Biofilm Associated Genotypes of Multidrug-Resistant *Pseudomonas aeruginosa*

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Abstract

Pseudomonas aeruginosa is a ubiquitous environmental microorganism that is also a common cause of nosocomial infections that vary in severity from chronic wound infections to pneumonia, bloodstream infections and sepsis. Its ability to survive in many different environments and persistently colonize human tissue is linked to its presence within biofilms that form on indwelling device surfaces such as plastics and stainless steel. Biofilm promotes bacterial adhesion and survival on surfaces, reduces susceptibility to desiccation, and the actions of antibiotics and disinfectants. Recent genome sequencing studies demonstrate that *P. aeruginosa* is a highly diverse species with a very large pan-genome consistent with its adaptability to differing environments. However, most MDR infections are caused by a small number of "high-risk" clones or lineages that recently emerged and spread globally.

In our 2017 study of the resistome of *P. aeruginosa* we confirmed the power of genome-wide association (GWAS) techniques to explore the genetic basis of several antibiotic resistance phenotypes and discovered 46 novel putative resistance polymorphisms. In this study we sought to examine genetic associations within a subset of these isolates with simple biofilm phenotypes. We examined the genetic basis for biofilm production on polystyrene at room temperature (22°C) and body temperature (37°C) within a total of 280 isolates. 69% of isolates (*n*=193) produced more biofilm mass at 22°C, whilst those producing more biofilm at 37°C had reduced optical density $_{540}$ variation. We found statistically significant associations with *lpxO* and other genes associated with arsenic resistance to be significantly associated with this trait. *lpxO* which encodes a lipid A hydroxylase and arsenic reduction genes have previously been found to be associated with biofilm production in this species. We analyzed 260 ST111 and ST235 genomes and found considerable genetic variation between isolates in their content of genes previously found associated with biofilm production. This is indicative of a highly variable and flexible population within these clades with frequent emergence of successful sub-lineages. Analysis

of 48 of these isolates' ability to form biofilm on stainless steel surfaces showed that a 'good' biofilm-forming phenotype had significant intra-clone variation, independent of core genome phylogeny with pan-genome analysis, suggesting a possible association and involvement of components of the type IV secretion system. However, GWAS and pan-GWAS analyses yielded weaker statistical significance. This study confirms GWAS and pan-GWAS trait associations can be performed for biofilm phenotype and produce data in agreement with each other. This panel of 280 study isolates, matched to genomic data has potential for the investigation of other phenotypes in *P. aeruginosa* perhaps as part of a growing database / collection. A representative, curated, genome sequenced collection should increase in usefulness as it grows offering increasing statistical power.

Importance

P. aeruginosa is a major cause of multiply antibiotic infections worldwide but it is also found in many hospital and natural environments, especially aquatic ones. In this study we examined genetic polymorphism associated with biofilm production at room temperature and at body temperature, the biofilm associated gene repertoire of two major MDR clones and also genetic polymorphisms associated with biofilm production on stainless steel. Using these genome-wide and pan-genome wide association methods we identified / confirmed potential key genes involved in biofilm production and survival of *P. aeruginosa*. The study demonstrates the potential usefulness of large, genome sequenced isolate collections such as ours, to better understand the genetics underlying phenotypic diversity in this species.

Introduction

Pseudomonas aeruginosa is a mono-flagellate, Gram-negative bacterium that is present in most environments. A frequent coloniser of humans, other animals and plants, it also is a very common opportunistic pathogen able to grow at a variety of temperatures[1]. It is a leading cause of severe, life-threatening nosocomial human infections and the major pathogen associated with lung infections of patients with cystic fibrosis. It is has been classified as one of the major species associated with multiple antimicrobial resistance of urgent public health concern by the Infectious Diseases Society of America[2], Centers for Disease Control and Prevention and the World Health Organization.

A variety of typing methods including multilocus sequence typing (MLST)[3] and more recently, genomic sequencing studies[4-7] have shown that *P. aeruginosa* has a non-clonal,

epidemic population structure in which successful clones occasionally arise and are globally transmitted[8]. Multidrug resistant (MDR) isolates of clones associated with outbreaks of infection belonging to MLST Sequence Type (ST) 111 and ST235 are two such 'high risk' clones that have global distributions and numerous and transferable antibiotic resistances[9]. Isolates of these clones contain a large number of horizontally transferred β-lactamase genes[4] and are highly virulent in comparison to a third, less significant clone - ST175[10].

The antibiotic resistome of both ST111 and ST235 clones have been characterised in attempts to understand relationships between antimicrobial resistance (AMR) phenotype and genotype. A genomic analysis of 87 UK isolates belonging to ST111 demonstrated divergence from a common ancestor estimated to have emerged in approximately 1965, with the majority of isolates carrying the β-lactam gene VIM-2[5]. A similar analysis of 79 ST235 isolates predicted a more recently evolved clone, the ancestral genotype of which emerged around 1984, coinciding with the widespread use of fluoroquinolones for the treatment of gram-negative infections[11].

Whilst understanding the relationship between a pathogen's genome, epidemiological surveillance, clinical treatment and prevention success is critically important[12,13], *P. aeruginosa* has many phenotypic features that also promote its success as a pathogen. In common with other bacterial pathogens *P. aeruginosa* usually exists within biofilms. These are complex microbial communities associated with extracellular polymeric matrices that help bacteria resist desiccation, mechanical removal and the actions of antibiotics and disinfectants[14]. In human tissue, biofilm-associated *P. aeruginosa* are difficult to eradicate and represent infectious foci that can lead to serious systemic disease. *P. aeruginosa* biofilms are found at surface-liquid, surface-air and liquid-air interfaces, and are a significant clinical problem in wounds and the cystic fibrosis lung[15]. Polymicrobial biofilms are also recognized as important niches for MDR evolution, as they represent a significant reservoir for horizontal gene transfer within and between bacterial species[16].

