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Divergence of a strain of *Pseudomonas aeruginosa* during an outbreak of ovine mastitis

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Bacterial infections causing mastitis in sheep can result in severe economic losses for farmers. A large survey of milk samples from ewes with mastitis in Sardinia, Italy, indicated an increasing prevalence of *Pseudomonas aeruginosa* infections. It has been shown previously that during chronic, biofilm-associated infections *P. aeruginosa* populations diversify. We report the phenotypic and genomic characterisation of two clonal *P. aeruginosa* isolates (PSE305 and PSE306) from a mastitis infection outbreak, representing distinct colony morphology variants. In addition to pigment production, PSE305 and PSE306 differed in phenotypic characteristics including biofilm formation, utilisation of various carbon and nitrogen sources, twitching motility. We found higher levels of expression of genes associated with biofilm formation (*pelB*) and twitching motility (*flgD*) in PSE305, compared to the biofilm and twitching-defective PSE306. Comparative genomics analysis revealed single nucleotide polymorphisms (SNPs) and minor insertion/deletion variations between PSE305 and PSE306, including a SNP mutation in the *pilP* gene of PSE306. By introducing a wild-type *pilP* gene we were able to partially complement the defective twitching motility of PSE306. There were also three larger regions of difference between the two genomes, indicating genomic instability. Hence, we have demonstrated that *P. aeruginosa* population divergence can occur during an outbreak of mastitis, leading to significant variations in phenotype and genotype, and resembling the behaviour of *P. aeruginosa* during chronic biofilm-associated infections.
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1. Introduction

Mastitis is an inflammation of the mammary gland and is a common condition in many sheep-rearing countries (Conington et al., 2008). The economic losses due to mastitis are of major consequence for sheep farmers. In particular, mastitis can result in poor milk yields, changes in milk composition, premature culling of ewes and poor growth rates of lambs (Conington et al., 2008). Mastitis is frequently caused by the introduction and multiplication of pathogenic bacteria in the mammary glands. Causative bacteria include the Coagulase Negative Staphylococci, *Staphylococcus aureus*, Streptococci, Enterobacteriaceae and *Pseudomonas aeruginosa*. A large survey of 27,218 milk samples from ewes with mastitis (1214 flocks and 1634 mastitis events) in Sardinia, Italy indicated that the prevalence of *P. aeruginosa* cases increased over the 7 year sampling period (unpublished data). The management of *P. aeruginosa* infections represents an increased challenge compared to infections involving other aetiological agents because of the tendency for long persistence and spread from subclinically infected animals throughout a herd. Furthermore with no effective antimicrobials available, intervention strategies are limited.

As a consequence of its large genome, *P. aeruginosa* is a versatile and adaptable opportunistic pathogen that can cause infections of both medical and veterinary importance (Winstanley and Fothergill, 2009; Hertl et al., 2011). The ability of *P. aeruginosa* to colonise and infect a variety of sites has been attributed to the many virulence factors that it can produce. These include various exotoxins, and several secreted products that are under the control of complex interconnecting quorum-sensing (QS) regulatory networks, including pyocyanin, elastases, LasA, alkaline proteases and rhamnolipid (Winstanley and Fothergill, 2009). Biofilm formation and alginate overproduction, are also important aspects of *P. aeruginosa* pathogenicity, especially in the context of chronic lung infections of cystic fibrosis (CF) patients, where they contribute to the intrinsic resistance of *P. aeruginosa* infections to antimicrobials (Winstanley and Fothergill, 2009).

Although several *P. aeruginosa* genome sequences have been reported, these are mostly from human infection isolates (Silby et al., 2011). Genome sequencing studies on *P. aeruginosa* isolates from human CF lung infections have shown that mutation and population divergence is a common feature of bacteria during these chronic, biofilm-associated, infections, often leading to changes in pigmentation or colony morphology (Mowat et al., 2011). Similar diversification has been observed in experimental biofilm systems (McElroy et al., 2014).

Having observed colonial morphology variations amongst *P. aeruginosa* isolated from an outbreak of ovine mastitis, the aim of this study was to determine whether during mastitis *P. aeruginosa* populations show behaviour characteristic of chronic biofilm-associated infections by characterising the phenotypic and genotypic properties of two *P. aeruginosa* isolates representing distinct colony morphology variants.

2. Methods

2.1. Bacterial strains

The isolates chosen for this study were from an infected flock of Sardinian ewes of about 350 animals notified for cases of mastitis during the lactation period 2004–2005. In a 6 month period, more than 100 animals became culture-positive for *P. aeruginosa*. Towards the end of the lactation period, a group of seven sheep showing signs of mastitis were chosen for more extensive sampling. Duplicate milk samples and swabs were collected for each half udder. In four cases, cultures from ewes showing clinical signs of mastitis yielded a *P. aeruginosa* with green fluorescent pigmentation (example isolate PSE305). From two sheep, a red-pigmented pyorubin-positive, non-fluorescent *P. aeruginosa* was isolated (example isolate PSE306). From each sample all colonies were either red or green-pigmented, and PSE305-like isolates were also obtained from the milking machine.

