisingly, there was an overall trend for both AES-1 and NC isolates to undergo downregulation of gene expression in the transition from planktonic to biofilm growth (Table 3), although interestingly there were only two genes, the amino acid transporter ammonium aspartate lyase-encoding *aspA* (PA5429) and PA2937 (encoding a hypothetical protein), that were differentially expressed in common by both groups. Few QS or QS-regulated genes were differentially expressed in either the

Table 3. Differential gene expression during the switch from planktonic to biofilm growth

Genes shown in bold were differentially expressed in both AES-1 and NC.

AES-1: planktonic vs biofilm			NC: planktonic vs biofilm		
Gene	Fold change	Description of protein	Gene	Fold change	Description of protein
Upregulated genes					
Pae M57501cdsg	15.5	<i>P. aeruginosa</i> PAK flagellin <i>flaA</i>	PA5203 (<i>gshA</i>)	1.1	Glutamate–cysteine ligase
Downregulated genes					
PA0105 (<i>coxB</i>)	−9.2	Cytochrome <i>c</i> oxidase, subunit II	PA0200	−6.5	Hypothetical protein
PA0106 (<i>coxA</i>)	−7.4	Cytochrome <i>c</i> oxidase, subunit I	PA1404	−5.0	Hypothetical protein
PA0108 (<i>coIII</i>)	−7.0	Cytochrome <i>c</i> oxidase, subunit III	PA1571	−4.2	Hypothetical protein
PA0852 (<i>cpbD</i>)	−9.3	Chitin binding protein CpbD	PA2146	−14.2	Hypothetical protein
PA1617	−4.2	Probable AMP-binding enzyme	PA2171	−6.5	Haemerythrin-binding site
PA2194 (<i>hcnB</i>)	−3.4	Hydrogen cyanide synthase HcnB	PA2433	−5.9	Hypothetical protein
PA2195 (<i>hcnC</i>)	−3.4	Hydrogen cyanide synthase HcnC	PA2746	−9.2	Hypothetical protein
PA2937	−4.9	Hypothetical protein	PA2937	−8.7	Hypothetical protein
PA3049 (<i>rmf</i>)	−12.9	Ribosome modulation factor	PA3123	−1.2	Hypothetical protein
PA3724 (<i>lasB</i>)	−12.6	Elastase	PA3309	−2.1	Hypothetical protein
PA3922	−9.7	Hypothetical protein	PA3529	−3.8	Probable peroxidase
PA3923	−8.8	Hypothetical protein	PA3622 (<i>rpoS</i>)	−3.4	Sigma factor RpoS
PA4607	−3.4	Hypothetical protein	PA3986	−3.6	Hypothetical protein
PA4733 (<i>acsB</i>)	−4.5	Acetyl-coenzyme A synthetase	PA4922 (<i>azu</i>)	−2.1	Azurin precursor
PA5429 (<i>aspA</i>)	−4.1	Aspartate ammonia lyase	PA5178	−2.8	Hypothetical protein
			PA5208	−1.5	Hypothetical protein
			PA5429 (<i>aspA</i>)	−6.1	Aspartate ammonia lyase

AES-1 or NC transition to biofilm. Other studies have also found this to be the case in PAO1, where biofilm transcriptomics and proteomics studies have shown a lower level of downregulation of QS and anaerobic respiration genes (Nouwens *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003).

Genes involved in aerobic respiration, including *coxA*, *coxB*, *collI*, *hcnB* and *hcnC*, were heavily downregulated (by a mean of about eightfold). These genes were not downregulated in the NC or PAO1 biofilms, suggesting that AES-1 isolates may achieve an anaerobic micro-environment more rapidly than NC isolates or PAO1 as they rapidly formed larger biofilms. Other highly down-regulated genes included those encoding the QS-regulated virulence factors elastase *lasB* (PA3724) and chitin-binding protein *cpbD* (PA0852), the ribosome modulation factor *rmf* (PA3049), PA3922 [homologous to the poly(3-hydroxyoctanoic acid) depolymerase of *Pseudomonas fluorescens* GK13; Schirmer & Jendrossek, 1994] and PA3923 (putative type V secretory pathway adhesin AidA of *P. aeruginosa* MA; Lewis *et al.*, 2005). Downregulation of virulence genes suggests that biofilms of chronically infecting AES-1 isolates may be better adapted to persistence. The flagellin-encoding gene (*flaA*, *P. aeruginosa* strain PAK) was the only upregulated gene in AES-1 biofilms (Table 3), indicating that AES-1 biofilm cells may retain the ability to disperse more easily.

In NC biofilms (Table 3), three downregulated genes encoded proteins of known function: *rpoS* (PA3622), *azu* (PA4922) and *aspA* (PA5429). Downregulation of *rpoS*, which encodes the sigma factor responsible for activating stress response mechanisms (Suh *et al.*, 1999), occurred only in NC biofilms. As seen in this study, NC biofilms are significantly smaller than those of AES-1. It is likely that activation of stress response genes is more critical in larger, more complex biofilms such as those formed by AES-1.

Mature AES-1 and NC biofilms show no gene expression differences

No genes were differentially expressed between AES-1 and NC isolates in our comparative analysis of gene expression in biofilms. This is not unexpected as it is likely that gene expression differences occur at the initial stage of biofilm formation, rather than at the mature biofilm stage, giving AES-1 biofilms an advantage of growing more rapidly. This was supported by the biofilm images (Fig. 1b), which showed a slower rate of growth in NC compared with AES-1 isolates at all three time points.

