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Biofilms and type III secretion are not mutually exclusive in *Pseudomonas aeruginosa*

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Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that causes acute and chronic infections in immunocompromised individuals. It is also a model organism for bacterial biofilm formation. Acute infections are often associated with planktonic or free-floating cells, high virulence and fast growth. Conversely, chronic infections are often associated with the biofilm mode of growth, low virulence and slow growth that resembles that of planktonic cells in stationary phase. Biofilm formation and type III secretion have been shown to be reciprocally regulated, and it has been suggested that factors related to acute infection may be incompatible with biofilm formation. In a previous proteomic study of the interrelationships between planktonic cells, colonies and continuously grown biofilms, we showed that biofilms under the growth conditions applied are more similar to planktonic cells in exponential phase than to those in stationary phase. In the current study, we investigated how these conditions influence the production of virulence factors using a transcriptomic approach. Our results show that biofilms express the type III secretion system, whereas planktonic cells do not. This was confirmed by the detection of PcrV in the cellular and secreted fractions of biofilms, but not in those of planktonic cells. We also detected the type III effector proteins ExoS and ExoT in the biofilm effluent, but not in the supernatants of planktonic cells. Biofilm formation and type III secretion are therefore not mutually exclusive in *P. aeruginosa*, and biofilms could play a more active role in virulence than previously thought.

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INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen and a common cause of hospital-acquired infections in the Western world (Lyczak *et al.*, 2000). Its metabolism is preferentially aerobic and oxidative, but it is also well adapted to microaerophilic or anaerobic conditions, where it can use nitrogen as a terminal electron acceptor in a process termed denitrification (Williams *et al.*, 2007; Zumft, 2004). *P. aeruginosa* can cause chronic as well as acute infections, and these are notoriously difficult to clear (Lyczak *et al.*, 2000). This is partly due to the high innate resistance of the bacterium to clinically used antibiotics, and partly due to the formation of highly antibiotic-resistant biofilms (Drenkard, 2003; Hancock & Speert, 2000).

Perhaps the best known role of *P. aeruginosa* is in cystic fibrosis (CF) pathology, where it causes chronic pulmonary infections that can persist for decades, leading to irreversible tissue damage, decreased lung function and, eventually, respiratory failure (Collins, 1992; Lyczak *et al.*, 2002; Rowntree & Harris, 2003). In the CF lung, *P. aeruginosa* can grow to cell densities as high as 10^8–10^{10} cells ml^{-1} (Parsek & Singh, 2003), and the predominating mode of growth, which plays a significant role in persistence, is believed to be biofilms (Lam *et al.*, 1980; Singh *et al.*, 2002; Yoon *et al.*, 2002). CF sputum has been shown to support aerobic as well as anaerobic growth (Palmer *et al.*, 2005, 2007), and growth in CF airways has been reported to be microaerobic or anaerobic, probably using a combination of electron acceptors (Alvarez-Ortega & Harwood, 2007; Worlitzsch *et al.*, 2002). Furthermore,

Abbreviations: CF, cystic fibrosis; PCA, principal components analysis; PCC, Pearson's correlation coefficient; QS, quorum sensing; TTSS, type III secretion system.

The GEO accession number for the microarray dataset associated with this paper is GSE12207.

Two supplementary tables are available with the online version of this paper, showing the effect of surface-associated growth on gene expression, and MASCOT data from LC-MS/MS analysis of secreted proteins from planktonic cells in stationary phase and 3-day-old biofilms.

anaerobiosis has been reported to promote biofilm formation in *P. aeruginosa* (Yoon *et al.*, 2002).

P. aeruginosa produces a large array of toxins and virulence factors that cause immune evasion, tissue damage and haemorrhage (Van Delden, 2004), and many of these are controlled by quorum sensing (QS) (Rumbaugh *et al.*, 2000). *P. aeruginosa* virulence is multifactorial and combinatorial, and it varies substantially depending on bacterial physiology as well as on the strain involved (Lee *et al.*, 2006). The virulence factors can be chemical or proteinaceous, and either cell-associated or secreted. Proteinaceous virulence factors are often secreted through one of the five protein secretion systems so far described in *P. aeruginosa*: type I, II, III, V (Ma *et al.*, 2003) and the recently discovered type VI (Filloux *et al.*, 2008; Mougous *et al.*, 2006). Especially the type III secretion system (TTSS), which injects effector proteins directly into the eukaryotic host cell cytoplasm, has been associated with high virulence. Infection with a type III secreting isolate has been shown to correlate with severe disease (Hauser *et al.*, 2002), and type III secretion (TTS) in lower respiratory and systemic infections is associated with an increased mortality rate (Roy-Burman *et al.*, 2001).

In recent years, a model has emerged regarding bacterial growth mode and virulence during acute and chronic infection. Acute infection is believed to involve fast-growing planktonic or free-swimming cells that are highly virulent, whereas chronic infection is believed to involve biofilms consisting of slower growing and less virulent cells (Costerton *et al.*, 1999; Kuchma *et al.*, 2005; Yahr & Greenberg, 2004). In support of this view, the physiology of biofilm cells has been shown to resemble that of planktonic cells in stationary phase, and this has been suggested to be a major factor in the resistance of biofilms to antibiotics (Hentzer *et al.*, 2005; Spoering & Lewis, 2001; Waite *et al.*, 2005). Furthermore, less virulent strains appear to become more abundant over time in chronically infected CF patients, which in many cases is due to mutations of the primary QS regulator *lasR* (Jelsbak *et al.*, 2007; Lee *et al.*, 2005; Smith *et al.*, 2006). Two sensor systems have also been described that reciprocally regulate the expression of the TTSS and the production of exopolysaccharides that lead to biofilm formation (Goodman *et al.*, 2004; Ventre *et al.*, 2006). In line with this, mutation of the TTSS has been shown to enhance biofilm formation, leading to the suggestion that TTS may be detrimental to biofilm formation, and that factors required for acute infection may be incompatible with those required for chronic infection (Kuchma *et al.*, 2005).