The Istituto Zooprofilattico Sperimentale is the official Italian public body operating in the frame of National Health Service with duties related to animal health and welfare and food safety. Animal samples were taken as part of the normal surveillance of a regional programme in Sardegna for reduction of ovine mastitis.

2.2. ArrayTube genotyping

Isolates PSE305 and PSE306 were characterised using the ArrayTube (CLONDIAG, Alere Technologies, Köln, Germany) genotyping method (Wiehlmann et al., 2007). Data from the 13 SNPs, flagellin type (a/b), and the presence of the mutually exclusive type III secretion exotoxins (S or U), were converted into a "hexadecimal code" represented by four digits, allowing the published database to be searched as described previously (Wiehlmann et al., 2007).

2.3. Bacterial culture, RNA extraction and cDNA synthesis

Growth curves were performed on PAO1, PSE305 and PSE306 to confirm the stage at which the bacterial cells are at the mid-exponential and stationary phase. PAO1, PSE305 and PSE306 were inoculated into LB to give a starting A_{600} of 0.05. The cultures were grown to mid-exponential (A_{600} of 0.5) and stationary phase (A_{600} of 1.5) shaking at 180 rpm and at 37 °C. PSE305 and PSE306 were also grown statically in 6-well plates in LB at 37 °C for 24 h. RNA extractions were collected from these isolates at mid-exponential, stationary phase and during conditions that promote biofilm growth in PAO1 and PSE305 using the Nucleospin RNA II kit (Macherey-Nagel). The pellets were washed in 1 M Tris pH 8 (0.1 M EDTA) and resuspended in 100 μl lysis buffer (Nucleospin RNA II kit; Macherey-Nagel). Following RNA extraction, 1 μg of RNA was used as a template for cDNA synthesis, using the Transcriptor First Strand cDNA Synthesis kit (Roche Diagnostics). RNA samples that were not exposed to reverse transcriptase were used as controls, and these were confirmed as having negligible levels of genomic DNA present.

2.4. Gene expression in PSE305 and PSE306 using SYBR Green®

The real time PCR was performed in a volume of 20 μl containing 1× LightCycler DNA Master SYBR Green I master mix (Roche Diagnostics), 500 nM of each primer and 2 μl of the appropriate cDNA sample. Each cDNA sample (including reverse transcriptase negative controls) was diluted 1:2 with sterile, RNase and DNase-free water and analysed in duplicate. The reactions were performed in 96-well plates in a LightCycler 480 real time PCR machine (Roche diagnostics). Amplification was carried out for 45 cycles of 95 °C for 10 s, 60 °C for 5 s and 72 °C for 30 s. Fluorescence was recorded at a wavelength between 510 nm and 550 nm at the end of each PCR cycle. PCR products were subsequently analysed by melting curve analysis ranging from 65 °C to 95 °C. Fold changes in gene expression was calculated using the $2^{-\Delta\Delta Ct}$ (Schmittgen and Livak, 2008). The reference gene was *gyrB* and the reference condition was exponentially grown PSE305 or PSE306. The fold changes in gene expression during stationary phase and biofilm growth were compared to PSE305 and PSE306 grown to exponential phase. Genes selected for analysis were *pilT*, *algD*, *pslD*, *pelB*, *lasR*, *pslA* and *flgD* (for primer details, see Supplementary Table 1). To confirm suitability of using the $2^{-\Delta\Delta Ct}$ method to analyse the quantitative real time PCR data, a standard curve was constructed for each target and reference gene (Schmittgen and Livak, 2008). Serial diluted *P. aeruginosa* genomic DNA was amplified with the corresponding primer sets and the obtained threshold crossing point cycles (CT) were plotted against logarithms of initial genomic DNA amounts utilised in each reaction. All PCRs exhibited a strong linear correlation ($R^2 < 0.9$) between template amounts and CT values, with PCR efficiencies ranging from 95% to 110% confirming the suitability of the $2^{-\Delta\Delta CT}$ method for the analysis of relative gene expression (Schmittgen and Livak, 2008). Furthermore, all quantitative PCRs had single melting points following melting curve analysis.