In addition to comprehending *in vivo* biofilm formation, understanding *P. aeruginosa* biofilm development and persistence on other surfaces is required. Stainless steel is the most common surface material used in many industries where control of microorganisms is important, including healthcare. Properties of stainless steel include resistance to corrosion, easiness to clean and a level of hardness likely to limit scratches and other defects[17].

However, many of these surfaces have been shown to allow the formation of biofilms, for example in hard-to-clean locations, and those with a favourable environment, such as sinks and pipes [18].

Both ST111 and ST235 isolates have been shown to produce significantly more biofilm compared to those from non-high-risk clones[19] whilst being over-represented in locations such as hospital sink pipes[20]. A recent study strongly implicates the presence of *P. aeruginosa* biofilm in hospital waste-water pipes as being responsible for an outbreak of infection due to MDR ST111 and ST235 in a haematology unit[21]. A policy promoting replacement of sink units to reduce such sources of infection has now been rolled out in the United Kingdom.

There is a need to better understand the clinical importance of biofilms in hospital environments starting with how biofilm production changes when *P. aeruginosa* cells move from the hospital environment to the human body. Genome Wide Association Studies (GWAS) have been used to great effect in identifying genetic determinants contributing to disease pathology in human medicine. Thus far, microbial GWAS and more generic Whole Genome Sequencing (WGS) studies have focused on the molecular epidemiology of infectious disease and particularly on associations between the genome and antimicrobial resistance and / or pathogenicity. There is huge potential for such methods to help understand the genetic basis of other phenotypes, such as key processes that are important in microbial ecology or industrial microbiology[22,23].

In this study we examine the associations between aspects of the *P. aeruginosa* pan-genomes and their biofilm-producing activity at different temperatures or on stainless steel and examine the genetic attributes associated with biofilm production in MDR clones ST111 and ST235

Methods

Bacterial isolates and genome sequences

This study analyzed two different sets of *P. aeruginosa* isolates. The first set, here-on-in described as 'temperature-dependent biofilm analysis' contained 280 isolates (Supp Table 1), for which genomic data and antibiotic susceptibility phenotype has previously been described[7,8].

148

149 The second set, here-on-in described as 'stainless steel biofilm analysis' contained 260 *P.*
150 *aeruginosa* genomes (Supp Table 2), 153 of which belong to the ST111 clone and 107 to the
151 ST235 clone. Of these, 25 ST111 and 23 ST235 isolates were used to study biofilm
152 phenotypes. All genomic data were downloaded from Genbank as fasta nucleotide
153 sequences. Bacterial strains used for biofilm phenotyping were provided by bioMérieux
154 (France) and Synthetic Genomics (USA) and were analysed in two previously published
155 studies[6,7].

156

157 ***Construction of phylogenies***

158 Core Single Nucleotide Polymorphisms (SNPs) were identified using the KSNP3 pipeline[24].
159 Kmer lengths of 21 were used for temperature-dependent biofilm analysis, whilst for
160 stainless steel biofilm analysis, kmer lengths for ST111 and ST235 were 21 and 23
161 nucleotides respectively. Kmer lengths were selected using the KCHOOSER module within
162 the KSNP3 suite. Bayesian phylogenetic analysis was performed using BEAST 2[25]. The
163 following conditions were set: gamma site heterogeneity model, Hasegawa-Kishino-Yano
164 (HKY) substitution model, relaxed-clock log-normal, chain length 5,000,000. BEAST 2 output
165 was summarized using TreeAnnotator with a 5% burn in.

166

167 ***Construction and interrogation of pan-genome***

168 All genomes were annotated using PROKKA[26]. Using PROKKA output, pan-genomes were
169 constructed using the Roary pan-genome pipeline[27], and visualized with the interactive
170 visualization tool Phandango [28].

171

172 ***Biofilm phenotypes***

173 Temperature-dependent biofilm analysis was carried out using standard non-modified 96-
174 well plates (Starsted, Leicester, UK). Stainless steel biofilm analysis was performed on MBEC
175 biofilm 96-well plates (Innovotech, Edmonton, Canada), with pegged lids spray coated with
176 stainless steel. The sub-micron thick stainless steel coatings were deposited onto the lid by
177 the physical vapour deposition technique of magnetron sputtering, which is widely used in
178 industry to deposit thin (sub-micron to several micron thick) metallic or ceramic
179 coatings onto a wide range of components[29]. The coatings were sputtered from a 300mm x
180 100mm 304 grade stainless steel target in a Teer Coatings Ltd UDP350 coating rig.