However, the exact role of growth mode and virulence during infection is still unclear in many cases. Contrary to the model of slow-growing biofilms in chronic infection, Yang *et al.* (2008) recently showed that *P. aeruginosa* strains in long-term-infected CF patients grow at their maximum capacity in the CF lungs. These authors further showed that only the mucoid strains grow in clusters, whereas the non-mucoid strains are free-floating or planktonic Another report has shown that *P. aeruginosa* biofilms can form rapidly during acute infection in thermally injured mice (Schaber *et al.*, 2007), suggesting that the two different lifestyles are not mutually exclusive in all conditions.

Characterization of *in vivo* physiology, as well as the relationships between growth mode and virulence, is crucial for the understanding of and intervention in bacterial infections. There can be little doubt that biofilm formation and the expression of virulence determinants are highly influenced by nutrients and environment. Examples of this are variations in biofilm structure depending on carbon source (Klausen *et al.*, 2003), modulation of exotoxin A synthesis by nutrients (Somerville *et al.*, 1999) or induction of the TTSS by low calcium levels (Dasgupta *et al.*, 2006). In a previous proteomic study we showed that, under our growth conditions, biofilms in a continuous-flow system are more similar to planktonic cells in exponential phase than to those in stationary phase (Mikkelsen *et al.*, 2007). In order to investigate how this influenced the production of virulence factors, that study was complemented with a transcriptomic study. Our results show that biofilms in these conditions express the TTSS, whereas planktonic cells do not, indicating that biofilm formation and TTS are not mutually exclusive in *P. aeruginosa*.

METHODS

Strains and growth conditions. *Pseudomonas aeruginosa* PAO1 was grown in AGSY medium (56 mM alanine, 17 mM K_2HPO_4, 86 mM NaCl, 100 μM $CaCl_2$, 10 mM $MgSO_4$, 5 μM $FeCl_2$, 7.5 μM $ZnCl_2$, 0.5 %, v/v, glycerol, 3 g yeast extract l^{-1}, pH 7) at 37 °C as previously described (Mikkelsen *et al.*, 2007). Briefly, planktonic cells were grown with vigorous shaking for 3 h (late exponential phase) or 9 h (early stationary phase), colonies were grown on 1.5 % (w/v) agar for 15 or 40 h, and biofilms were grown for 3 days in silicone tubes, 6 mm i.d. (Hentzer *et al.*, 2005), with a continuous flow of 50 ml h^{-1}. Cells were harvested as previously described (Mikkelsen *et al.*, 2007).

RNA extraction. All cells were harvested directly into RNAlater (Ambion), kept at 5 °C overnight and stored at −80 °C until use. The culture/RNAlater mixture was thawed on ice, and a suitable amount of cells was sedimented by centrifugation. Cells were resuspended in water containing 1 mg lysozyme ml^{-1} (Sigma) and incubated for 13 min at room temperature. RNA was extracted using an RNeasy Mini Purification kit (Qiagen) following the supplier's instructions. DNA was removed using an on-column DNase I treatment (Qiagen). If this treatment was insufficient, the DNase I treatment was repeated in solution, and the RNA was repurified using the RNA cleanup protocol (Qiagen). For planktonic cells in stationary phase, RNA was concentrated by precipitation with sodium acetate (3 M, 1/10 volume) and 100 % ethanol (2.5 volume). Samples were kept at −80 °C overnight, and RNA was sedimented by centrifugation in a microfuge (5 min, 4 °C, maximum speed). Pellets were then dried, and RNA was redissolved in RNase-free water.

Microarrays and data analysis. The microarray dataset is publicly available from the Gene Expression Omnibus (ncbi.nlm.nih.gov/geo), accession number GSE12207. We analysed three biological replicates

for each of the five conditions. cDNA synthesis, fragmentation, labelling and microarray analysis were performed as previously described by Hentzer *et al.* (2005) using GeneChip *P. aeruginosa* Genome Arrays (Affymetrix). Arrays were scanned using a GeneChip Scanner 3000 (Affymetrix), and total target signal was scaled to 500 for each array. Following this, the raw data were pre-processed using Affymetrix GCOS 1.4. The resulting output data were analysed using R 2.5.0 (http://www.r-project.org) and the BioConductor packages affyPLM, GCRMA and LIMMA (http://www.bioconductor.org). Normalization was carried out using quantile normalization, no background correction, perfect match probes only and median polish summary. Significantly modulated genes were identified using the empirical Bayes (eBayes) statistic (Smyth, 2004). The standard *t*-test assumes that genes are independent. The eBayes statistic seeks to compensate for this by shrinking the estimated sample variance towards a common value and computes a moderated *t*-statistic with an adjusted *P*-value and a *B*-value that shows the odds of a gene being differentially expressed. Genes were deemed significantly modulated between two conditions if they passed the following criteria: adjusted *P*-value $\leqslant 0.01$, fold change $\geqslant 2$ and $B \geqslant \ln 99$ (i.e. the odds of the gene being differentially expressed are at least 99 %). A principal components analysis was performed on the normalized data using Simca-P + 11 (Umetrics). The analysis was performed using parito scaling and was unsupervised (i.e. performed without using information on the nature of the samples for the calculations). Hierarchical cluster analysis was performed in Java TreeView 1.1.1 (http://sourceforge.net/projects/jTreeView) using Pearson's correlation coefficient and complete linkage.