Microarray validation

The microarray expression ratios of the eight selected genes (see Methods) correlated with the mean quantitative RT-PCR ratios obtained (correlation coefficient $R^2=0.7914$).

Table 4. Comparison of microarray and quantitative RT-PCR fold values

Comparison (isolate no.)	Growth condition	Gene	Description of protein	Fold difference	
				Micro-array	Mean qRT-PCR
PAO1 vs AES-1 (1)	PL	<i>bdhA</i>	3-Hydroxybutyrate dehydrogenase	-5.1	-18.9
PAO1 vs AES-1 (3)	PL	<i>bdhA</i>	3-Hydroxybutyrate dehydrogenase	-13.9	-17.0
PAO1 vs AES-1 (1)	PL	<i>pvdA</i>	L-Ornithine N5 oxygenase	-4.8	-23.1
PAO1 vs AES-1 (3)	PL	<i>pvdA</i>	L-Ornithine N5 oxygenase	-14.8	-19.4
PAO1 vs AES-1 (1)	PL	PA0986	Probable transposase	8.4	11.3
PAO1 vs AES-1 (3)	PL	PA0986	Probable transposase	2.9	2.5
PAO1 vs AES-1 (1)	BIO	<i>pvdA</i>	L-Ornithine N5 oxygenase	5.1	14.5
PAO1 vs AES-1 (3)	BIO	<i>pvdA</i>	L-Ornithine N5 oxygenase	1.7	3.1
PAO1 vs AES-1 (1)	BIO	<i>prtB</i>	DksA, DnaK suppressor protein	-5.2	-2.3
PAO1 vs AES-1 (3)	BIO	<i>prtB</i>	DksA, DnaK suppressor protein	-11.2	-17.6
PAO1 vs AES-1(1)	PL vs BIO	<i>exoT</i>	Exoenzyme T	-8.0	-10.8
AES-1 vs AES-1 (1)	PL vs BIO	<i>pvdA</i>	L-Ornithine N5 oxygenase	-5.9	-7.2
AES-1 vs AES-1 (1)	PL vs BIO	<i>bdhA</i>	3-Hydroxybutyrate dehydrogenase	-3.7	-3.0
AES-1 vs AES-1 (1)	PL vs BIO	<i>lasB</i>	Elastase	-10.6	-12.8
AES-1 vs AES-1(3)	PL vs BIO	<i>lasB</i>	Elastase	-44.7	-39.4
AES-1 vs AES-1 (1)	PL vs BIO	<i>flaA</i>	Flagellin gene <i>P. aeruginosa</i> PAK	6.5	12.3
AES-1 vs AES-1 (3)	PL vs BIO	<i>flaA</i>	Flagellin gene <i>P. aeruginosa</i> PAK	8.8	14.1
PAO1 vs NC (5)	PL	<i>aprA</i>	Alkaline metalloproteinase precursor	-6.9	-8.2
PAO1 vs NC (7)	PL	<i>aprA</i>	Alkaline metalloproteinase precursor	-4.9	-7.0
PAO1 vs NC (7)	BIO	<i>exoT</i>	Exoenzyme T	-18.3	-21.4
AES-1 (3) vs NC (5)	PL	<i>prtB</i>	DksA, DnaK suppressor protein	-4.9	-7.7
AES-1 (3) vs NC (7)	PL	<i>prtB</i>	DksA, DnaK suppressor protein	-5.4	-7.1

All genes evaluated showed either upregulation or down-regulation consistent with the microarray results (Table 4). Large variations in fold difference between microarray and RT-PCR were observed in two genes only, *pvdA* and *bdhA*, in planktonic AES-1 versus PAO1.

Genetic comparison of MA and AES-1

We attempted to amplify putative transmissibility markers identified in the MA strain (Lewis *et al.*, 2005) with the aim of identifying common markers of transmissibility between AES-1 and MA. PCR of two of the five gene island markers identified in MA (MA18 and MA21; Lewis *et al.*, 2005) failed to amplify either of the expected products in any AES-1 and NC isolates. Additionally, no MA gene island was found in AES-1 using primers modelled on sequences adjacent to its insertion point in MA (PA1013 and PA1014; data not shown). PCR of two genes highly conserved in MA (PA0724 and PA2032; Table 2) showed that PA0724 was absent in all AES-1 and NC isolates, whilst PA2032 was absent in one AES-1 but present in three of four NC isolates. The results suggested that these genes are not suitable as transmissibility markers in AES-1 as significant differences exist between the two clonal strains within these gene regions.

Conclusions

In summary, this study extends our understanding of the properties of both clonal and NC *P. aeruginosa* in the chronically infected CF lung. The clonal strain AES-1 shares a number of genetic characteristics with the LES and MA strains, as well as differences, and has a differential-expression profile distinct from that of NC isolates. AES-1 also expressed the Pf4 bacteriophage gene PA0729, which may play a role in its enhanced biofilm formation compared with NC isolates. No particular set of differentially expressed genes in AES-1 could definitively be ascribed a role in its infectivity, thus increasing the likelihood that infectivity is multi-factorial. However the data from biofilm growth as well as gene expression suggest a need for follow-up studies to confirm the role of candidate motility and respiration genes in enhanced biofilm formation and increased infectivity. Our findings also need validation in clonal isolates from newly acquired infections where transmissibility determinants are intact. The study was also limited by the fact that we were unable to investigate gene expression of novel AES-1-specific genes. As *P. aeruginosa* is capable of considerable genetic exchange, it is highly likely that there are AES-1 genes not present in the PAO1 genome that contribute to AES-1 transmissibility.

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