181

182 The method for biofilm growth was a modified version of that described by Coffey and
183 Anderson[30]. *P. aeruginosa* strains were maintained on Luria-Bertani (LB) agar (BD, Sparks,
184 MD) at 4°C. Liquid cultures were prepared by inoculating a single colony into 10 ml of LB
185 broth (BD, Sparks, MD), and incubated at 37 °C with agitation (180 rpm). An overnight
186 culture of each strain was diluted to an optical density (OD) of 1.0_{540}. A 1:100 inoculation in
187 sterile LB broth was made in a well of the MBEC plate base. Four wells per strain were
188 inoculated. For temperature-dependent biofilm analysis, plates were incubated without
189 shaking at either 22°C or 37°C for 48 hours.
190
191 For stainless steel biofilm analysis, the MBEC lid was replaced and the plate was incubated at
192 37°C with agitation (110 rpm) for 48 hours, with a change to a new 96-well plate with 200 µl
193 of sterile LB broth in each well after 24 hours. To quantify biofilms, plates or pegs were
194 gently immersed in water to remove non-adhered cells, placed in 0.1% crystal violet (Sigma,
195 Dorset) for 10 minutes then gently washed in water, twice, to remove excess stain. The
196 plates for temperature-dependent biofilm analysis had 200µl of 30% acetic acid added to
197 each well to solubilize stain, whereas the lid with stained pegs was placed into of a 96-well
198 plate containing 200µl per well of 30% acetic acid (Fisher Scientific, Loughborough) for 10
199 minutes with agitation (110 rpm). Optical density of each well was measured at 540 nm with
200 a FLUOstar Omega plate reader (BMG Labtech, Aylesbury).
201
202 ***Trait definition***
203 In order to carry out GWAS and pan-GWAS (below), categorization of biofilm production was
204 required. For temperature-dependent biofilm analysis, isolates were split into one of two
205 traits: either producing more biofilm at 37°C compared to growth at 22°C, or producing
206 more biofilm at 22°C compared to 37°C. For stainless steel biofilm analysis, a "good" biofilm
207 formation trait was considered to be exhibited in strains providing an OD reading >= 0.5_{540},
208 whilst poor biofilm formation was considered as an OD reading of < 0.5_{540}.
209
210 ***Pan-genome-wide association analysis***
211 Scoary is a software tool able to provide pan-genome associations (pan-GWAS) based on
212 user-defined traits using an input (gene presence / absence) file generated by Roary[31].
213 Analysis was performed independently for temperature-dependent biofilm traits as for both
214 ST111 and ST235 genomes in the stainless steel biofilm analysis. Statistical significance of
215 the associations was tested by Scoary with a Fisher's exact test, and a pairwise comparisons

algorithm to control spurious associations from stratified populations. *P* values were corrected for multiple comparisons using the Bonferroni adjustment. Where Scoary analysis provided genes significantly ($p<0.05$) associated with the biofilm phenotype that had not previously been annotated in PROKKA, and therefore considered hypothetical / putative, the amino acid sequence was searched in BLASTP, and if no hit was found, assessed using the sensitive protein homology detection, function and structure prediction tool HHPred (MPI Bioinformatics Tool Kit)[32]. The top three predictions were considered for assumed protein identity and function, assuming a match probability of at least 80%.

SNP genome-wide association study

We used PLINK v1.90b5.3[33] to explore SNP-based associations with user-defined traits. Analysis was performed in PLINK to test case / control associations with Fisher's exact test, and return Bonferroni adjustments for statistical significance. Analysis was performed independently for temperature-dependent analysis as before for both ST111 and ST235 genomes in the stainless steel dataset using core SNP data produced by the KSNP3 pipeline. SNPs significantly associated with phenotype ($p<0.05$) where located in the genome using Artemis genome browser[34]. Genes containing SNPs of interest were interrogated using BLASTP and HHpred, with the top three predictions with a probability match of over 80% considered for assumed protein function.

Results and discussion

Temperature-dependent biofilm analysis

Biofilm formation varied greatly between isolates at both 22°C and 37°C (Figure 1). Of the 280 isolates studied, 69% (n=193) produced more biofilm at 22°C (average OD_{540} of 1.98) compared to 37°C (OD_{540} measurement of 1.29). When grown at 37°C, isolates demonstrated less variation in OD_{540} measures than at 22°C (Figure 2). Increased biofilm growth phenotype at 37°C was widely distributed across the phylogeny (Figure 3).

Scoary analysis yielded 3,431 genes associated with the phenotypic trait of producing more biofilm at 22°C compared to 37°C, with only one gene passing with a significant Bonferoni *p* value (Table 1), annotated using BLASTN as *lpx*O, a lipid A hydroxylase gene. Lipid A is one of three distinct regions of the lipopolysaccharide of *P. aeruginosa*, and although literature on the gene is limited, it has been suggested that LpxO plays an essential part in the success of chronic infections (e.g. cystic fibrosis)[35,36]. More interestingly still, *lpxO* has been shown to

251 be upregulated in *P. aeruginosa* when grown at a suboptimal temperature (22°C) compared
252 to growth at 37°C[37]. Scoary analysis also provided other genes with significance, although
253 with a Bonferonni *p* value of just above the 0.05 cut off (Table 1). These included *acr3*, *arsC*,
254 *arsR* and *arsH*, four genes involved in arsenic resistance / reduction. Presence of arsenic in
255 bacterial cells has been demonstrated to affect biofilm synthesis as well as chemotaxis and
256 motility[38], with suggestion that arsenic can prevent the switch between planktonic and
257 sessile lifestyle[39].
258
259 PLINK analysis yielded eleven significant SNPs (p<0.001 after Bonferroni adjustment)
260 associated with increased biofilm production at 37°C compared to 22°C (Table 2). The most
261 significant was a non-synonymous SNP found in the *lpxO* gene, the lipid A gene described
262 earlier. Four other non-synonymous SNPs were identified as significant (p<0.001 after
263 Bonferroni adjustment) and are present in a type 1 fimbrial gene, the *marC*, *pilW* and a
264 hypothetical gene (*ydcV*). It is believed that type 1 fimbrial protein plays an essential role in
265 the development of fimbrial structures, facilitiating bacterial attachement and promoting
266 biofilm formation[40]. The *pilW* is involved in the development of the type-IV pilus, widely
267 demonstrated as integral to twitching, swarming and biofilm production in *Pseudomonas*
268 spp [41,42]. *marC* encodes a multidrug resistance protein (homologous to *tspR*), deletion of
269 which reduces type III secretion systems and motility whilst increasing biofilm production[43].
270 Of the remaining SNPs, four, whilst all present within a gene, were synonymous. One SNP
271 was found in *arsH*, a gene which the Scoary gene presence/absence analysis also found to be
272 of significant interest with the temperature-dependent biofilm phenotype (as described
273 earlier). Another synonymous SNP of interest was located in a gene annotated as *smf-1*, a
274 fimbriae-1 gene found in *Stenotrophomonas maltophilia*, which shares significant similarities
275 to fimbrial adhesins found in the *cupA* fimbriae of *P. aeruginosa,* and has been
276 demonstrated to be important in biofilm formation[44].
277
278 ***Biofilms on stainless steel***
279 Of the isolates assessed for biofilm production on stainless steel (Figure 3), 40 % (n= 10 out
280 of 25) ST111 and 52% (n=13 out of 23) ST235 were considered high biofilm producers. This
281 demonstrates a large intra-clone variation of the biofilm phenotype. However, there was no
282 significant difference between inter-clone variation, suggesting the intra-clone variation may
283 be a common feature of other clones.
284