Protein extraction and Western blot analysis. Intracellular protein was extracted as previously described (Mikkelsen *et al.*, 2007). The secretome of planktonic cells and biofilms was prepared essentially as described by Corbett *et al.* (2005). Planktonic cells were grown with shaking for 9 h, cultures were harvested and cells were sedimented by centrifugation (8300 ***g***, 30 min, 4 °C). Biofilm effluent was collected on ice on day 3 after inoculation, and planktonic cells were removed by centrifugation as before. Supernatants were passed through a 0.2 μm filter, and protein was precipitated by adding solid trichloroacetic acid to a final concentration of 12.5 % (w/v). Protein was left to precipitate at 4 °C overnight and was sedimented by centrifugation (8300 ***g***, 30 min, 4 °C); pellets were washed three times in 1 ml 80 % (v/v) acetone and redissolved in ASB14 buffer (Mikkelsen *et al.*, 2007). pH was adjusted to 8.5 with NaOH, and protein was quantified using a detergent-compatible protein assay kit (Bio-Rad), following the supplier's protocol. Equal amounts of protein were separated on a 12 % (v/v) SDS-polyacrylamide gel as indicated for the individual experiments and detected by Western blotting. Primary antibodies were a gift from Arne Rietsch, Case Western Reserve University. Secondary antibodies were ECL peroxidase-labelled anti-rabbit antibodies (Amersham), and detection was achieved using Immobilon Western Chemiluminescent HRP Substrate (Millipore).

LC-MS/MS. Secretome samples were prepared as described above, and protein pellets were redissolved in 0.1 % (w/v) PPS Silent Surfactant (Protein Discovery) in ammonium bicarbonate (50 mM, pH 8). Protein (100 μg) was digested twice with 2.5 μg modified porcine trypsin (Promega) at 37 °C overnight. LC-MS/MS analysis was conducted using a nanoAcquity UPLC coupled to a QToF Premier (Waters). The amount injected of each sample was determined by optimizing sensitivity against saturation/overloading to maximize the protein identification and best characterize the sample. Tryptic digests were injected and trapped to a pre-column (50 mm × 75 μm i.d., 5 μm particle size) and washed for 5 min with buffer A (0.1 %, v/v, formic acid) at 5 μl min^{-1}. Peptides were eluted and separated across an analytical column (100 mm × 75 μm i.d., 1.7 μm particle size) by applying a linear gradient of 0–40 % buffer B (acetonitrile, 0.1 % formic acid) over 70 min (300 nl min^{-1}). To prevent carry-over between injections, the system was washed with 90 % buffer B for 10 min prior to a 10 min equilibration with 100 % buffer A. Each sample was injected in duplicate to achieve maximal proteome coverage. Each data-dependent acquisition allowed the selection of five precursor ions from each MS spectrum for subsequent tandem MS; each spectrum was constructed from at least 30 000 counts (TIC). Post-acquisition, precursor ions were mass corrected with an external lock mass (Glufibrogen peptide), the spectra were smoothed (Savitzky–Golay, four channels, two iterations) and centroided (80 % top), and used to generate .pkl files (ProteinLynx Global Sever), which were then searched against the *Pseudomonas* protein database (http://www.pseudomonas.com; Mar 2006, 5571 sequences) using Mascot (Matrix Science, version 2.2.0). Key search parameter values were: 20 p.p.m. precursor ion mass tolerance, 2 ^{13}C, 1 missed cleavage. Peptide sequences which matched spectra with an expect value <0.01 were used to support protein identifications, and proteins were deemed to be present in a sample if at least two different peptides could be detected in all three biological replicates.

RESULTS

In the current study, analyses were performed on cells grown in five different conditions. These will hereafter be referred to as PE (planktonic cells in exponential phase), PS (planktonic cells in stationary phase), C15h (colonies grown for 15 h), C40h (colonies grown for 40 h) and B3d (biofilms grown in a continuous flow system for 3 days).

Growth phase makes more difference than growth mode

Microarray data were processed and normalized as described in Methods; the number of significantly modulated genes is shown in Table 1. In accordance with our previous proteomic study (Mikkelsen *et al.*, 2007), the largest number of significant differences was found to be between PE and PS, confirming that growth phase makes a bigger difference than growth mode under these conditions. Furthermore, there were fewer differences between biofilms and exponentially growing planktonic cells than between biofilms and stationary-phase planktonic cells, confirming that biofilm cells in this system do not, on average, resemble planktonic cells in stationary phase. The apparent discrepancy between this study and that of Hentzer *et al.* (2005), who found biofilms to be more similar to planktonic cells in stationary phase, can probably be explained by the use of an undiluted rich medium for biofilm growth in our study as opposed to a more minimal medium in the study of Hentzer *et al.* (2005).

Colonies are something in between planktonic cells and biofilms

In order to get an overview of the largest source of variation in the multi-dimensional microarray dataset, a principal components analysis (PCA) was performed. This yielded a model consisting of four principal components (PCs) that explained 91 % of the total variation ($R^2=0.91$).

Table 1. Number of genes significantly modulated between the five conditions tested

Numbers in parentheses indicate (upregulated, downregulated) with respect to condition 1 compared to condition 2. For example, 607 genes were modulated between PE and PS, and 392 of these were more highly expressed in PE.

Condition 1	Condition 2			
	B3d	C40h	C15h	PS
PE	253 (107, 146)	272 (121, 151)	140 (20, 120)	607 (392, 215)
PS	391 (156, 235)	276 (113, 163)	521 (153, 368)	
C15h	158 (90, 68)	115 (72, 43)		
C40h	135 (74, 61)			

The majority of this was accounted for by PC1 and PC2, representing 53 % and 18 % respectively. As expected, PC1 separated exponentially growing planktonic cells from stationary-phase planktonic cells (Fig. 1), confirming that growth phase was the largest source of variation. PC2 on the other hand separated biofilms, colonies and planktonic cells, irrespective of incubation time in the latter two, with planktonic cells and biofilms at the extremities and colonies more centrally located. Small colonies (C15h) were most similar to planktonic cells in exponential phase (PE), whereas large colonies (C40h) were more similar to biofilms (B3d).

Surface-associated growth is oxygen limited

In order to investigate the effect of surface-associated growth on gene expression, we compared colonies with planktonic cells in their respective growth phase (C15h vs PE and C40h vs PS; see Supplementary Table S1, available with the online version of this paper). Of the 34 genes that were modulated in the same way between colonies and planktonic cells in their respective growth phase, 33 were more highly expressed in colonies. Furthermore, 20 of these genes have previously been shown to be activated in biofilms compared to planktonic cells or during biofilm development (Hentzer *et al.*, 2005), and 12 have been shown to be activated in low-oxygen conditions or during growth with nitrogen as a terminal electron acceptor (Alvarez-Ortega & Harwood, 2007; Filiatrault *et al.*, 2005; Platt *et al.*, 2008).