2.5. Cell attachment assay

P. aeruginosa isolates PSE305 and PSE306 were inoculated into PM1F-0 solution (Biolog) until the solution was at 42% transmittance (Biolog turbidimeter). This solution in a volume of 15 ml was further diluted in 75 ml of PM1F-0 solution. The diluted bacterial suspension, in a volume of 100 μl, was added to each well of PM1 and PM2A 96-well plates (Biolog) and incubated for 24 h at 37 °C. This was performed in triplicate. Following incubation, the media and planktonic cells were removed and the adhered biofilms were washed three times with 150 μl of phosphate-buffered saline. The biofilms were allowed to dry and 125 μl of 0.5% (w/v) crystal violet in water was added to each well and stained for 15 min at room temperature. The biofilms were washed thoroughly with water to ensure excess stain was removed. Following which, 125 μl of ethanol was added to each well to dissolve the crystal violet. The solution was transferred to a new microtiter plate and read at A_{550nm}.

2.6. Twitching motility assay

P. aeruginosa strains were stabbed into LB agar using a sterile toothpick, through to the bottom of the petri dish. The plates were incubated overnight at 37 °C. Following incubation, the agar was removed from the petri dish and the dish was stained with 0.5% (w/v) crystal violet for 20 min at room temperature. The stain was washed off and the diameter of the stained zones was measured. The twitching motility assay was performed six times. For complementation, the plasmid pDFR1C (Martin et al., 1995), containing *pilM*, *pilN*, *pilO* and *pilP*, was kindly supplied by Professor John Mattick, University of Queensland. The plasmid was introduced into *P. aeruginosa* PSE306 by electroporation, selecting for carbenicillin resistance (400 μg/ml).

2.7. Genome sequencing

DNA was extracted from PSE305 and PSE306 grown planktonically in LB broth overnight at 37 °C using the Wizard® Genomic DNA Purification Kit (Promega). The purity of the extracted bacterial DNA was assessed using the Nanodrop ND-1000 and the quantity of bacterial DNA was measured using the Qubit® 2.0 Fluorometer. Strain PSE305 was sequenced using the Roche 454 Genome Sequencer FLX (GS-FLX) by preparing a 6 kb paired-end library using the standard titanium chemistry for 454. Contigs were assembled from the reads, and scaffolded using the Roche 454 Newbler assembler (version 2.5) with default settings. The scaffolds were ordered and orientated with respect to *P. aeruginosa* PAO1 (AE004091) using Abacas (http://abacas.sourceforge.net/) and the alignment programme MUMmer (Kurtz et al., 2004). Glimmer v3.02 was used to call putative ORFs (Delcher et al., 2007). A putative function was then assigned to each gene by BLAST (Altschul et al., 1990) comparison with a database of sequences generated from previously annotated *P. aeruginosa* genomes. In order to identify regions of difference, the pseudochromosome of strain PSE305 was aligned to the genome of PAO1 and analysed using the ARTEMIS Comparison Tool (Carver et al., 2005). In addition, the genome of strain PSE306 was sequenced using the SOLiD v4.0 system in order to identify single nucleotide polymorphism (SNP) mutations and insertions/deletions (INDELs) within this genome in comparison to PSE305. The SOLiD reads were mapped using BWA (Li and Durbin, 2009) in colorspace using sequence quality. The resulting BAM was then processed using GATK (McKenna et al., 2010) base quality score recalibration, indel realignment, and duplicate removal. SNP and INDEL discovery was performed using standard hard filtering parameters (DePristo et al., 2011). The VCF output was filtered using VCFTOOLS (Danecek et al., 2011) and viewed in ARTEMIS (Carver et al., 2005). Genome sequencing was performed by the Centre for Genomics Research, University of Liverpool.

2.8. Multi-locus sequence typing (MLST)

For MLST typing, regions of the *acsA*, *aroE*, *guaA*, *mutL*, *nuoD*, *pspA* and *trpE* genes were extracted directly from

genome sequence data. Sequence types were assigned using the available database (http://pubmlst.org/paeruginosa/).

2.9. BIOLOG phenotypic assays

Isolates PSE305 and PSE306, and the control *P. aeruginosa* strains PAO1 and PA14 were subjected to a phenotypic microarray (PM) using BIOLOG PM1 and PM2 microtiter plates, which together test the ability to utilise 190 different carbon sources, and PM3, which tests for the ability to utilise 95 different nitrogen sources. The *P. aeruginosa* were prepared as described previously (Viti et al., 2009). Data were read by the OmniLog PM Software and a *t*-test was applied to three independent replicates to determine statistical significance.

2.10. Genome sequence accession

The genome sequence data for strain PSE305 has been deposited in the ENA-EMBL database under accession number HG974234.