285 The BEAST 2 analysis suggests ST111 isolates diverged from a common ancestor ≅ 43 years
286 ago (Figure 4.a), with a pan genome of 15,488 genes. The 107 ST235 isolates had a pan-
287 genome of 15,178 genes and diverged from a common ancestor ≅ 28 years ago (Figure 4.b),
288 suggesting that the two clones emerged within approximately 15 years of each other. When
289 considered alongside biofilm phenotype, those isolates sharing phylogenetic similarity
290 display similar biofilm phenotype, except for ST235 isolates WH-SGI-V-07622 and WH-SGI-V-
291 07625, as well as isolates WH-SGI-V-07498 and WH-SGI-V-07624. Nevertheless, this analysis
292 suggests many good and poor biofilm formers are closely related, and that biofilm
293 production on stainless steel is not necessarily predictable based only on genomic analysis.
294
295 The pan-genome of biofilm genes widely described in the literature (Figure 5) has a different
296 core vs accessory structure in each of the clones, with the majority of genes (≈54%) in both
297 clones present in less than 15% of genomes. ST235 has a higher percentage of core genes
298 compared to ST111 (61.7% vs 31.34%), whilst ST111 has a larger cloud genome compared to
299 ST235 (40.3% vs 17.02%). This variation is down to a number of homologous genes in the
300 ST111 biofilm pan-genome *e.g. pelA*, *pslA*, *pslB*. The genes *pilA* and *fimT* were both identified
301 in the ST111 pan-genome, but not found in the ST235 pan-genome, whilst *pslC* was found in
302 ST235 but not in the ST111 pan-genome.
303
304 Scoary analysis provided 77 and 107 genes of interest for association with biofilm phenotype
305 in ST111 and ST235 respectively although none had a Bonferonni of $p<0.05$. Of these genes,
306 24 ST111 and 30 ST235 genes did not provide a homology match of over 80% using HHpred,
307 and would require further functional investigation to understand their potential association
308 with biofilm phenotype. The remaining genes were considered for predicted function that
309 might be obviously associated with biofilm formation (Table 3). ST111 had six genes with
310 potential function associated with biofilm (7.8% of the ST111 Scoary output). Predicted
311 homology of two of these genes suggest association of the flagellum system, while one gene
312 shared 99.67% homology with *motB*, an essential gene of the *P. aeruginosa* flagellar motor.
313 Mutants containing defects in the *mot* gene complex have been shown to exhibit a reduced
314 or abolished motility[45]. Another gene shared 83.03% homology with *qseC*, a flagellum
315 regulator gene with demonstrated importance in biofilm production in gram negative
316 bacteria [46,47]. Two further genes suggest association with the type-IV pili system,. These two
317 genes share homology to a type-IV pili peptidoglycan endopeptidase (RipA – 92.55%) and a
318 geopilin domain 1 protein (PilA – 92.32%). Of the two remaining ST111 genes from the

319 Scoary analysis, one gene provided 100% homology to a subtilisin-like serine protease, a
320 cellular mechanism which, if absent, has been shown to produce lower biofilm formation
321 and motility function in *P. aeruginosa*[48]. The last of the ST111 Scoary genes shares 90.64% to
322 BamC outer membrane protein, a lipoprotein likely involved in the outer-membrane
323 biogenesis [49].
324
325 Of the ST235 Scoary analysis, five genes (4.7% of the ST235 Scoary output) had predicted
326 function that might be obviously associated with biofilm formation (Table 3). Three of the
327 genes share structural homology with type-IV secretion systems, two as hydrolases (one
328 more specifically sharing homology with peptidoglycan endopeptidase RipA), and a third
329 sharing homology to a type-IV secretion system-like gene IcmL-like,[50]. The fourth gene
330 shared 98.12% homology with PilP, an essential part of the type-IV pili complex [51], whilst the
331 fifth shared 95.85% homology with a flagellar transcriptional activator FlhD found in gram-
332 negative bacteria [52,53]. Whilst the Scoary pan-genome association analysis does not suggest
333 an exact gene shared between the two clones in relation to the biofilm phenotype, it does
334 emphasize the importance of the Type IV secretion systems (T4SS), with multiple genes
335 present in both analyses. It is widely accepted and demonstrated in the literature that T4SS
336 plays an important role in twitching motility (important for initial biofilm formation) as well
337 as biofilm maturation[54,55]. It is acknowledged that biofilm production can be a highly specific
338 trait, with environmental and mechanical changes and stresses altering the biofilm
339 performance of a particular isolate, and so continued analysis of biofilm phenotypes in
340 different models / environmental conditions would be beneficial.
341
342 PLINK analysis provided three SNPs of significant association with biofilm phenotype in
343 ST235, and no significant SNPs in ST111 (Table 4). Cross checking SNPs with the relevant
344 genome and BLASTP of the protein sequence shows that two of the SNPs were located in the
345 same gene, a phage virion morphogeneisis protein, whilst the third was a non-coding SNP
346 downstream of the viron morphogenesis protein. PaperBLAST[56] searches provides on record
347 of the gene or a homologue having already been explored in the literature.
348
349 Whilst the phenotype and genotype analysis and associations described in this paper
350 provide novel insight into the relationship between non-discreet phenotypes and genotype,
351 standardization of such phenotypes are required. Currently, no agreed method exists for the
352 categorization of good or poor biofilm formers, and much of the wider biofilm literature
353 uses a dynamic range of methods and approaches to biofilm studies [57]. This will be essential