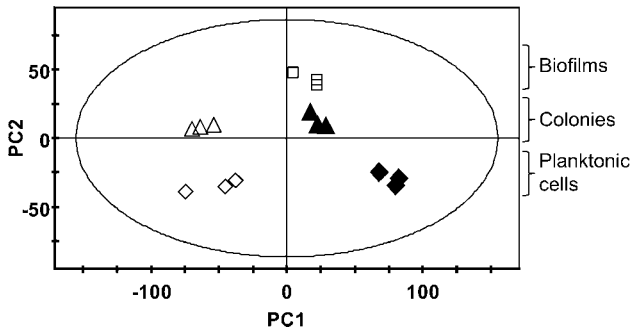

Fig. 1. PCA scores plot of PC1 vs PC2. PC1 accounted for 53 % of the total variation in the dataset, whereas PC2 accounted for another 18 %. The indicated samples are: planktonic cells in exponential phase (◇) or in stationary phase (◆), colonies incubated for 15 h (△) or 40 h (▲), and biofilms incubated for 3 days in a continuous-flow system (□).

This difference was even more pronounced in biofilms. Table 2 shows the 101 genes that were modulated between biofilms and planktonic cells in both exponential and stationary phase. Many of these genes were also significantly modulated between biofilms and colonies, and these data have been included in the table. Genes involved in energy conversion under low-oxygen conditions or during denitrification were more highly expressed in biofilms than in planktonic cells or colonies. The genes encoding phenazine and hydrogen cyanide biosynthetic proteins were also induced in biofilms compared with planktonic cells.

Biofilms express the TTSS

In addition to genes involved in low-oxygen metabolism, 21 genes involved in TTS were induced in biofilms compared with planktonic cells and colonies. The TTSS is encoded by 36 genes (PA1690–PA1725), and 18 of these were significantly increased in biofilms compared with planktonic cells. Moreover, three genes located elsewhere on the chromosome (encoding the secreted products ExoT and ExoS and PA3842, a probable chaperone in TTS) were also significantly induced in biofilms compared to planktonic cells and colonies.

In order to investigate if expression of the TTSS was correlated with other cellular functions, a hierarchical cluster analysis of samples as well as expression profiles was performed. This analysis revealed that the expression profiles of 31 genes involved in TTS clustered together with a Pearson's correlation coefficient (PCC) of 0.78. The total number of genes in this cluster was 61 and, interestingly, 14 of these were involved in nitrogen metabolism (data not shown). The expression of these genes was in all cases highest in biofilms, lowest in

Table 2. Genes that were significantly modulated between B3d and PE, as well as between B3d and PS

Some of these genes were also significantly modulated between B3d and C15h or C40h, and these data have been included in the table. Annotation according to http://www.pseudomonas.com.

Locus tag	Gene name	Fold change				Product name	Ref.
		B3d-PE	B3d-PS	B3d-C15h	B3d-C40h		
Energy metabolism, energy production and conversion							
PA0836	*ackA*	2.4	2.5		2.1	Acetate kinase	
PA0918		3.0	4.2	3.6	2.7	Cytochrome b_{561}	
PA1555		11.9	12.3	3.6	2.3	Probable cytochrome *c*	*†
PA1556		10.3	11.4	2.9		Probable cytochrome *c* oxidase subunit	*†
PA1557		8.9	8.9	3.1		Probable cytochrome oxidase subunit (cbb_3-type)	*†
PA4133		3.8	3.1			Cytochrome *c* oxidase subunit (cbb_3-type)	*
PA4571		2.8	2.4	2.3	2.3	Probable cytochrome *c*	
PA4587	*ccpR*	12.4	11.4	4.4	2.6	Cytochrome c_{551} peroxidase precursor	*‡
PA5427	*adhA*	9.5	7.4	4.3	2.3	Alcohol dehydrogenase	*†§
Nitrogen metabolism							
PA0510		3.6	2.9	2.5	2.4	Probable uroporphyrin-III c-methyltransferase	*§‖
PA0511	*nirJ*	3.7	2.8	2.3		Haem d1 biosynthesis protein NirJ	*‡§
PA0513		2.2	2.0			Probable transcriptional regulator	*§‖¶
PA0514	*nirL*	3.4	3.0	2.3	2.2	Haem d1 biosynthesis protein NirL	*§‖¶
PA0515		6.9	6.0	2.9	3.1	Probable transcriptional regulator	*†§
PA0516	*nirF*	3.7	3.4	2.4	2.5	Haem d1 biosynthesis protein NirF	*†§‖¶
PA0517	*nirC*	6.9	6.1	2.9	3.7	Probable *c*-type cytochrome precursor	*†§
PA0518	*nirM*	12.5	9.9	4.3	4.4	Cytochrome *c*-551 precursor	*†§‖¶
PA0519	*nirS*	22.9	22.2	3.7	4.6	Nitrite reductase precursor	*†§‖
PA0523	*norC*	6.5	5.9	2.5	4.7	Nitric-oxide reductase subunit C	*†§¶
PA0524	*norB*	4.6	4.2		3.6	Nitric-oxide reductase subunit B	*†‡§¶
PA0526		3.9	3.2	3.2	2.6	Hypothetical protein	‖
PA3392	*nosZ*	5.0	4.3	3.3	3.8	Nitrous-oxide reductase precursor	*§¶#
PA3872	*narI*	2.4	2.2		2.0	Respiratory nitrate reductase gamma chain	‖¶
Secreted factors (toxins, enzymes, alginate)							
PA0263	*hcpC*	4.8	2.9		5.0	Secreted protein Hcp	‡
PA1901	*phzC2*	4.0	4.0			Phenazine biosynthesis protein PhzC	†
PA1902	*phzD2*	2.5	2.4			Phenazine biosynthesis protein PhzD	†
PA1903	*phzE2*	3.5	3.8			Phenazine biosynthesis protein PhzE	†
PA1904	*phzF2*	3.9	3.4			Probable phenazine biosynthesis protein	†
PA1905	*phzG2*	4.1	3.5			Probable pyridoxamine 5′-phosphate oxidase	†
PA2193	*hcnA*	3.1	4.7			Hydrogen cyanide synthase HcnA	*†
PA2194	*hcnB*	3.1	3.2			Hydrogen cyanide synthase HcnB	*†
PA2195	*hcnC*	2.6	2.9			Hydrogen cyanide synthase HcnC	*†
PA4211	*phzB1*	5.0	8.1			Probable phenazine biosynthesis protein	†
PA4217	*phzS*	5.4	6.6			Flavin-containing monooxygenase	†
Motility, chemotaxis and attachment							
PA2128	*cupA1*	2.9	2.6	3.0	2.8	Fimbrial subunit CupA1	*
PA5042	*pilO*	2.1	2.1			Type 4 fimbrial biogenesis protein PilO	
PA5043	*pilN*	2.1	2.1			Type 4 fimbrial biogenesis protein PilN	
PA5044	*pilM*	2.9	2.4		2.0	Type 4 fimbrial biogenesis protein PilM	
Type III secretion (TTS)							
PA0044	*exoT*	5.8	5.4	4.2	5.0	Exoenzyme T	
PA1699		2.2	2.3		2.1	Conserved hypothetical protein in TTS	
PA1700		2.4	2.2		2.1	Conserved hypothetical protein in TTS	
PA1701		3.0	3.1		2.8	Conserved hypothetical protein in TTS	
PA1705	*pcrG*	2.4	2.1			Regulator in TTS	
PA1706	*pcrV*	3.1	2.7	2.4	2.6	TTS protein PcrV	
PA1707	*pcrH*	5.1	4.9	3.0	3.9	Regulatory protein PcrH	