3. Results

3.1. Genotyping of mastitis-associated isolates representing the two colony morphology variants

The ArrayTube genotyping method and MLST (derived from genome sequence data) were used to confirm that PSE305 (green-pigmented isolate) and PSE306 (the red-pigmented isolate) were of the same clone type. The ArrayTube genotyping method (Wiehlmann et al., 2007), which gives a genotypic profile that can be used to search a large database of *P. aeruginosa* strains, showed that PSE305 and PSE306 have an identical profile, which they also share with *P. aeruginosa* clone V (hexadecimal code: 0812). Clone V is not one of the major clones in the database, but it has been found amongst the collection tested, mostly associated with isolates from human bacteraemia cases (Wiehlmann et al., 2007). Previous analyses of clonal complex structures based on the genotyping data identify clone V as an outlier rather than a member of a major cluster (Wiehlmann et al., 2007). In addition the analysis of the PSE305 genome sequence identified the strain as MLST type ST-244.

3.2. Phenotypic variation between isolates PSE305 and PSE306

In addition to pigment production, there were a number of phenotypic differences between the *P. aeruginosa* isolates PSE305 and PSE306. PSE306 did not form stable biofilms in the presence of various substrates, unlike PSE305 (Fig. 1). There were statistically significant differences in the levels of crystal violet staining between PSE305 and PSE306, following growth in conditions that promote biofilm formation and in the presence of various substrates (two-tailed Students *t*-test, no additional substrate: $t[4] = 3.13$, $p < 0.05$; with α-D-glucose: $t[4] = 10.68$, $p < 0.001$; with butyric acid: $t[4] = 39.62$, $p < 0.001$; with caproic acid: $t[4] = 7.051$, $p < 0.01$; with capric acid: $t[4] = 51.28$,

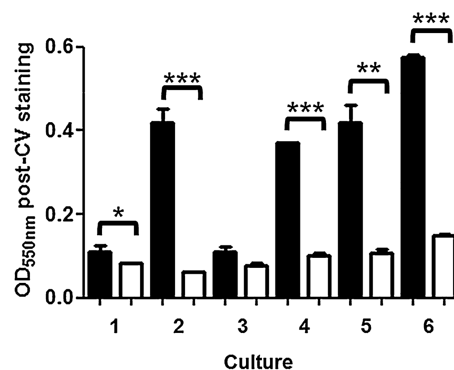

Fig. 1. PSE305 forms stable biofilms, unlike PSE306. PSE305 and PSE306 were statically grown in the presence of various substrates, and the resultant biofilms were stained with crystal violet (CV) to assess the extent of biofilm formation in these cultures. 1. No extra substrate, 2. α-D-glucose, 3. D9 α-D-lactose, 4. butyric acid, 5. caproic acid, 6. capric acid (all groups: $n = 3$). The black and white bars represent PSE305 and PSE306, respectively. Error bars represent the standard error of the mean. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

$p < 0.001$). PSE305 grown in the presence of α-D-glucose, butyric acid, caproic acid and capric acid significantly increased biofilm formation in this strain, when compared to growth without additional substrates (two-tailed Students *t*-test, with α-D-glucose: $t[4] = -8.24$, $p < 0.001$; with butyric acid: $t[4] = -18.87$, $p < 0.001$; with caproic acid: $t[4] = -6.53$, $p < 0.01$; with capric acid: $t[4] = 30.00$, $p < 0.001$).

PSE305 and PSE306 differed in their utilisation of certain nitrogen and carbon sources (Supplementary Table 2). In particular, PSE305 exhibited more metabolic activity in the presence of α-D-glucose, butyric acid, caproic acid, capric acid and L-histamine, than PSE306 grown under the same conditions. This was true of other nitrogen and carbon sources that were assayed (data not shown). However, interestingly both mastitis strains did not utilise lactose to a significant extent (Supplementary Table 2).

One way ANOVA (Bonferroni adjusted) indicated that PSE306 was deficient in twitching motility compared to PSE305 (Fig. 2) ($p < 0.001$).

3.3. Comparative genomics of isolates PSE305 and PSE306

We obtained the genome sequence of isolate PSE305 using 454 pyrosequencing and the pseudochromosome of strain PSE305 was aligned to the genome of strain PAO1. Insertions and deletions in the genome of strain PSE305 in comparison to the genome of strain PAO1 are summarised in Supplementary Tables 3 and 4. Many of these were located in previously reported regions of genome plasticity (Mathee et al., 2008). Most of the larger insertions corresponded to known genomic islands or prophages. The largest additional region in the genome of strain PSE305 was a 115 kb genomic island.