354 if GWAS and pan-GWAS studies are to be utilized to better understand the genetics of
355 biofilm production.
356
357 **Conclusions**
358 In this study we were able to identify candidate genes in which polymorphism (or deletion)
359 was associated with differences in temperature-dependent biofilm phenotype for 280 *P.*
360 *aeruginosa* isolates. The two different GWAS approaches used in this paper (SNP-based and
361 gene presence / absence) both resulted in the identification of similar genes, namely *lpxO*,
362 fimbrial genes and genes involved in arsenic resistance. The relationship between biofilm
363 phenotypes on stainless steel and genotype in *P. aeruginosa* was less clear, likely due to the
364 smaller subset of isolates used in this study. Further work should seek to utilize GWAS and
365 pan-GWAS studies to identify new gene targets / cellular machinery alongside functional
366 studies such gene expression (for example RNA-seq). Our study demonstrates the
367 importance of understanding the complex nature of *P. aeruginosa* biofilm, and that
368 phenotypic studies in isolation and/or assumptions that biofilm phenotype is consistent
369 across *P. aeruginosa* offers little in the way of meaningful data. Unraveling the phenotype-
370 genotype relationship of *P. aeruginosa* will be an essential next step in understanding and
371 preventing biofilm in the era of AMR.
372
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574 **Figures and tables**
575

576 **Table 1. Genes associated with greater biofilm growth at 22 °C compared to 37 °C as**
577 **determined by Scoary pan-genome-wide association analysis.**

578

Gene	Bonferroni corrected P value	Naive P value	Annotation	Function
lpxO_1	0.03419409	1.08E-06	Lipid A hydroxylase LpxO	Integral component of cellular membrane
lpxO_2	0.05942534	1.88E-06	Lipid A hydroxylase LpxO	Integral component of cellular membrane
lpxO	0.06039183	1.91E-06	Lipid A hydroxylase LpxO	Integral component of cellular membrane
arsC_1	0.07764851	2.45E-06	Arsenate reductase	reduces arsenate to arsenite Integral component of cellular membrane
acr_3	0.11612236	3.67E-06	Arsenical-resistance protein	Part of integral component of membrane Involved in transmembrane transport
arsR	0.11612236	3.67E-06	transcriptional regulator	DNA-binding transcription factor activity
lpxO	0.11612236	3.67E-06	Lipid A hydroxylase LpxO	Integral component of cellular membrane
arsH	0.11612236	3.67E-06	Arsenical resistance NADPH-dependent FMN reductase ArsH	Involved in oxidation-reduction process Enables oxidoreductase activity

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582 **Table 2. SNPs found statistically (p<0.05 using the Fisher exact test with Bonferroni**
583 **adjustment) associated with more biofilm growth at 22 °C compared to 37 °C using PLINK.**
584 **Base number relates to SNP position in isolate WH-SGI-V-07049.**

585

SNP	Bonferroni corrected P value	Classification	Base number (direction)	SNP variant one	SNP variant two	Blast result for coding region containing the SNP
CCCGATGCGC.GGCGCCGTTC	1.52E-07	Non-synonymous	5,340,240 (reverse)	G	T	lpxO
CCTGCAGAAC.CCGGGAAGAA	9.77E-06	Non-synonymous	3,288,081 (forward)	G	A	Type I Fimbrial protein
AACTGGTCAG.TCGGTCGGAC	1.27E-05	Non-synonymous	1,327,097 (reverse)	C	T	ydcV
GCAGCCCTTG.GGCGACGGGA	4.18E-05	Non-synonymous	4,997,1780 (reverse)	G	T	marC family protein
AGGAGGCCTA.CAGCGTCTCC	7.56E-05	Non-synonymous	5,995,683 (reverse)	T	C	PilW
CGAGGCGCTG.AAGAGCGCTC	8.92E-05	Not in a gene	1,418,740 (forward)			
CGCCCTGCTG.TGAACAAGCA	9.33E-05	Synonymous	5,974,394 (reverse)	T	C	hypothetical
GCGCAGCGCC.GCCATAGGCA	0.0003472	Not in a gene	4,211,288 (forward)			
AGATCAAGGA.GTGCGCGCCG	0.0003916	Synonymous	6,237,835 (forward)	A	G	fusA 2 elongation factor
AGGGCAAGAC.CTGGCGGTGA	0.000442	Synonymous	2,169,567 (forward)	C	G	arsH arsenical resistance
AGCATTTCAC.ACCCTGTTCC	0.0007363	Synonymous	6,299,006 (forward)	C	T	smf-I major fimbrial subunit

586

587 **Table 3. Genes found statistically associated (unadjusted p<0.05) with biofilm phenotype**
588 **using Scoary pan-genome-wide association analysis, for both ST111 or ST235.**

589

Gene ID	Predicted protein homology	Percentage similarity
ST111		
Group_6254	Subtilisin-like serine protease	100
Group_8277	PomB/MotB/ompA-like – involved in flagellar motor	99.67
Group_6216	Type-IV pili peptidoglycan endopeptidase (RipA)	92.55
Group_1372	Geopilin domain 1 protein; pilin, PilA, bacterial nanowire	92.32
Group_2185	BamC outer membrane protein assembly factor	90.64
Group_8212	Sensor protein qseC	83.03
ST235		
Group_107	Type-IV secretory hydrolase	99.95
Group_752	Type-IV pili peptidoglycan endopeptidase (RipA)	95.1
Group_3943	PilP protein	98.12
Group_859	IcmL-like; type IV secretion system protein	97.13
Group_5868	Flagellar transcriptional activator flhD/AcaD	95.85