Table 2. cont.

Locus tag	Gene name	Fold change				Product name	Ref.
		B3d-PE	B3d-PS	B3d-C15h	B3d-C40h		
PA1708	*popB*	10.0	9.0	4.9	5.4	Translocator protein PopB	
PA1709	*popD*	7.3	6.1	3.8	4.3	Translocator outer-membrane protein PopD precursor	
PA1710	*exsC*	3.5	3.7	2.2	2.3	ExsC, exoenzyme S synthesis protein C precursor.	
PA1711	*exsE*	3.9	3.6	2.4	2.3	ExsE	
PA1712	*exsB*	2.6	2.4		2.0	Exoenzyme S synthesis protein B	
PA1713	*exsA*	2.8	2.6			Transcriptional regulator ExsA	
PA1714	*exsD*	2.9	2.8		2.1	ExsD	
PA1716	*pscC*	2.2	2.0			TTS outer-membrane protein PscC precursor	
PA1718	*pscE*	4.6	6.6		4.0	Type III export protein PscE	
PA1719	*pscF*	2.7	2.8		2.3	Type III export protein PscF	
PA1721	*pscH*	2.3	2.2			Type III export protein PscH	
PA1722	*pscI*	3.0	2.7		2.5	Type III export protein PscI	
PA3841	*exoS*	8.1	6.9	5.8	5.6	Exoenzyme S	
PA3842		4.0	4.0	2.3	2.8	Probable chaperone	
PA3843		2.1	2.0			Hypothetical protein	
Phage							
PA0616		−2.3	−2.4	−4.1		Hypothetical protein	§
PA0620		−2.2	−2.3	−3.9		Probable bacteriophage protein	*§¶
PA0621		−2.7	−2.7	−4.3		Conserved hypothetical protein	*§
PA0633		−2.8	−3.1	−4.9		Hypothetical protein	*§‖
Other							
PA1546	*hemN*	2.5	2.7			Oxygen-independent coproporphyrinogen III oxidase	*†‡
PA2119		2.4	2.1			Alcohol dehydrogenase (Zn-dependent)	#
PA2826		3.2	3.1	3.5	4.2	Probable glutathione peroxidase	
PA2931		2.5	2.1	2.7	2.2	Probable transcriptional regulator	
PA3126	*ibpA*	4.6	4.5	5.0	2.5	Heat-shock protein IbpA	†‡
PA3190		−3.4	−3.0			Probable binding protein component of ABC sugar transporter	
PA3337	*rfaD*	2.4	2.2			ADP-L-glycero-D-mannoheptose 6-epimerase	†
PA4067	*oprG*	8.2	11.7	2.1		Outer-membrane protein OprG precursor	†
PA4236	*katA*	2.9	3.0			Catalase	*
PA5348		−3.3	−3.8	−2.1		Probable DNA-binding protein	
PA5429	*aspA*	4.6	3.5			Aspartate ammonia-lyase	
Hypothetical							
PA0141		4.5	4.1	2.3		Conserved hypothetical protein	†
PA0200		3.5	3.0			Hypothetical protein	‡‖
PA0534		2.6	2.5	2.7	2.6	Conserved hypothetical protein	
PA0614		−2.1	−2.3	−3.6		Hypothetical protein	§‖¶
PA0713		8.9	4.8	3.1		Hypothetical protein	*†§
PA1029		2.3	2.5	2.2	2.6	Hypothetical protein	
PA1123		2.3	2.0			Hypothetical protein	*†
PA1673		4.0	3.5	3.1		Hypothetical protein	*†‡
PA1746		7.9	5.5	4.2	2.0	Hypothetical protein	*†
PA2501		4.7	2.7	3.0	2.8	Hypothetical protein	
PA2753		3.4	3.0	2.7		Hypothetical protein	†‡
PA2781		2.9	2.6	3.5	3.1	Hypothetical protein	‡
PA2782		4.2	4.1		2.4	Hypothetical protein	†
PA2783		3.6	3.2		2.3	Hypothetical protein	
PA3278		3.5	2.7		2.1	Hypothetical protein	†
PA3309		11.6	4.6	3.4		Conserved hypothetical protein (UspK)	*
PA3572		8.8	5.8	5.1	3.3	Hypothetical protein	†

Table 2. cont.