In addition we sequenced the genome of strain PSE306 using the SOLiD v4.0 system in order to identify SNP mutations and INDELs within this genome in comparison to PSE305. Three large gene clusters identified as present in the genome of strain PSE305 but absent from the genome

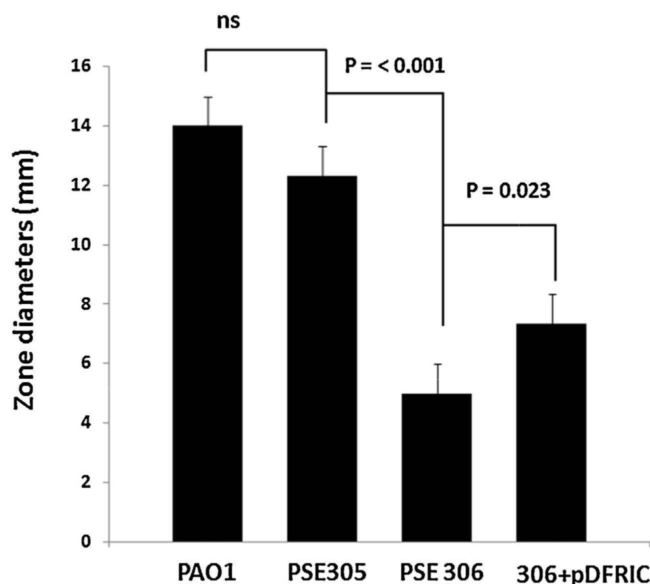

Fig. 2. Comparison of twitching motilities. Twitching motility was compared between strains PAO1 (control strain), PSE305, PSE306 and PSE306 containing the plasmid pDFRIC. Error bars represent the standard error of the mean. *p* values one-way analysis of variance (Bonferroni adjustment) comparisons are shown ($n = 6$); ns: not significant.

of strain PSE306 were identified. Two of these (insertions E and N in Supplementary Table 3) contained several ORFs sharing similarity with phage proteins, and are putative prophages. A third large deletion (approximately 91 kb; position 2070401 to 2161555) was of a cluster of genes corresponding to the PAO1 genes PA1981-2059. This latter region has not previously been reported as divergent. PCR assays have been designed to these three deleted regions. In all cases, strain PSE305 was PCR-positive and PSE306 was PCR-negative, confirming the genome sequence data (data not shown).

SNP and minor INDEL variations in the genome of strain PSE305 when compared to the genome of strain PSE306 are summarised in Table 1. Only six SNPs passing the quality threshold were found in the genome of PSE306. These included SNPs in a number of ORFs with putative functions that might impact on important phenotypes such as a sensor histidine kinase, siderophore receptor, type IV pilus biogenesis protein, alginate regulatory protein, permease and phenazine biosynthesis protein. Only a small number of minor INDELs were detected, one of which was in an ORF with a putative role in heme utilisation or adhesion (Table 1).

3.4. Gene expression variations between PSE305 and PSE306

Because PSE305 and PSE306 exhibited different phenotypic properties with regards to biofilm-forming ability and twitching motility, we used gene expression analysis to ascertain whether differences in gene expression could have contributed to these observed phenotypic differences. Therefore, the expression of genes involved in biofilm formation and twitching motility in PSE305 and PSE306 were quantitatively assessed (Fig. 3). Specifically, the expression profile in different *in vitro* models of growth (planktonic and biofilm mode of growth) were characterised to identify whether there are differences between the PSE305 (which can undergo twitching motility and biofilm formation) and PSE306 (which cannot). The expression profiles of the genes *pilT* and *flgD* both of which are involved in type IV fimbrial biogenesis and motility (Bertrand et al., 2010) was characterised. In addition, *pslD*, *pslA*, *pelB and algD* expression profiles, which are involved in biofilm architecture and formation (Campisano et al., 2006; Ghafoor et al., 2011) and *lasR* which is involved in the regulation of QS network (Winstanley and Fothergill, 2009) were determined.

The expression of *flgD* was significantly lower in PSE306 at stationary phase and under conditions that promote biofilm formation, compared to expression in PSE305 (two-tailed Students *t* test; stationary phase: $t(6) = 2.54$, $p = 0.04$. Biofilm growth: $t(6) = 2.87$, $p = 0.03$). In addition, the expression of *pelB* was also significantly lower in PSE306 at stationary phase and under conditions that promote biofilm formation (two-tailed Students *t* test; stationary phase: $t(6) = 2.72$, $p = 0.03$. Biofilm growth: $t(6) = 2.99$, $p = 0.03$). This difference between the two isolates was also true of *algD* (two-tailed Students *t* test; stationary phase: $t(6) = 6.86$, $p = 0.0005$. Biofilm growth: $t(6) = 3.96$, $p = 0.007$). There was no statistical significant difference between the gene expression profiles of *pilT*, *pslD*, *pslA* and *lasR* between PSE306 and PSE305 grown in either stationary phase or under conditions that promote biofilm formation. There was, however, a significant increase in *pslD* expression in PSE306 between stationary phase and during growth in conditions that promote biofilm formation (two-tailed Students *t* test; $t(6) = 3.57$, $p = 0.01$). This difference in *pslD* expression was not observed in PSE305.