590
591

592 **Table 4. SNPs and related genes belonging to ST235 which have been found statistically**
593 **(p<0.05 using the Fisher exact test with Bonferroni adjustment) associated with biofilm**
594 **phenotype using PLINK genome-wide association analysis. Base number relates to SNP**
595 **position in WH-SGI-V-07627.**

SNP	Bonferroni corrected P value	Classification	Base number (direction)	SNP base for good biofilm formers	SNP base for bad biofilm formers	Amino acid coded for in good biofilm formers	Amino acid coded for in good biofilm formers	Blast result for coding region containing the SNP
CATGCTCAGG.TGGAATGTGA	0.006687	Non-synonymous	657,864 (f)	T	C	Threonine	Alanine	Pseudomonas phage tail fiber protein
GACGCCGCTG.GCATAACGGA	0.006687	Synonymous	657,937 (f)	C	T	Alanine	Alanine	Pseudomonas phage tail fiber protein
AGCGGCCACG.GTACATGCTC	0.04681	Noncoding	657,850 (f)					

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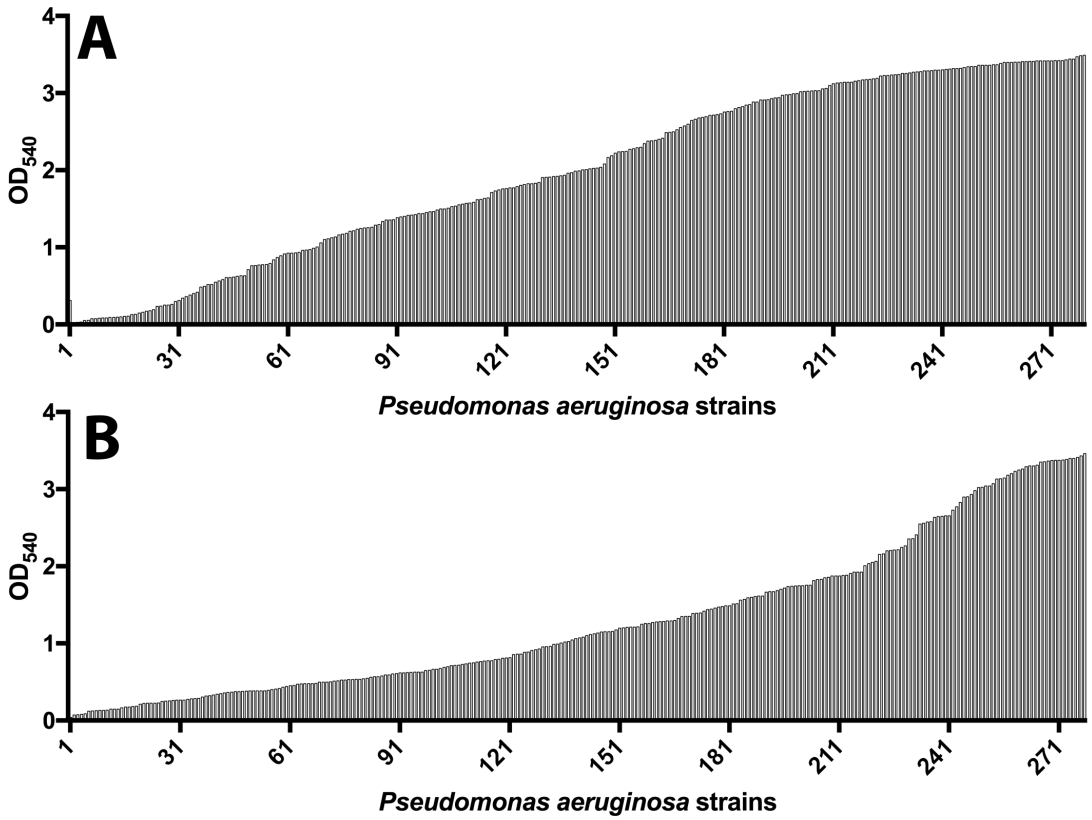


Figure 1. Optical density related to biofilm stained with 0.1% crystal violet and solubilised in 30% acetic acid at either 22 °C (A) or 37 °C (B) for 280 *Pseudomonas aeruginosa* isolates.

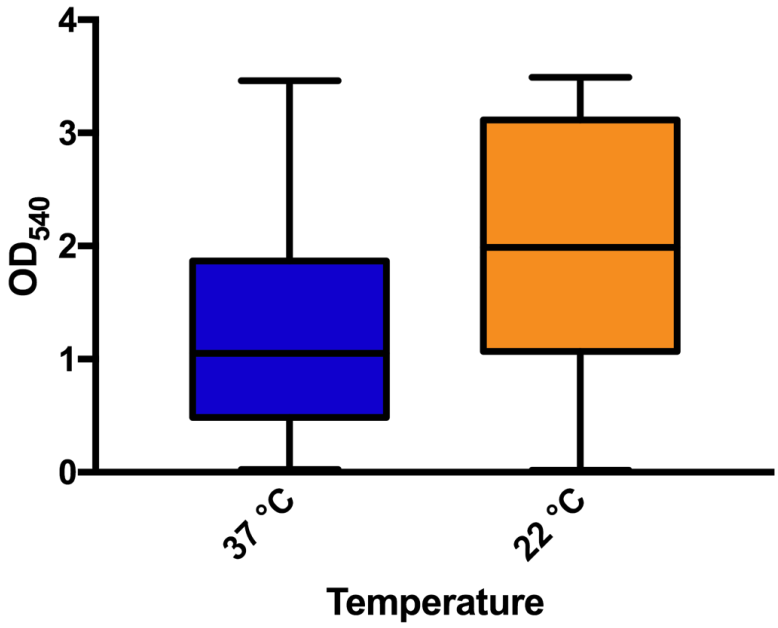


Figure 2. Box plot demonstrating the variation in biofilm formation for 280 *Pseudomonas aeruginosa* isolates at two different temperatures as measured by optical density.