Locus tag	Gene name	Fold change				Product name	Ref.
		B3d-PE	B3d-PS	B3d-C15h	B3d-C40h		
PA3613		2.9	2.1			Hypothetical protein	
PA3880		3.8	3.9	2.2	2.6	Conserved hypothetical protein	ll#
PA4352		9.8	7.9	3.0		Conserved hypothetical protein (Usp-type)	*†
PA4577		4.1	2.1	2.9		Hypothetical protein	
PA4610		3.6	2.9	3.5	2.5	Hypothetical protein	†ll
PA5027		3.4	2.6	2.2		Hypothetical protein (Usp-type)	*†
PA5446		5.9	2.7	3.0		Hypothetical protein	
PA5475		6.6	5.4	3.8	2.0	Hypothetical protein	*†
PA5494		2.0	2.1		2.2	Hypothetical protein	

*Genes activated in response to decreased oxygen tension (Alvarez-Ortega & Harwood, 2007).
†Genes differentially expressed between planktonic cells and biofilms (Hentzer *et al.*, 2005).
‡Genes differentially expressed during biofilm development (Hentzer *et al.*, 2005).
§Genes activated in cells grown anaerobically with NO_2^- (Platt *et al.*, 2008).
llGenes differentially expressed in aerobic cultures with and without NO_3^- (Filiatrault *et al.*, 2005).
¶Genes activated in cells grown anaerobically with NO_3^- (Platt *et al.*, 2008).
#Proteins with increased abundance during anaerobic growth (Wu *et al.*, 2005).

planktonic cells and intermediate in colonies. Other virulence factors displayed similar expression patterns, such as the *hcnABC* genes, which were highly correlated with *oprG* (PCC 0.95). Furthermore, the *phzC2D2E2F2G2* genes were highly correlated with *phzS*, *pilWX*, *pilY2*, PA0391 and the secreted protein gene PA0572 (PCC 0.91). Examples of these expression profiles can be seen in Fig. 2(a, b).

Some virulence factors vary according to growth phase

The previously mentioned virulence genes varied according to the growth mode, whereas other genes encoding virulence-related functions had a more growth-phase-dependent expression pattern. The *aprDEF* genes, which encode the type I secretion system, and *aprA*, which encodes the type I-secreted alkaline protease, were most highly expressed in slow-growing cells (large colonies and planktonic cells in stationary phase). Closely correlated with these expression profiles (PCC 0.92) was the adjacent gene PA1245, as well as *rhlA*, *rhlB* and *rhlR*, the protease gene *lasA* and the probable toxin transporter gene PA4143 (Fig. 2c). Apart from PA4143, all these genes have previously been shown to be modulated by QS (Hentzer *et al.*, 2003; Schuster *et al.*, 2003; Wagner *et al.*, 2003). In accordance with this, their expression levels in planktonic cells and colonies increased with prolonged incubation time, whereas the expression in biofilms was relatively low. The latter was in agreement with the transcriptomic and proteomic data that showed biofilms to be more similar to fast-growing cells than to slow-growing cells.

TTS can be detected in biofilms, but not in planktonic cells

In order to verify the expression of the TTSS in biofilms, intracellular protein of planktonic cells, colonies and biofilms was subjected to Western blotting using a PcrV antibody. PcrV, which is a component of the type III translocation machinery, could be detected in the cellular fraction of biofilms and, in smaller amounts, in large colonies, but not in planktonic cells (Fig. 3). This protein could also be detected in the secretome of 3-day-old biofilms, but not in that of stationary-phase planktonic cells.

In order to investigate if other related proteins could be detected in the biofilm effluent, secreted protein from 3-day-old biofilms and stationary-phase planktonic cells was analysed in triplicate by LC-MS/MS (Table 3). PcrV was detected in the secretome of all biofilm samples, but not in any of the planktonic cell samples, and the same was the case for the type III effector proteins, ExoS and ExoT. Comprehensive MASCOT data for these proteins can be seen in Supplementary Table S2. These results showed that both structural components and effectors of the TTSS were present in biofilms under these conditions, whereas they could not be detected in planktonic cells.

DISCUSSION

In this study, we investigated the interrelationships between planktonic cells, colonies on agar plates and biofilms in a continuous-flow system, using a microarray approach. In a previous proteomic study, we showed that