3.5. Partial complementation of twitching motility

Having identified a mutation in the *pilP* gene (Table 1) in the twitching motility-defective strain PSE306, we

Table 1
Non-synonymous SNPs/INDELs in PSE306 relative to the chromosome of PSE305 The position refers to the location of the SNP and INDEL in the PSE305 pseudochromosome. Comp. indicates that the gene is on the complementary strand and only SNPs with a minimum quality score of 100 are shown.

Position	Description	Quality	PSE305 CDS	PAO1 ORF	Comments
1051625	SNP T → C	246.81	PSE305_10100	PA1098	Flagellar sensor histidine kinase FleS (402 AA in length); change of $Val_{57} \rightarrow Ala_{57}$; database *P. aeruginosa* sequences are all Ala in this position
3880538	SNP A → G (comp. T → C)	128.06	PSE305_36890c	PA3408	Hemophore HasA outer membrane receptor HasR; iron siderophore receptor protein (883 AA in length); change of $Ser_{860} \rightarrow Pro_{860}$; database *P. aeruginosa* sequences are all Pro in this position
5237941	SNP T → G (comp. A → C)	157.63	PSE305_49740c	PA5041	Type IV pilus biogenesis protein PilP (174 AA in length); change of $Thr_{107} \rightarrow Pro_{107}$; database *P. aeruginosa* sequences are all Thr in this position
5521552	SNP G → A (comp. C → T)	253.13	PSE305_52460c	PA5261	Alginate biosynthesis regulatory protein AlgR (248 AA in length); change of $Pro_{16} \rightarrow Leu_{16}$; database *P. aeruginosa* sequences are all Leu in this position
6105549	SNP G → A	241.13	PSE305_58030	PA0220	Amino acid permease (477 AA in length); change of $Gly_{33} \rightarrow Asp_{33}$; database *P. aeruginosa* sequences are all Gly in this position
6752225	SNP T → G (comp. A → C)	122.6	PSE305_64430c	PA1905 and PA4216	Phenazine biosynthesis protein PhzG1/PhzG2 (95 AA in length); change of $Lys_{24} \rightarrow Thr_{24}$; database *P. aeruginosa* sequences are all Arg in this position. Poor database matches in the N-terminal first 24 residues
2300849	INDEL GC → G	16/16	PSE305_22350	N/A	Possible RBS region mutation upstream of ORF21710c (putative NADH-flavin reductase); matches PAO1 ORF PA2176
3166266	INDEL CT → C	11/11	–	N/A	Between ORFs 28800 and 28810
3297412	INDEL G → GC (comp. C → CG)	11/11	PSE305_31300c	PA2885	Acyclic terpenes utilisation regulator, AtuR, TetR family
4564584	INDEL CAGGT → C	Poor	PSE305_43480	PA3980	(Dimethylallyl)adenosine tRNA methylthio-transferase; frameshift mutation from position 125 in a 446 AA protein
4981298	INDEL A → AC	7/7	–	N/A	Between ORFs 46340 and 46350
5153865	INDEL GC → G (comp. CG → C)	Poor	PSE305_49100c	PA4981	Amino acid transporter, AAT family; frameshift mutation from position 327 in a 470 AA protein
5907354	INDEL C → CG	8/8	PSE305_56120	PA0041	Large exoproteins involved in heme utilisation or adhesion
6245439	INDEL TC → T	10/10	PSE305_59200	PA0299	Omega-amino acid-pyruvate aminotransferase
6733229	INDEL CA → C	Poor	–	N/A	Between ORF 62780 and 62790
6738774	INDEL GT → G	Poor	–	N/A	Between ORF 62800 and 62810

introduced plasmid pDFR1C, containing an intact *pilP*, into the strain. The presence of the plasmid led to partial complementation of the loss of twitching motility (Fig. 2).

4. Discussion

Little is known about the behaviour of *P. aeruginosa* during mastitis infections in sheep. Having observed variations in colony morphology amongst isolates, we hypothesised that during such infections *P. aeruginosa* show behaviour characteristic of chronic, biofilm-associated infections. In this study, we focused on representatives of the two major colony morphology types identified during the mastitis outbreak and have demonstrated considerable variations between them. The two isolates (PSE305 and PSE306) from the same outbreak of ovine mastitis in a flock of Sardinian ewes were confirmed as being of the same clone-type, both genotype 0812 (clone V), which has been found in a number of different clinical sources and amongst isolates from water (Cramer et al., 2012). Previous analyses of clonal complex structures based on the genotyping data identify clone V as an outlier rather than a member of a major cluster (Wiehlmann et al., 2007). MLST analysis showed that PSE305 belonged to the subtype, ST-244. This subtype has been identified in a collection of human wound isolates from the Mediterranean, and in cases of bacteraemia in Asia (Maatallah et al., 2011; Woo et al., 2011). Hence, it is likely that the two isolates compared in this study are derived from the same outbreak. Despite being of the same clone-type, PSE305 and PSE306 showed evidence of both phenotype and genotype variation. PSE305 and PSE306 differed in pigment production, twitching motility and in their ability to form stable biofilms, suggesting that strain diversification occurred within this outbreak of ovine mastitis.