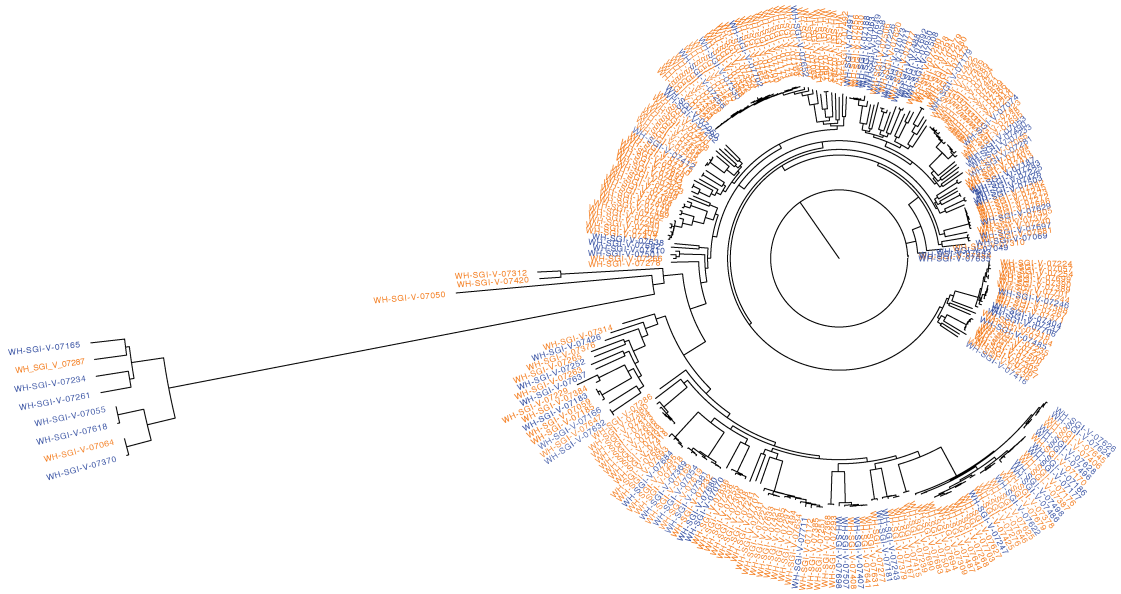
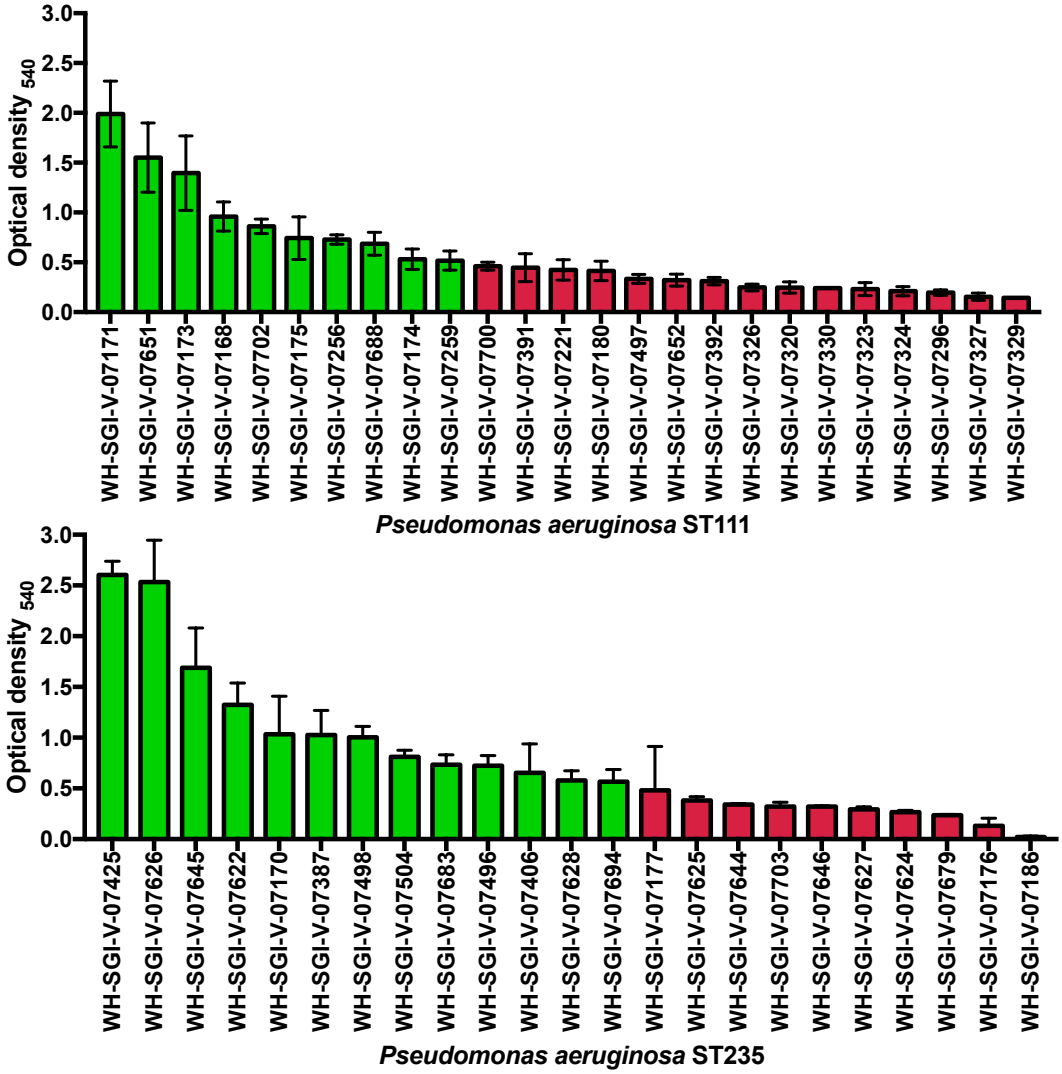


Figure 3 -Maximum parsimony tree (from KSNP3 analysis) of 280 Pseudomonas aeruginosa isolates. Names highlighted orange represent an isolate that produced more biofilm at 22 °C and names highlighted blue represent an isolate that produced more biofilm at 37 °C.