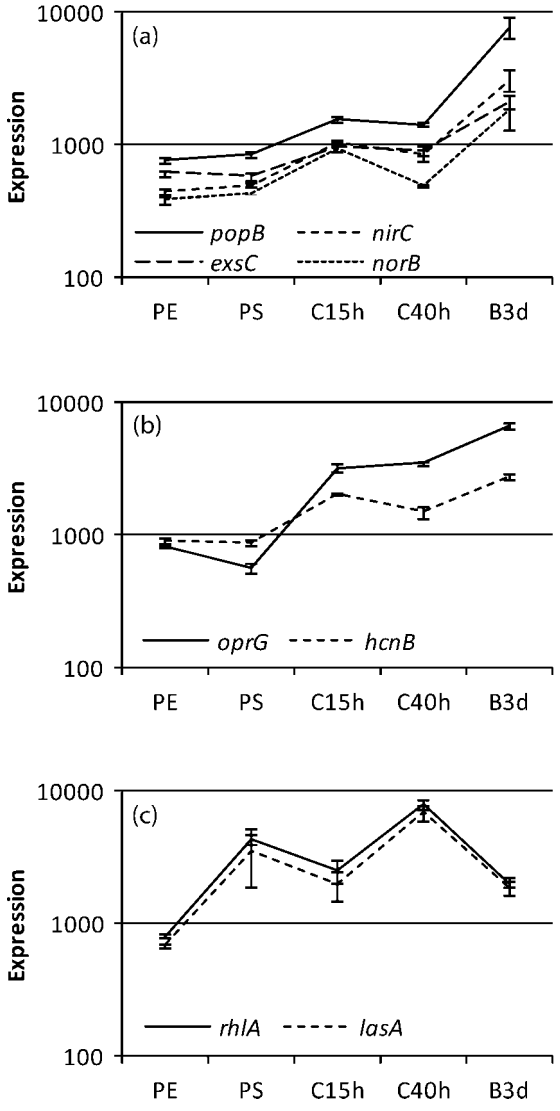

Fig. 2. Examples of DNA array expression profiles of genes encoding virulence factors, as well as some of the genes that were closely correlated in a hierarchical cluster analysis. (a) *popB* and *exsC* are involved in TTS, whereas *nirC* and *norB* are involved in denitrification. (b) *hcnB* is involved in hydrogen cyanide biosynthesis, and *oprG* encodes a major outer-membrane component. (c) *rhlA* and *lasA* are examples of genes that were most highly expressed in planktonic cells in stationary phase and in large colonies.

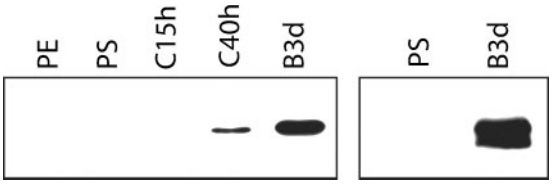

Fig. 3. Western blot of intracellular protein (left, 5 μg protein per lane) and extracellular protein (right, 20 μg protein per lane) using a PcrV antibody.

growth phase makes a bigger difference than growth mode with respect to number of differentially expressed gene products, and that biofilms in our conditions are metabolically active structures that are very different from planktonic cells in stationary phase (Mikkelsen *et al.*, 2007). These findings were confirmed in the current study, both by Student's *t*-test (Table 1) and by PCA (PC1, Fig. 1). The second-largest variation in the dataset (PC2, Fig. 1) separated planktonic cells, colonies and biofilms, irrespective of growth phase, with biofilms and planktonic cells at the extremities and colonies more centrally located. This suggested that colonies in this system are something in between planktonic cells and biofilms.

An analysis of the significantly modulated genes between biofilms and planktonic cells in both growth phases (Table 2) revealed that oxygen limitation was one of the major factors that separated the different growth modes. This was evident in colonies already after 15 h of growth (Supplementary Table S1), but was more pronounced in biofilms. Several of the modulated gene products have previously been shown to be induced during oxygen-limited conditions, during growth with nitrogen as a terminal electron acceptor or during biofilm growth (Alvarez-Ortega & Harwood, 2007; Filiatrault *et al.*, 2005; Hentzer *et al.*, 2005; Platt *et al.*, 2008; Wu *et al.*, 2005). Genes encoding three Usp-type universal stress proteins, which have been shown to be induced in low-oxygen conditions, were also induced in biofilms (Table 2) (Alvarez-Ortega & Harwood, 2007; Boes *et al.*, 2006; Schreiber *et al.*, 2006). Table 2 further shows that some virulence factors, such as pyocyanin, hydrogen cyanide and type IV pili, are highly expressed in biofilms.

In addition to genes associated with metabolism under low-oxygen conditions, 21 genes involved in TTS were significantly increased in biofilms compared to planktonic cells. Biofilms are often believed to be less virulent than planktonic cells, and biofilm formation versus expression of the TTSS has been described as a lifestyle choice that represents divergent roads leading to either acute or chronic infection (Furukawa *et al.*, 2006; Goodman *et al.*, 2004; Yahr & Greenberg, 2004). It has even been suggested that TTS may be detrimental to biofilm formation (Kuchma *et al.*, 2005). However, chronic infections do not necessarily involve biofilm formation (Yang *et al.*, 2008), and biofilms have also been associated with some acute infections (Schaber *et al.*, 2007). Our finding that the TTSS is induced in biofilms compared with planktonic cells was confirmed by the detection of PcrV, a part of the type III translocation apparatus, in the cellular fraction as well as in the secreted fraction of the biofilms by Western blotting. The presence of PcrV in the biofilm effluent was confirmed by LC-MS/MS analysis of the planktonic cell and biofilm secretome. The type III effector proteins, ExoS and ExoT, were also detected in the biofilm effluent,

Table 3. Proteins identified in the secreted fractions of planktonic cells in stationary phase (PS) and 3-day-old biofilms (B3d), analysed in triplicate by LC-MS/MS and MASCOT searching

Annotation according to http://www.pseudomonas.com. +, Detected by at least two different peptides in all three biological replicates; (+), matching peptides detected, but not two different peptides for every sample; −, no matching peptide detected.

Subcellular localization	PA no.	Gene name	Product name	PS	B3d
Extracellular	0041		Probable haemagglutinin	+	(+)
	0044	*exoT*	Exoenzyme T	−	+
	0423	*pasP*	PasP	+	+
	0852	*cbpD*	Chitin-binding protein CbpD precursor	+	+
	1094	*fliD*	Flagellar capping protein FliD	+	+
	1706	*pcrV*	Type III secretion protein PcrV	−	+
	3407	*hasAp*	Haem-acquisition protein HasAp	+	(+)
	3841	*exoS*	Exoenzyme S	−	+
Outer membrane	0973	*oprL*	Peptidoglycan-associated lipoprotein OprL precursor	+	−
	1086	*flgK*	Flagellar hook-associated protein 1 FlgK	+	(+)
	1777	*oprF*	Major porin and structural outer-membrane porin OprF precursor	+	(+)
	2462		Hypothetical protein	+	−
	2853	*oprI*	Outer-membrane lipoprotein OprI precursor	+	−
Periplasmic	0888	*aotJ*	Arginine/ornithine binding protein AotJ	+	+
	1074	*braC*	Branched-chain amino acid transport protein BraC	+	(+)
	1092	*fliC*	Flagellin type B	+	+
	4739		Conserved hypothetical protein	+	(+)
	4922	*azu*	Azurin precursor	+	+
	5489	*dsbA*	Thiol : disulfide interchange protein DsbA	+	+
Cytoplasmic	0456		Probable cold-shock protein	(+)	+
	1800	*tig*	Trigger factor	−	+
	3162	*rpsA*	30S ribosomal protein S1	−	+
	4265	*tufA*	Elongation factor Tu	(+)	+
	4385	*groEL*	GroEL protein	+	(+)
	4386	*groES*	GroES protein	+	+
	5240	*trxA*	Thioredoxin	(+)	+
	5505		Conserved hypothetical protein	+	(+)
Unknown	0315		Hypothetical protein	+	(+)
	0388		Hypothetical protein	+	(+)
	0623		Probable bacteriophage protein	+	(+)
	2659		Hypothetical protein	+	(+)
	3785		Conserved hypothetical protein	+	+
	3931		Conserved hypothetical protein	+	−
	4761		DnaK protein	+	(+)
	5505		Probable TonB-dependent receptor	+	(+)