Some of these phenotypic differences could be explained by the SNP mutations present in PSE306 and the other genomic differences between these two isolates. In particular, the SNP in *pilP* of PSE306 could explain the deficiency in twitching motility seen in this isolate. PilP is a lipoprotein that localises to the inner membrane of *P. aeruginosa* and promotes the assembly and function of

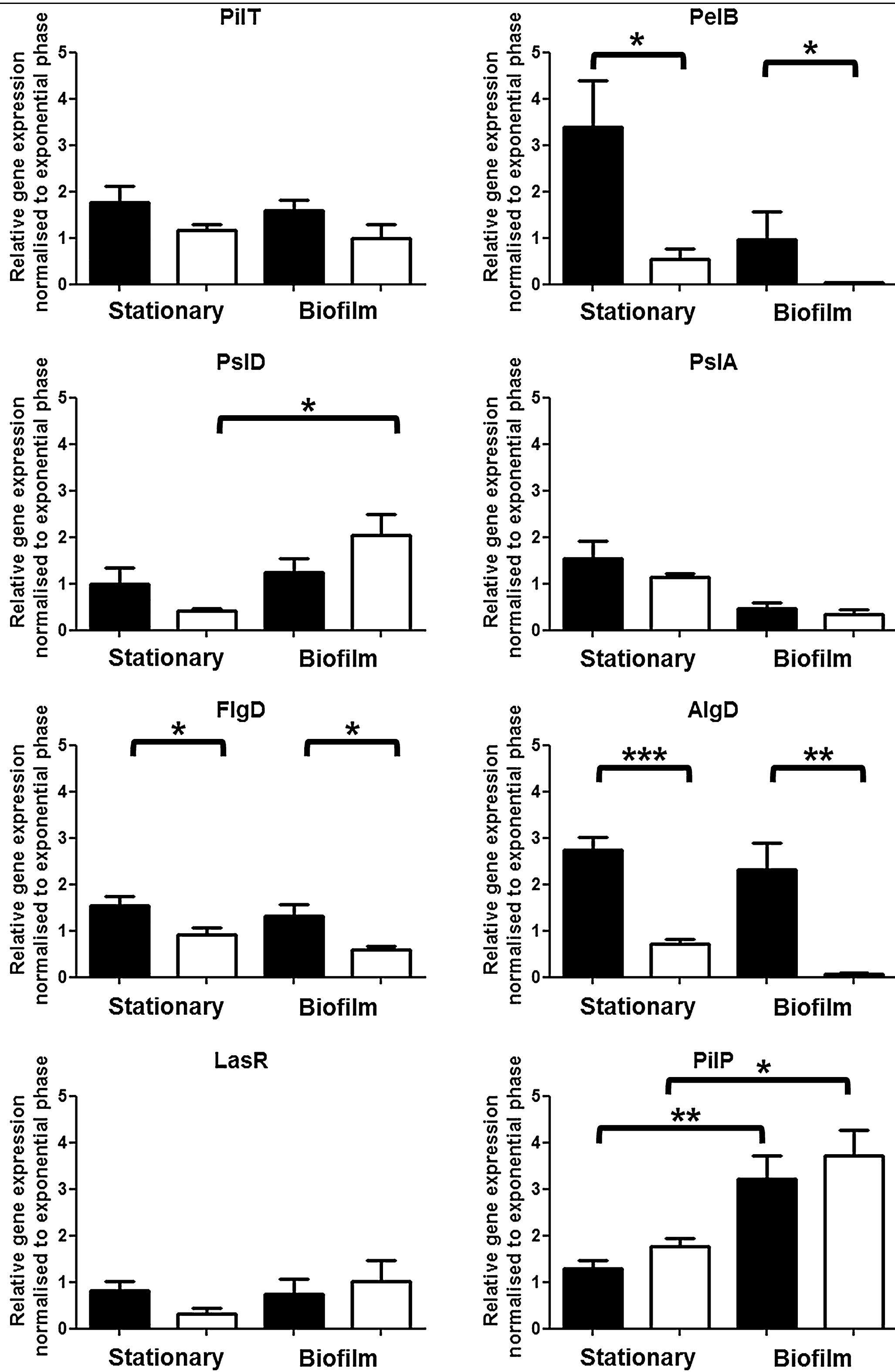

Fig. 3. Relative levels of gene expression in PSE305 and PSE306. Relative expression levels of biofilm and motility genes between PSE305 and PSE306 grown to stationary phase and under conditions that promote biofilm formation, compared to exponential phase ($n = 3–4$). The reference gene was *gyrB*. Black and white bars represent PSE305 and PSE306, respectively. Error bars represent the standard error of the mean. (* $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$).

the type IV pilus (Ayers et al., 2009). Studies have shown that disruption of the *pilP* gene, results in a loss of surface type IV pili and a deficiency in twitching motility (Ayers et al., 2009). We demonstrated partial restoration of twitching motility following the introduction of a wild-type *pilP* on a plasmid, implicating this mutation in the defective twitching motility of PSE306.