614
615 **Figure 4. Optical density readings at 540nm of Pseudomonas aeruginosa biofilm after**
616 **staining with 0.1% crystal violet and solubilised in 30% acetic acid. Biofilms were grown on**
617 **a modified MBEC assay plate, of which the pegs had been coated in stainless steel. Data**
618 **represent 25 ST111 (A) and 23 ST235 (B) strains. An optical density cut off of 0.5_{540} was**
619 **used to differentiate high (green) and poor (red) biofilm producers.**

620

621 **Figure 5. Bayesian evolutionary analysis sampling tree (BEAST 2) analysis representative of**
622 **the ST111 (A) and ST235 (B) strains included within analysis. Horizontal distance is**
623 **indicative of time in years, with 0 representing the most recent isolate (2017). Strains**
624 **included in the biofilm on stainless steel phenotype assay (figure 4) are overlaid with their**
625 **designated phenotype – high biofilm producers (green) and poor biofilm producers (red).**

626

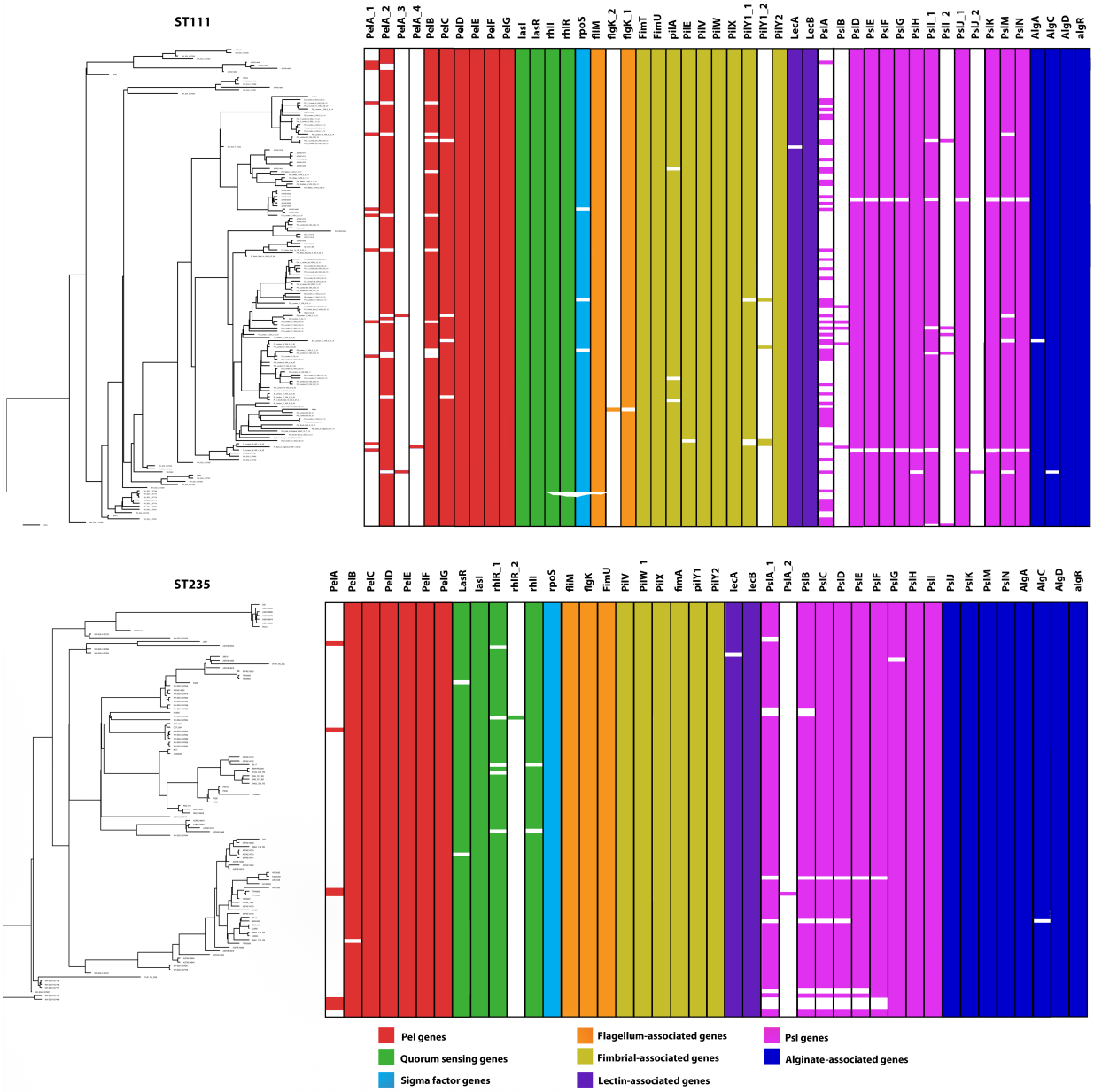


Figure 6. Biofilm gene presence/absence aligned with BEAST 2 analysis for ST111 (top) and ST235 (bottom) isolates (from Figure 2). Coloured blocks represent 48 ST111 and 42 ST235 genes that have previously been linked to biofilm formation in the literature, whilst white space represents gene absence. Colour of each block indicates gene families or biofilm features described in figure legend.