whereas these proteins were not detected in the supernatants of planktonic cells (Table 3). This suggests that not only are the genes for the TTSS induced in biofilms compared to planktonic cells, but the corresponding proteins are also produced in a similar pattern. In addition to this, a number of proteins that are known to be abundant in the bacterial cytoplasm and periplasm were detected in the biofilm effluent. This indicates a certain degree of cell lysis in planktonic cells as well as in biofilms. We can therefore not be certain if PcrV, ExoS and ExoT are released due to active secretion or due to lysis. Nevertheless, the analysis confirms that these proteins are produced in biofilms, and that the TTSS is, if not actively secreting, then at least ready to do so in biofilms, whereas planktonic cells show no evidence of TTS.

The regulation of TTS in *P. aeruginosa* is known to be closely linked to the metabolic state of the cells (Rietsch & Mekalanos, 2006), and a number of external factors have been shown to induce TTS in *P. aeruginosa*, such as low calcium, high salt concentrations and host-cell contact (Dasgupta *et al.*, 2006; Hornef *et al.*, 2000). Apart from perhaps high salt, none of these inducing factors were present in our growth conditions. Furthermore, media components are not sufficient to explain the induction of TTS in biofilms and not in planktonic cells. Absence of the LPS O-antigen has previously been shown to facilitate TTS in *P. aeruginosa* in planktonic cells (Augustin *et al.*, 2007), but no differences were observed in the LPS biosynthetic genes or in the O-antigen banding patterns as determined by SDS-PAGE and silver staining (data not shown). The *rhl*

QS system and RpoS have been shown to negatively regulate TTS in *P. aeruginosa* (Bleves *et al.*, 2005; Hogardt *et al.*, 2004), and in accordance with this the expression of *rhlIR* and *rpoS* was low in biofilms. For the QS systems this was unlikely to be due to signal washout (Kirisits *et al.*, 2007), since the flow was laminar (Reynold's number 3.3). Moreover, two hybrid sensor kinases, LadS and RetS, have been described that reciprocally regulate TTS and the production of the exopolysaccharide Pel that leads to enhanced biofilm formation (Goodman *et al.*, 2004; Ventre *et al.*, 2006). In accordance with the report by Sakuragi & Kolter (2007) that the Pel genes have very low expression levels at 37 °C, the seven Pel biosynthetic genes (PA3058–64) were hardly expressed under our conditions. Conversely, the genes encoding the exopolysaccharide Psl (Ma *et al.*, 2006, 2007) were all expressed, but the levels changed very little between the five groups of samples. Similarly, the expression of *ladS* and *retS* hardly changed between the samples, although the gene encoding RetS, which has been shown to be required for TTS, was approximately twice as highly expressed as the gene encoding LadS, which promotes biofilm formation (Goodman *et al.*, 2004; Ventre *et al.*, 2006).

The induction of TTSS in biofilms in our system therefore does not seem to be due to currently known environmental factors, although low expression of QS genes, *rpoS* and *ladS*, may allow this to take place. Whereas genes involved in oxygen limitation and denitrification have previously been shown to be induced in biofilms (Hentzer *et al.*, 2005; Sauer *et al.*, 2002; Waite *et al.*, 2006), the same is not true for TTSS. One plausible explanation could be that the researchers employed either minimal or reduced-strength media for biofilm growth (Hentzer *et al.*, 2005; Sauer *et al.*, 2002; Ventre *et al.*, 2006; Waite *et al.*, 2006; Whiteley *et al.*, 2001). Under these conditions, factors like QS and RpoS are likely to repress TTSS, and the biofilm cells may adopt a stationary-phase-like lifestyle.

Matz *et al.* (2008) recently showed that the *P. aeruginosa* clinical isolate PA99 employs the TTSS to kill biofilm-associated amoebae in mixed-species biofilms. Even though the induction in this case occurred in the presence of a strong inducing factor (eukaryotic cells), this is clear evidence that TTS is indeed possible in biofilms. Interestingly, expression of the TTSS in our system was closely correlated with genes involved in denitrification, which can serve as an energy source in a low-oxygen environment. Bearing in mind that the microarray data are an average of sometimes very heterogeneous bacterial populations, this could suggest a link between TTS and oxygen limitation. A similar observation has been made in enterohaemorrhagic *Escherichia coli* (Ando *et al.*, 2007). Certain mutants of *P. aeruginosa* that are unable to grow anaerobically using nitrogen as a terminal electron acceptor have also been shown to be attenuated in infection models (Filiatrault *et al.*, 2006; Van Alst *et al.*, 2007). However, further studies are required to confirm where in the biofilms the TTSS is expressed, and whether this occurs in the same cells as those that use nitrogen as a terminal electron acceptor. This could give new insight into the regulation of TTS in *P. aeruginosa*.

In conclusion, we have shown that biofilm formation and TTS are not mutually exclusive in *P. aeruginosa*. Biofilms under our conditions express the TTSS, whereas planktonic cells do not. This could suggest that biofilms in some conditions play a more active role in virulence than previously thought.

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