Furthermore, there were also differences in the utilisation of histamine as a nitrogen source between PSE305 and PSE306. PSE305 utilised histamine to a greater extent compared to PSE306. This could have been attributed to the presence of a SNP mutation in PSE306, in a region with homology to the open reading frame, PA0220 in PAO1. PA0220 codes for an amino acid permease, which when knocked out in PAO1 caused a defect in histamine utilisation (Johnson et al., 2008). Biolog analysis also showed the absence of lactose utilisation by both PSE305 and PSE306. Although lactose is a major component of ovine milk (Jandal, 1996), the absence of lactose utilisation was not surprising as *Pseudomonas* sp. does not typically ferment lactose. Glucose appears to be the dominant carbon source of PSE305, unlike in PSE306. Interestingly, capric acid utilisation by PSE306 appeared to be greater than by PSE305. This fatty acid is also a significant component of ovine milk (Breckenridge and Kuksis, 1967) and could be a source of carbon for PSE306. Studies have shown that the fatty acid profile of ovine milk can be manipulated by using different feeding regimens (Addis et al., 2005). It is possible that by changing the diet of the ovine herd in order to reduce the fatty acid components of the milk, the *P. aeruginosa* populations that utilise these components for growth could be controlled. In contrast, capric acid has been shown to have antimicrobial properties against other Gram-negative bacteria, including *Campylobacter jejuni*, *Salmonella* spp. and *Escherichia coli* (Thormar et al., 2006).

The three major deletions from the genome of PSE306 compared to PSE305, and the insertions/deletions in PSE305 compared to PAO1, suggest considerably genomic instability during the outbreak. Genomic instability in *P. aeruginosa* CF infections has been widely reported (Cramer et al., 2011). However this is the first evidence of genomic instability during acute *P. aeruginosa* mastitis infections.

Besides the genomic differences between PSE305 and PSE306, there were also significant differences in the gene expression profiles between these two isolates. These gene expression differences could also explain the observed phenotypes of PSE305 and PSE306, in particular the deficiency in twitching motility and the absence of stable biofilm formation in PSE306. There was a significant difference between the expression of *pelB* between PSE305 and PSE306 under the two growth conditions tested. The *pel* genes are involved in the production of a glucose-rich polysaccharide that contributes to the formation of a matrix that surrounds the cells in a biofilm; PelB in particular has been predicted to be a transmembrane protein located within the cytoplasmic membrane (Vasseur et al., 2005). *P. aeruginosa* with mutations in *pelB* are deficient in their ability to form biofilms, in particular during early stages of biofilm formation (Vasseur et al., 2005). This suggests that the reduced expression of *pelB* in PSE306 compared to PSE306, could have contributed to the deficiency of PSE306 to form stable biofilms. As with *pelB*, *algD* gene expression was also reduced in PSE306 compared to PSE305, under the growth conditions tested. The *algD* gene is part of an operon that is responsible for the synthesis of alginate and contributes to biofilm architecture (Stapper et al., 2004). Expression of the *flgD* gene, involved in flagella assembly, was reduced in PSE306. It has been shown that flagella are necessary for *P. aeruginosa* biofilm development and motility (O'Toole and Kolter, 1998). Therefore, the reduced expression of *algD*, *flgD* and *pelB* could contribute to the inability of PSE306 to form stable biofilms. Furthermore, reduced *flgD* expression in PSE306 could have resulted in reduced flagella-dependant motility in this isolate. Interestingly, *pilP* transcription was not significantly reduced in PSE306 compared to PSE305 despite PSE306 containing a SNP mutation in this gene. Therefore it is possible that the SNP mutation affects the function of PilP, but not the level of *pilP* transcription. Overall, some key differences in gene expression between PSE305 and PSE306 were identified that could have contributed to the phenotypic differences between these two isolates.

5. Conclusion

PSE306 and PSE305 represent isolates of the same clone that vary in both genotype and phenotype. This provides evidence that strain divergence occurred during this infection outbreak. *P. aeruginosa* population divergence has previously not been reported in mastitis infections. The further understanding of population behaviour of *P. aeruginosa* during mastitis infections, in particular regarding biofilm forming ability, may aid the development of novel therapeutic strategies to treat mastitis infections.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.vetmic.2014.11.011.

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